

PURCHASED BY DR N B SRIDHAR
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Veterinary Toxicology

Edited by: Patricia Marques

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VETERINARY TOXICOLOGY

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LIST OF ABBREVIATIONS

APAP	Acetaminophen
AIC	Akaike Information Criterion
ALS-PDC	Amyotrophic Lateral Sclerosis-Parkinsonism-Dementia complex
ADC	Analog to Digital Converter
ANOVA	Analysis of variance
AR	Androgen receptor
AEDs	Antiepileptic drugs
AMH	Antimullerian hormone
AFC	Antral follicle count
BER	Base excision repair
BCFs	Bioconcentration factors
BM	Bone marrow
CNT	Carbon nanotubes
CCRK	Cell cycle-related kinase
CAS RN	Chemical Abstract Services registry number
CI	Combination index
CAES	Connecticut Agricultural Experiment Station
DM	Deltamethrin
DCFH-DA	Dichlorodihydrofluorescein diacetate
DMRs	Differentially methylated regions
DMSO	Dimethyl sulfoxide
DPA	Diphenylamine
DTXSID	Distributed Structure-Searchable Toxicity substance identifier
EM	Electron microscopy
EDCs	Endocrine disrupting chemicals
FPG	Fasting Plasma Glucose
FET	Fish Embryo Toxicity
FMD	Flow mediated dilation

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FDA	Food and Drug Administration
FFQ	Food Frequency Questionnaire
FcRs	Fragment crystallizable region receptors
FNPP	Fukushima Daiichi Nuclear Power Plant
GFR	Glomerular filtration rate
GR	Glucocorticoid receptor
GSSG	Glutathione disulfide
GSH	Glutathione
GPx	Glutathione peroxidase
GR	Glutathione reductase
GRd	Glutathione reductase
GST	Glutathione S-transferases
GDP	Gross domestic product
GH/IGF	Growth hormone/insulin-like growth factor 1
HAB	Harmful algal blooms
hsps	Heat shock proteins
HSC	Hematopoietic stem cells
H&E	Hematoxylin and eosin
HCC	Hepatocellular carcinoma
HFD	High-fat-diet
HFC	High fish consumers
HPAI	Highly pathogenic avian influenza
HAT	Histone acetylases
HDAC	Histone deacetylases
HPAA	Hypothalamic-pituitary-adrenal axis
IS	Immune system
ICPAES	Inductively coupled plasma atomic emission spectroscopy
ICP-MS	Inductively coupled plasma mass spectrometry
InChI	International Chemical Identifier
LAL	Limulus amoebocyte lysate
LPO	Lipid peroxidation
LPS	Lipopolysaccharide
LC/MS/MS	Liquid chromatography/mass spectrometry/mass spectrometry
LCS	Local Citation Score

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LOEC	Lowest observed effect concentration
LT-HSC	Long-term repopulation
LBW	Low-birthweight
LDL	Low-density lipoprotein
LFC	Low fish consumers
LMP	Low Melting Point
LNs	Lymph nodes
MDA	Malondialdehyde
MAGIC	Meta-analysis of the Global Impact of Chemicals
MOA	Mode of action
MDRD	Modification of Diet in Renal Disease study
MPS	Mononuclear phagocytes system
NAMPs	Nanoparticle-associated molecular patterns
NHIS	National Health Interview Survey
NIM	Nuclear Instrument Modules
OR	Odds ratios
OGTT	Oral glucose tolerance test
OECD	Organization for Economic Cooperation and Development
OC	Osteocalcin
OTC	Over-the-counter
ppb	Part per billion
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern-recognition receptors
PCP	Pentachlorophenol
PFCs	Perfluorinated compounds
PFOA	Perfluorooctanoic acid
PBTK	Physiologically Based Toxicokinetic
PBDEs	Polybrominated diphenyl ethers
PCOS	Polycystic ovary syndrome
PEG	Polyethylene glycol
PEIC	Potential environmental impact chemicals
PSU	Practical salinity units
PP	Precocious pubarche
PI	Propidium iodide

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PSA	Prostate specific antigen
QDs	Quantum dots
ROS	Reactive Oxygen Species
SCLs	Salivary cotinine levels
SMILES	Simplified Molecular Input Line Entry Specification
SW	Single-walled
SES	Socio-economic status
SD	Standard deviation
SD	System dynamics
TJ	Tight junction
TAC	Total antioxidant capacity
TK	Toxicokinetics
TEM	Transmission electron microscopy
UV	Ultra violet
UPR	Unfolded protein response
USPSTF	U.S. Preventive Services Task Force
VMCUMN	Veterinary Medical Center, Universityof Minnesota
WB	Western blot
ZO-1	Zonula occludens-1

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PREFACE

Veterinary Toxicology refers to the study of environmental and food hazards that affect animals both domestic and wild.

This book is a useful source of information for the veterinary clinician, student, and toxicologist. The book has 8 sections. The book is divided into many sections, including sections on basic toxicological concepts (section 1), neurotoxicity (section 2), imunotoxicology (section 3), radiation and radioactive materials toxicity (Section 4), and toxicity of metals (section 5). Other sections cover pesticides, insecticides, rodenticides and herbicides (section 6); and environmental toxicology (section 7); The book ends with sections on diagnostic toxicology and therapeutics (section 8).

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Sections 1 discuss the general concepts in Veterinary science, like the one-health concept and veterinary pathogens; Translational and application of toxicology studies.

Section 2 concentrates on the study of Neurotoxicity, a form of toxicity in which a biological, chemical, or physical agent produces an adverse effect on the structure or function of the central and/or peripheral nervous system.

Section 3 is dedicated to Immunotoxicity related with nanoparticles. This section includes general chapters dedicated to nanoparticles of different materials, metallic nanoparticles, zebrafish as a model for nanoparticles toxicity and reduce quantity of nanoparticles to reduce toxicity.

Another important subject in veterinary toxicology is the one caused by radiation and radioactive materials (section 4), due to exposer during nuclear radiation accidents or due to radiation treatments.

Metals toxicity is the subject of section 5, which is separated in chapters that analyse different metals (zinc oxide, aluminium, arsenic, chromium, cadmium, copper, mercury, selerium and gold) in different animals.

Section 6 studies the toxicity of pesticides, insecticides, rodenticides and herbicides.

Environmemtal toxicology (section 7) which includes ecotoxicology is the next subject. Ecotoxicology is the study of the effects of toxic chemicals on biological organisms, especially at the population, community, ecosystem

and biosphere levels. Ecotoxicology is a multidisciplinary field, which integrates toxicology and ecology.

The last section (section 7) is about diagnostic and therapeutics.

The strengths of this book comprise the inclusion of large and small animal species; the broad range of topics encompassing regulatory, diagnostic, pathologic, and clinical toxicology.

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SECTION I: CONCEPTS IN VETERINARY TOXICOLOGY

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Chapter 1

A System Dynamics Approach to Understanding the One Health Concept

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ABSTRACT

There have been many terms used to describe the One Health concept, including movement, strategy, framework, agenda, approach, among others. However, the inter-relationships of the disciplines engaged in the One Health concept have not been well described. To identify and better elucidate the internal feedback mechanisms of One Health, we employed a system dynamics approach. First, a systematic literature review was conducted via

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searches in PubMed, Web of Knowledge, and ProQuest with the search terms: 'One Health' and (concept* or approach*). In addition, we used the HistCite® tool to add significant articles on One Health to the library. Then, of the 2368 articles identified, 19 were selected for evaluating the inter-relationships of disciplines engaged in One Health. Herein, we report a visually rich, theoretical model regarding interactions of various disciplines and complex problem descriptors engaged in One Health problem solving. This report provides a conceptual framework for future descriptions of the interdisciplinary engagements involved in One Health.

INTRODUCTION

The One Health concept is not new [1, 2] perhaps dating back to the late nineteenth century when medical observations were made that human and animal health were closely linked [3, 4]. Today the One Health concept has often been cited an effective approach to complex public health problems that involve multiple disciplines. Often human health, animal health and environmental health are examined as closely linked. Examples of such complex problems include emerging infectious diseases, food safety, and selection of antimicrobial resistant pathogens. In particular, emerging zoonotic disease problems are now remarkable common [5] and very complex. Controlling these complex problems often requires interdisciplinary approaches such as *One Health* [6, 7]. Despite the One Health concept's growing popularity and acceptance by the professional community, the definition of the term remains imprecise. For example, One Health has been variously defined as an initiative [8], a movement [9], a strategy [10], a framework [11], an agenda [12, 13], an approach, a collaborative effort, among others. As a milestone in the history of One Health, the symbolic 'umbrella', developed by One Health Sweden in cooperation with the One Health Initiative Autonomous Pro Bono Team, illustrates the broad One Health scope. According to the 'umbrella', the goal of One Heath is to promote a worldwide strategy for the well-being in all aspects of health of people, animals and the environment [2, 14]. As this definition is broad, many disciplines might contribute One Health efforts [15]. Yet, the internal relationships of these disciplines remain a bit amorphous. To better understanding the One Health concept, we sought to review the literature and study key One Health publications with a *system dynamics approach*, providing a systematic representation of the numerous components and how they interact.

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MATERIALS AND METHODS

System Dynamics

System dynamics (SD) is a methodology first developed by J.W. Forrester [16] that uses visual and mathematical modeling to simulate and understand complex system problems. Systems thinking is the notion that the system must have structure and the structure determines the system's patterns of behavior. As such, the patterns of behavior will lead to the system's output. Therefore, an understanding of system structure helps to better deliver the desired results. Or alternatively stated, thoroughly understanding a complex problem involves discovering the system's structure, which often involves feedback connections between interdependent components. *Qualitative system dynamics* is used to explain the system's internal feedback loops to make its relationships easier to understand. This approach has also been successfully used to enhance the development of health policies and programs [17, 18], enable modeling of health care systems [19], and guide chronic disease prevention and interventions [20–22].

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A system dynamics theoretical model can be constructed using the following series of steps:

- Step 1. **Problem Identification.** Make clear the problem you want to study.
- Step 2. **Behavior Analysis.** Carefully identify the elements or components of the system. Analyze the influence of the most crucial behavioral factors involved in the system, either positive or negative, and attempt to explain the cause of the identified problem.
- Step 3. **Model Hypothesis.** Propose a hypothesis explaining the results of the second step, and illustrate the model hypothesis in the form of a causal loop, showing the clear mathematical feedback relationships.
- Step 4. **Model Evaluation.** Evaluate the model and make sure that it reflects the behaviors and relationships presenting in the system. If it does not, revisit the steps above, else proceed to step 5.
- Step 5. **Conclusion and Discussion.** Draw conclusions from the system dynamics model.

Systematic Literature Search for One Health Articles

To identify scientific articles pertaining to the One Health concept, we performed searches in multiple abstract databases, including PubMed, Web of Science, and ProQuest, in February and March 2017. In PubMed the query string used was '("one health") AND (concept* OR approach*)'. In Web of Science, it was 'TS = ("one health") AND TS = ((concept*) OR approach*)' with the language restriction—English-language only. In ProQuest, we used 'all("one health") AND all(concept* OR approach*)' with the language restriction—English-language only and exclusion source types from newspapers and social media reports. All references were extracted into a reference manager—EndNote (version X7.4, Bld 8818) and screened independently by two researchers (TX and WL). This systematic review conforms to the PRISMA statement. Articles were included if they were peer-reviewed and met four additional criteria: 1) addressed the concept of 'One Health', not one health care plan, one health center, or other unrelated topics described by the words "one health"; 2) focused on the concept of One Health overall, not just briefly mentioned the term or only covered a particular practice of One Health; 3) were in the form of an article or review; 4) were written in English. In full test screening, the exclusion criteria were: 1) One Health practice in a particular disease or district; 2) One Health concept or approach mentioned in limited sentences.

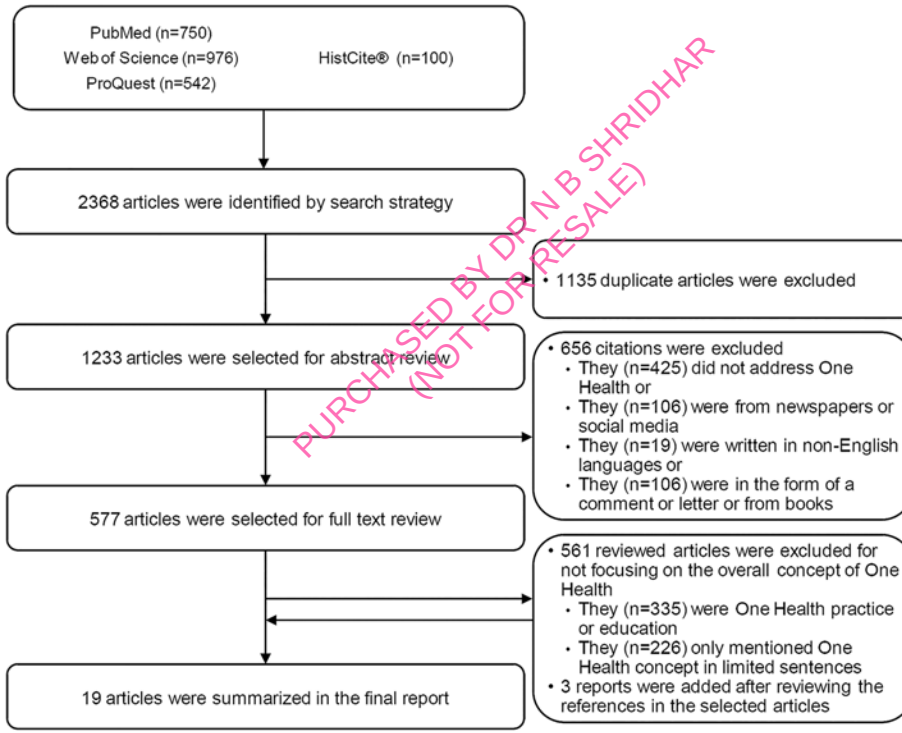
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To ensure that we did not omit important literature using the aforementioned search strategy, we also used the HistCite® tool to identify significant articles in One Health. HistCite® is a science tool that visualizes the citation relationships between papers to identify key publications in the results returned by a Web of Science search. We used the query string 'One Health'. Articles were ranked by each publication›s Local Citation Score (LCS), which describes how many times it has been cited by the other publications returned by the search. The results were analyzed with HistCite® software version 12.3.17 (Thomson Reuters, New York City, NY, USA). The default setting for searched articles in the HistCite® software is 30 but we increased this default number to 100 articles.

RESULTS

The initial search identified 2368 articles: 750 from PubMed, 976 from Web of Science, 542 form ProQuest, and 100 from the HistCite® analysis (Fig 1). One thousand and one hundred thirty-five duplicate reports were

manually excluded using reference management software. The abstracts of the remaining 1233 articles were reviewed. Of these, 550 articles were excluded for not addressing the concept of One Health, for originating from newspapers or social media platforms, or for being written in a language other than English. An addition, 106 articles were excluded for being comments or letters or from books. The full texts of the remaining 577 articles were reviewed, after which 561 reports were excluded for lacking a primary focus on One Health or the One Health concept. Three additional report were added during the full text review of articles. In total, 19 remaining publications were included in the subsequent analyses (Table 1).



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Figure 1: Flow diagram of the literature search process.

Using the search strategy, a total of 2368 articles were identified. Duplicate articles were removed. Nineteen publications were used in the final analyses. See text for more details.

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Table 1: Nineteen key One Health articles selected for component analyses

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Publications	Components
Cardiff et al, 2008 [24]	pathology, comparative pathology, genomic biology, food safety, emerging infectious disease
Frank, 2008[25]	veterinary medicine, food security, food safety, emerging infectious diseases, ecosystem protection, comparative medical, human physical/mental health, public health, human medicine, bio-engineering, animal science, environmental science, wildlife
Kahn et al, 2008[4]	veterinary medicine, food supply, intensive agricultural practices, exotic animals global trade, consumption of "bush meat", human population pressures, comparative medicine
Gibbs et al, 2009[9]	emerging disease, medicine and veterinary medicine, wildlife, public health, education, cross-species disease transmission, media publications
Monath et al, 2010 [10]	zoonotic agents, veterinary medical, laboratory animal research, public health, environmental health, biomedical,research, clinical medicine and surgery, biomedical research, ecosystem management, public health, food and agricultural systems safety, biosecurity
Coker et al, 2011[11]	public health, economic security, social stability, health service organisation, patterns and provision and access, fiscal systems, regulation and governance, information systems
Zinsstag et al, 2011 [26]	veterinary medicine, public health, animals and wildlife
Rabozzi et al, 2012 [27]	public health, clinicians, public health workers, veterinary medicine and veterinary public health officials
Zinsstag et al, 2012 [28]	zoonotic infections, livestock and wildlife, global public health, livestock production and wildlife conservation
Calistri et al, 2013 [29]	infectious diseases, globalization, animal products trade, wild animals, bush meat, zoonoses agents, public health policy, food-agricultura, veterinary medicine
Atlas, 2013[3]	infectious diseases, vaccination, epidemiology, medical education and clinical care, global travel and commerce, medical education and clinical care
Gibbs, 2013[7]	public health, biomedical research, global food safety and security, ecosystem health, caring for animals, public health, food safety, epidemiology, population medicine, foreign animal diseases
Bidaisee et al, 2014 [30]	zoonoses, food safety, agriculture, infectious diseases, livestock into wildlife, scientific and policy challenges, the social, cultural, political norms, veterinary medicine, public health, clinical practice
Evans et al, 2014[6]	epidemiological globalisation, pathogen adaptation, changing human demographics, evolving animal production systems, climate change, water, pollution and environmental contaminants, food safety, food sufficiency and insecurity, the universal global condition of rapid environmental change, new drugs, biodiversity, epidemics, pests, food security, economic prosperity
Gibbs, 2014[2]	veterinary profession, medical profession, wildlife specialists, environmentalists, health policy analysts, social scientists, humanities scholars
Stephen et al, 2014 [31]	infectious disease, veterinary, medical and environment sectors, wildlife, animal welfare, food safety and security, drugs and vaccines, public health, training and research
Lerner et al, 2015 [15]	infection biology, contagious diseases, zoonotic infections, evolutionary medicine, comparative medicine, translational medicine, biology, human medicine, veterinary medicine, public health, environmental chemistry, health economy, zoonotic diseases, epidemics and toxicants, food-producing animals, pet ownership, education
Stadtlander, 2015 [32]	medicine, veterinary medicine, microbiology, public health, biogeography, ecology, environmental, biology
Kingsley, 2016[33]	parasitologists, biosecurity, infection control risks, zoonotic infections, human and veterinary medicine, food security, healthy diets, climate change

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Problem Identification (Step 1)

From the articles identified by the literature review, it was difficult to determine a generally accepted definition for the One Health concept, including the scope and breadth of its practice. This lack of consensus supported the value of our system dynamics theoretical model in developing a conceptual framework to better understand One Health. This concept of One Health served as the root or first level of the theoretical model.

Behavior Analysis (Step 2)

All 19 analyzed reports agreed that the One Health concept contained at least three primary domains—human, animal, and environment/ecosystem. These three domains were added as the second level of the theoretical model. However, the articles markedly differed regarding their description of the scope of One Health. This discrepancy is likely due to the ongoing rapid development of One Health and the addition of new disciplines that are embracing it as a way forward [23]. Table 1 summarizes the various and diverse disciplines or scopes mentioned in each of the 19 publications, which highlights the broad interpretation that could result in defining the One Health concept. These disciplines and scopes were included as the third level of the theoretical model.

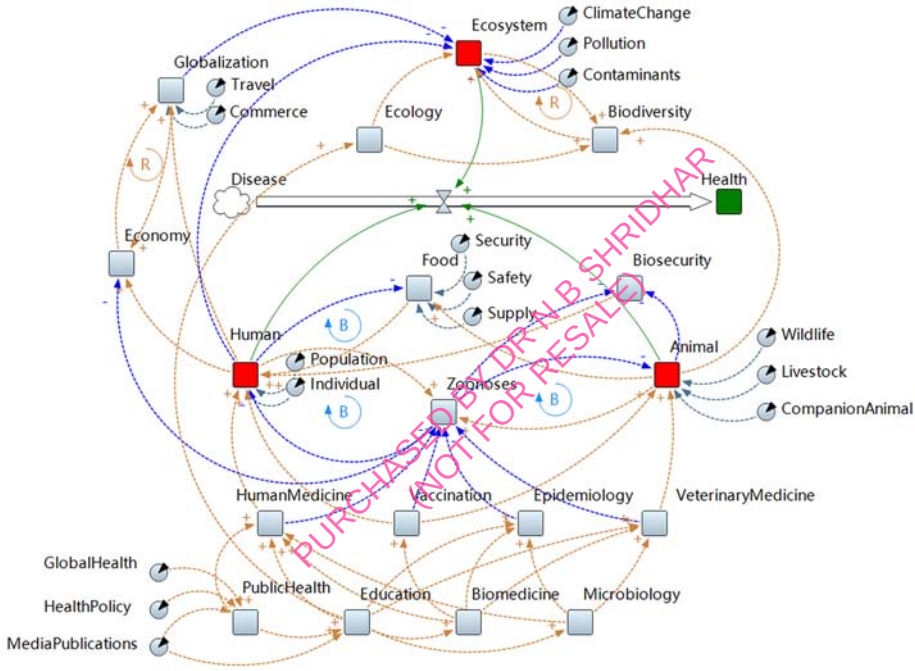
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Model Hypothesis (Step 3)

In the theoretical model, the direction of influence is indicated by the polarity of a causal loop, represented by connecting arrows, which may be positive ("+") or negative ("-"). A positive causal link indicates that both the causative and the resultant factors increase or decrease in the same direction. A negative causal link indicates that the two linked factors change in opposite directions. Additionally, should two or more elements in the model have a reinforcing or non-reinforcing relationship, the model can be annotated such that these associations can be visualized. This was done by placing a "R" within a clockwise cycling arrow between elements that did have a reinforcing relationship, and a "B" for those elements that did not.

In the first level of our model, we demonstrate the connection between disease with health using an arrow that shows the direction of this relationship. We then added the human, animal, and ecosystem domains (second level) to the model. These terms are represented by the red squares which are used to denote stock elements. Next, the third level elements were added, denoted by squares with grey color, which were also derived from the

reviewed articles. Then, variable elements associated with each of the three One Health domains were added, denoted by circular shapes. These variable elements are assigned as descriptive components of the stocks. Last, causal links as previously described, were drawn between the elements. This model (we have learned the "One Health Cosmos", Fig 2) was constructed using AnyLogic®PLE software, version 7.3.4 (AnyLogic North America, LLC, Chicago, IL, USA).



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Figure 2: "One Health Cosmos".

One Health Cosmos shows the relationships between the various disciplines and complex problem descriptors that are reported to fall within the One Health concept. Squares and circles represent nodes, and the arrows connecting nodes represent causal links. Brown color is used to show positive causal link which also have a "+" sign besides the arrowhead. Negative causal link is portrayed with a blue color and "-" sign besides the arrowhead. A positive causal link means that both the causative and the resultant factors increase or decrease in the same direction. A negative causal link indicates that the two linked factors change in opposite directions. The positive reinforcing loop has a "R" in the clockwise cycle. A negative reinforcing loop opposite has a "B" in the counterclockwise cycle. A big arrow shows

the direction of this relationship between disease and health through One Health.

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Model Evaluation (Step 4)

Our analysis focused upon peer-reviewed articles specifically discussing the One Health concept, which were selected as described by the search criteria. Some articles were excluded because they were not focused on the overall concept of One Health, just briefly mentioned the concept, or only covered a particular practice of One Health (exclusion criteria). While these papers may have been excluded, it is important to note that many of the specific topics they discuss, such as zoonoses and food safety, in the context of One Health practice, are still accounted for in our model. Thus, the model likely benefits from the removal of those reports so that the conceptual framework is centered on more specific interpretations of the One Health concept.

A term that has been used synonymously to One Health is comparative/translational medicine, which in fact was used before the One Health term was introduced [2, 3, 6, 25]. However, comparative/translational medicine was not used as a search term in this model given that the One Health concept has instead been more readily accepted as the more common term to use. Furthermore, compared to comparative/translational medicine, One Health has a broader application in scope [15].

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DISCUSSION

From our analysis of the One Health literature and through the construction of the One Health Cosmos using a system dynamics theoretical model, we were able to derive multiple inferences.

Our results make it clear that One Health represents a wide-ranging synergistic field that is rapidly growing. This is reflected in the number of One Health articles that have been published in the past 25 years, which has seen an average publication increase of 14.6% per year during this time [23]. As a result, the interpretation of the One Health concept remains unclear because its internal relationships between this growing list of various components have not been systematically described.

Our model indicates that humans and animals are closely connected by zoonoses and the food supply chain [34, 35]. Some common zoonoses

include avian influenza [36, 37], leishmaniasis [38], Middle East respiratory syndrome coronavirus prevention [39], rabies [40, 41] etc. Additionally, the rising demand for animal protein for human consumption, resulting in a shift towards larger, more complex animal production operations, has spurred the increased use of antimicrobials. In fact, experts estimate that from 2010 and 2030, the global consumption of antimicrobials will increase by 67% [42]. As such, investing more resources into the research and control of zoonotic diseases and complex food production systems could be an effective strategy for improving the overall health of humans and animals.

Our model also shows that zoonoses are closely associated with the economy, and can result in extensive economic losses when large zoonotic diseases outbreaks among humans and animals occur. For example, after the 2014 outbreak of Ebola virus, it was estimated that the affected countries of Guinea, Liberia, and Sierra Leone alone lost $2.2 billion in gross domestic product (GDP) the following year [43]. Additionally, the 2014 highly pathogenic avian influenza (HPAI) H5N2 outbreak in the United States, resulted in an estimated economic loss of $3.3 billion [44]. Hence, given the strength of association between zoonoses and the economy as demonstrate by the model, investing in prevention measures could result in an overall cost savings as new potential outbreaks are avoided [45].

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Also, the health of companion animals is a growing concern. Pet keeping is a traditional custom for Western countries and becoming a new trend for non-Western countries. Pets could also bring disease to pet owners or non-pet owners who lives in close with them [46], such as echinococcosis, toxocarosis [47–49]. The One Health concept is required for a good understanding of the parasites' biology and epidemiology and the education of pet owners.

We also found that education plays a foundational role in the development of the One Health concept. One Health education can be divided into education of those already working in the relevant professional disciplines, and of students seeking professional qualifications to enter one of these disciplines [2]. As One Health continues to be accepted as a way forward, there will be an increasing need to integrate the One Health concept into related disciplines' education [50, 51]. Our model currently links education to epidemiology, human medicine, veterinary medicine, ecology, microbiology, and biomedicine. However, the depth of current education in One Health is unknown. Seems likely that education is not standardized across disciplines.

This report has a number of limitations. Despite our comprehensive approach we likely missed some important One Health publications in the literature review. Likewise, we may have failed to identify some important factors or components engaged in One Health activities.

To date, our analyses is the first to apply a system dynamics approach to understanding the One Health concept. Such an approach is useful in further elucidating the complex connections and causal factors that currently comprise One Health. By showing the inter-relationships between the various components of One Health, offering a visual example of the conceptual framework, the One Health Cosmos can help facilitate a more concise interpretation of the One Health concept for the development of future research and training programs.

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Chapter 2

Translational Toxicology: A Developmental Focus For Integrated Research Strategies

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ABSTRACT

Background

Given that toxicology studies the potential adverse effects of environmental exposures on various forms of life and that clinical toxicology typically focuses on human health effects, what can and should the relatively new term of "translational toxicology" be taken to mean?

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DISCUSSION

Our assertion is that the core concept of translational toxicology must incorporate existing principles of toxicology and epidemiology, but be driven by the aim of developing safe and effective interventions beyond simple reduction or avoidance of exposure to prevent, mitigate or reverse adverse human health effects of exposures. The field of toxicology has now reached a point where advances in multiple areas of biomedical research and information technologies empower us to make fundamental transitions in directly impacting human health. Translational toxicology must encompass four action elements as follows:

- Assessing human exposures in critical windows across the lifespan
- Defining modes of action and relevance of data from animal models
- Use of mathematical models to develop plausible predictions as the basis for
- Protective and restorative human health interventions.

The discussion focuses on the critical window of *in-utero* development.

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SUMMARY

Exposure assessment, basic toxicology and development of certain categories of mathematical models are not new areas of research; however overtly integrating these in order to conceive, assess and validate effective interventions to mitigate or reverse adverse effects of environmental exposures is our novel opportunity. This is what we should do in translational toxicology so that we have a portfolio of interventional options to improve human health that include both minimizing exposures and specific preventative/restorative/mitigative therapeutics.

Keywords
Translational toxicology, Environmental health, Biomarkers

BACKGROUND

In the current genomics era, gene-environment interactions provide the key to understanding health and disease. Human exposures to chemicals, physical agents and social factors are inevitable and determining what their subtle

biological effect means to human disease is difficult. Absence of evidence of (effect or harm) is not evidence of absence (of effect or harm), particularly if adverse outcomes are ecologically apparent in environmental settings or in experimental animals following known exposures. To minimize adverse health outcomes, regulators, commercial entities, health care providers and the general public need to know what exposures pose a significant risk that merits intervention(s) to either reduce exposure or mitigate effects.

Multiple factors in the environment impact human development and subsequent health and disease. It has long been appreciated that vitamins, minerals and other essential nutrients play an important role in fetal growth and development. For example, the essential role of folate (folic acid) in pregnancy and human development is well known. But apart from a modest number of other well-known deficiencies, there is still limited understanding of the impact of micronutrient deficiencies or inadequacies on the fetus and subsequent long-term health outcomes [1]. Additionally, several socioeconomic and societal factors are known to have important effects. An unfolding example is the effect of maternal stress on pregnancy outcome which is still being elucidated.

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The appreciation of maternal stress and other aspects of the social environment on the fetus has only recently gained some mechanistic explanation. Animal research suggesting transgenerational epigenetic programming of genes (maternal imprinting) has been seen as an attractive and plausible explanation. Recent reports in humans and nonhuman primates show that epigenetic effects may be sufficient explanations for such social effects. Radtke et al. [2] analyzed the methylation status of ten promoter sites for the glucocorticoid receptor (GR) gene in mothers and their children 10 to 19 years after birth. They paired those findings with the retrospective assessment of maternal exposure to intimate partner violence. Although the mother's GR gene was not affected by that profile of intimate partner violence, the methylation status of the GR gene promoters of the adolescent children was influenced by their mother's experience during pregnancy. If maternal exposure to intimate partner violence occurred during pregnancy, methylation of GR gene promoters was increased three-fold in the offspring [2]. In a broader profile of differential methylation of adult DNA, Borghol et al. [3] found that disadvantaged socioeconomic position in childhood was reflected in a change in the level of methylation for over 1200 gene promoters. While such a broad pattern of molecular effect does not explicitly link a disadvantaged background to the well-known adult mortality and morbidity risks, the findings certainly sustain the view that this is a

biologically plausible mechanism. In nonhuman primates, Tunga et al. [4] used the social status (dominance rank) of female macaques to demonstrate that dominance rank imposed a widespread imprint on gene regulation such that gene expression data could (in reverse) predict social status with 80% accuracy. These remarkable correlations between methylation patterns and dominance rank strongly suggest that epigenetic molecular marks are a key response to social status effects during development. Taken together these human and nonhuman primate studies imply a distinct plausible association between social influence and individual physiology at a molecular level.

DISCUSSION

Transformation of Human Toxicology into a Translational Discipline: the Translational Gap

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At present there is still a critical gap between laboratory toxicology research and testing (i.e., molecular, cellular and pathological outcomes in cell systems and animal models) and epidemiological and ecosystem studies, which examine exposed populations for disease. The current bipartite approach fails to make adequately overt the link between exposure(s) and clinical outcome(s). In fact, most such claims of cause-and-effect are no better than a tentative association of exposure and effect. So we are typically left with both, a) inadequate evidence of a plausible explicit risk due to an exposure, and b) inadequate evidence that there is no plausible explicit risk due to an exposure and therefore limited intervention possibilities. It is our objective to discuss current knowledge from research toxicology and epidemiological studies on the occurrence of adverse events triggered during critical life-span periods focusing on *in-utero* development and how to plausibly mitigate adverse health effects of these exposures particularly in terms of endocrine/metabolic, neurodevelopment and reproductive consequences across the ensuing lifespan.

The public health imperative of endocrine and metabolic disorders

If chemical exposures and stress influence the occurrence of endocrine mediated diseases, what is the potential public health impact? Golden [5] comprehensively reviewed the clinical and public health significance of 54 endocrine and metabolic disorders in the US, and the importance of any early

in-life events that might impact subsequent endocrine or metabolic function associated with these disorders. Disorders with at least 5% prevalence among adults included diabetes mellitus, impaired fasting glucose, impaired glucose tolerance, obesity, metabolic syndrome, osteoporosis, osteopenia, mild-moderate hypovitaminosis D, erectile dysfunction, dyslipidemia, and thyroiditis. Even if the less common endocrinopathies are not considered, the high prevalence and incidence of common endocrine and metabolic disorders demonstrates the profound adverse impact of endocrine diseases on longevity, quality-of-life, public health and health care utilization.

Endocrine and metabolic disorders: childhood obesity As the world has moved from agrarian, to industrial, then information ages, the level of sedentary activities open to humans have increased exponentially, which has contributed to a global rise in obesity rates, affecting both low and high income countries, as well as creating wide variations within countries. In high income countries, the poor population is most affected while in low income countries, the most affected are the middle class [6]. This rise in obesity within populations forecasts an increased burden from several diseases, most notably cardiovascular diseases, diabetes, and cancers. Wang et al. [7] used a simulation model to project an increase of 65 million more obese adults in the US by 2030, coupled with in obesity-related medical costs reaching as high as $66 billion per year.

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Obesity seems to activate disease by acting through sympathetic nervous activation. While the mechanisms responsible for initiating the pathway remain to be unequivocally elucidated, hyperinsulinemia, obstructive sleep apnea, increased circulating cytokines, stress and beta adrenergic receptor polymorphism are implicated in the causal pathway to diabetes and most of the other obesity related diseases [8]. For example, Chouchane et al. [9] found expression of TNF-alpha and stress protein (hsp70-2) associated with obesity and that these, and other endocrine disruptors, are most likely activated along the pathway leading to disease; thus they are potential targets for intervention. While diet and exercise is still an important therapy, the fact that these two simple interventions have not driven down the rising wave of obesity means that additional interventions are needed. Pharmacological interventions targeting the sympathetic nervous system, either directly or indirectly, may reduce disease outcomes and be useful along with improved nutrition and increased exercise.

As childhood obesity has steadily increased, legitimate public health concerns have been broadly raised. While there is great concern, there also is uncertainty as to the long-term health impact of this reality. On one hand,

Birch et al. [10] suggest that the common notion that children will, "grow out of obesity," is not true and that critical environmental factors that affect children's development and obesity risks in their early years should be modified in order to beneficially influence health and well-being throughout life. On the other hand, there remains a significant challenge to document whether childhood obesity is or is not an independent risk factor for adult metabolic syndrome, type II diabetes, or biomarkers of the same (blood lipid status, insulin levels, etc.) [11].

Childhood obesity and *In-utero* environment Over the last 20 years, childhood obesity has dramatically increased globally with accompanying increases in childhood illnesses associated with obesity. Childhood obesity leads to increased use of hospital services for several obesity-related conditions when compared to normal weight children [12]. More than a decade ago, Wang and Deitz [13] estimated that the cost associated with hospitalization for children due to obesity associated diseases at around 127 million for the two year period ranging from 1997 to 1999. Although obesity rates seem to have stabilized among females 2 to 19 years since 1999, it is still rising for males [14]. The associated health costs will continue to go up because the effects of obesity are cumulative causing more diseases and ill-health over time if not reversed. There is evidence that adult obesity is influenced by environmental factors and the same is probably true for childhood obesity, including prenatal and early postnatal environment. Salois [15] describes an association between the built environment (i.e., measures relating to food choice and physical activity) and prevalence of obesity in low income preschool children. There is an interaction between modern sedentary lifestyles, nutrition and *in utero* and early post-natal environments that influence the probability of childhood obesity. The *in utero* influences are likely the result of sympathetic nervous system activation in mothers leading to disruption of various endocrine pathways in the developing fetus. The *in utero* environment that triggers childhood obesity is not well understood. Various sympathomimetic compounds may be disrupting the *in-utero*environment and causing receptor changes that interact with the postnatal environment to produce phenotypic obesity. Such changes possibly could be detected in amniotic fluid. Possible sympathomimetic candidate compounds include environmental phenols, fungicides, pesticides and their metabolites, perfluorochemicals, phthalate metabolites and some metals.

Childhood obesity and nutritional or metabolic imprinting Epidemiological studies in human and animal experiments have shown that nutrition during fetal and neonatal life may lead to obesity and its related

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disorders in adulthood. There are no adequate models to reproduce human situations. For example, milk formula taken by infants during neonatal life may contain trace compounds of sympathomimetics that may produce subtle changes in receptor activation. Low birth weight is considered the result of an adverse *in utero* growth trajectory and is often associated with later metabolic diseases in adult age. The hypothalamic-pituitary-adrenal axis (HPAA) and growth hormone/insulin-like growth factor 1 (GH/IGF) axis may have a crucial role in the regulation induced by nutritional programming. The persistent alterations seem to be a consequence, at least in part, of elevated insulin levels during "critical periods" of pre- and early postnatal development. Likewise, leptin seems to play an important role in this complex system. New knowledge about these mechanisms suggests the development of new, rational, and effective preventive and/or therapeutic options before and/or after birth. Thus, early infancy may provide an opportunity for intervention aimed at reducing later disease risk [16].

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Effects of drugs and environmental chemicals on the reproductive system

Clearly, drugs and environmental chemicals can adversely affect the reproductive system. Available data indicate that the consequences of exposure depend on the nature of the chemical, the dose of the chemical, its target, and the timing of exposure relative to critical windows in development of the reproductive system [17]. Toxicant exposures may affect critical events in reproductive development, ranging from early primordial germ cell determination to gonadal differentiation, gametogenesis, external genitalia, or signaling events regulating sexual behavior. Although there are differences between the human reproductive system and that of laboratory animal models, such models have been extremely useful in assessing risks for key human reproductive and developmental processes. The objectives for future studies should include the elucidation of the specific cellular and molecular targets of known toxicants; the design of a systematic approach to the identification of reproductive toxicants; and the development of sensitive, specific, and predictive animal models, minimally invasive surrogate markers, and/or *in vitro* tests to assess reproductive system function during embryonic, postnatal, and adult life. Integrated testing strategies will be required to account for the many mechanisms associated with reproductive development that occur *in vivo*. Evidence from animal models and human studies indicates that many specific events can be perturbed by a variety of toxicants, with endocrine-mediated mechanisms being the more widely

studied. Prioritized research needs include basic studies on the cellular-molecular and endocrine regulation using expanded animal studies on different classes of chemicals, comparing responses during development (prenatal and postnatal) with responses in adults; and, more extensive explorations regarding the reproductive biology and toxicology of puberty in humans [18].

Folic acid and autism Autism is a heterogeneous group of complex developmental disabilities that result from a number of possible etiologies. There is some debate about the increasing prevalence of autism and whether diagnosis of autism is increasing due to changing definitions and parents influencing the medical system for autism diagnosis for their children to access treatment for other ailments [19]. The 2010 prevalence estimate from meta-analyses of prevalence studies, 25 per 10,000, is up from the 1970 estimate of 1 per 10,000 [19]. Diagnostic and Statistical Manual of Mental Disorders (DSM) II was in use in 1970 and had different criteria for diagnosing autism from DSM IV-TR® in use in 2010. Diagnosis of autism is complicated because patients often have other co-morbidities such as seizures, GI problems, sleep disorders and attention deficit disorders. The causes of autism are still under active investigation with genetics, *in utero* exposure to antiepileptic drugs (valproate) [20] and GI bacteria being actively investigated as potential causes [21]. *In utero* hormonal disturbances are also indicted in causation of autism [22] and this is thought to explain the higher diagnosis of autism in males. High socio-economic status (SES) has consistently been found to be associated with autism but this could be due to under diagnoses in families with low SES [23]. Wang and Leslie [24] found that total expenditure for autism per child rose by 3.1% to $22,772 between 2000 and 2003 using Medicaid data from 42 states.

In summary, developmental exposure to some environmental chemicals appear to directly adversely affect neurodevelopment while other exposures that increase the incidence of metabolic conditions in mothers may be considered as an indirect cause as well. A recent publication indicates folic acid supplementation by mothers around conception and during pregnancy reduces risk of autism in children [25].

Use of biomarkers as endpoints

Numerous outcomes can be measured in human clinical trials. Particularly in therapeutic clinical trials (such as studies of pharmaceutical agents), investigators have the luxury of designing the studies to permit measurement

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of disease outcomes that would irrefutably demonstrate efficacy or lack of efficacy of the study drug. Hand-in-hand, alternative outcomes can be measured which are plausibly linked to the disease outcomes but may or may not be reliably associated with the ultimate outcome of interest. This conundrum has led to a classification of study outcomes (endpoints) that aids in the interpretation of study results. The National Institutes of Health have recommended definitions of outcome measures and these were nicely summarized by Grimes et al. [26] (See Table 1).

Table 1: National Institutes of Health–recommended definitions of outcome measures[26]

Outcome	Definition
Clinical end point	"A characteristic or variable that reflects how a patient feels, functions, or survives."
Surrogate end point	"A biomarker that is intended to substitute for a clinical end point. A surrogate end point is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence."
Biological marker (biomarker)	"A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."

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In our effort to develop a comprehensive approach to translational toxicology, we cannot anticipate relying on clinical endpoints that occur as a result of environmental exposures as the primary sensor for determining cause and effect relationships. Application of the precautionary principle has a strong ethical basis when the exposures of interest are unintended or unavoidable as occurs with numerous environmental health concerns. A study subject can give informed consent to take a study drug but individuals in an exposed population have given no such consent. Therefore in translational toxicology studies we can often expect to necessarily rely on surrogate endpoints or even biomarkers to drive decision-making and interventions.

The spectrum of potential biomarkers that may have utility in translational toxicology research is too broad to list. Beyond chemical and molecular markers such as clinical chemistry and hematology profiles, hormone levels [27], a multitude of imaging techniques (radiology images, ultrasound, CT, MRI, PET, etc.) can also be used. Histopathology remains a key component in assessing target tissue responses to exposures and new "-omics"

technologies such as transcriptomics, proteomics and metabolomics as well as next generation sequencing for mutations present data sets that can serve as biomarker outcomes in various toxicologic investigations across the lifespan. For example, metabolomics have been applied to human embryonic stem cell test systems to begin identifying biomarkers of developmental toxicity [28]. Global metabolite profiling (untargeted metabolomics) allows the measurement of thousands of compounds in minimal amounts of biological material [29] and this technology can be applied broadly to any number of toxicity research inquires. These same "old" and "new" classes of biomarkers should be useful tools as we strive to assess the impact of interventions that may be implemented to mitigate the effects of prior or ongoing toxicant exposure.

Within the realm of genomics and RNA signaling, novel opportunities continue to arise. For example micro RNAs that are known to be key regulators of metabolism have been shown to circulate and therefore are available in accessible human specimens (blood) to quantify as either signaling molecules or disease markers [30]. Additionally, at the interface of molecular and cellular biomarkers, the recent report by Kim et al. [31] that the low levels of leukocyte mitochondrial DNA copy number are reduced in postmenopausal women with metabolic syndrome suggests that overt links between molecular markers, cellular markers, target tissue function and disease outcomes can be explicitly linked.

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Key developmental periods for interventions

Key intervals in the lifespan that may be taken as Windows of Susceptibility (of adverse effects due to an exposure) are also likely to be the key intervals for intervention (Windows of Responsivity) to minimize, negate or reverse antecedent or ongoing exposure effects. Those key intervals of dynamic biological change during the lifespan that may be impacted by environmental exposures are:

- early development [pre-conception, *in utero*, neonatal, early childhood]
- peri-pubertal, and
- albeit in women only, the menopausal transition.

Effects of exposures can be one or more of the following:

- Create a new syndrome
- Recreate a known "old" syndrome

- Increase the prevalence or severity of some extant disease in an exposed population
- Exposure effects can interact with pre-existing unrecognized (such as common under-diagnosed) diseases, deficiencies, and other stressors to exacerbate later disease outcomes, either prevalence or severity.

There is also the possibility of unintended side-effects of interventions during the windows of susceptibility and this has contributed to reluctance to intervene during these periods.

Early development More than a decade ago, the U.S. Environmental Protection Agency convened a conference that addressed the concept of critical windows during development (preconception through puberty) when exposure to xenobiotics may have the greatest adverse impact on subsequent health. The potential impact of developmental exposures on reproductive health later in life [17, 32] was judged to be especially vulnerable due to the multitude of potential cellular and molecular targets, the time dependency of essential developmental events and the numerous modes of toxicant action that can be anticipated. Key events occur within gametes before fertilization, across embryonic and fetal life, with additional maturational events occurring in the perinatal period, and at puberty. Complex genomic, hormonal, metabolic and growth signals are involved at all stages. The workshop group advised that future studies should include the elucidation of the specific cellular and molecular targets of known toxicants; the design of a systematic approach to the identification of reproductive toxicants; and the development of sensitive, specific, and predictive animal models, minimally invasive surrogate markers, or *in vitro* tests to assess reproductive system function during embryonic, postnatal, and adult life. Additionally, the panel emphasized that maternal exposures or stressors that needed to be further assessed included the following:

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- Psychosocial
- Nutritional
- Chemical exposures
- Infectious disease exposures
- Inter-current diseases (in mother)
- Concomitant medications
- Maternal body composition (obesity/fitness).

All of these themes persist today as important and inadequately defined

areas of concern. As a single illustration, there is a strengthening view that several developmental exposures are causally associated with obesity later in life. Recent and ongoing epidemiological and animal studies have shown that up to 20 chemical endocrine disrupters are potential causes of obesity in children and adults if exposure occurs in early development [33]. Stress on the mother and nutrition of the mother have also been established to alter endocrine receptors and lead to obesity in children [9, 16] from epidemiological and animal studies.

There is a need for further investigation to understand where effective interventions could be applied such as targeted nutritional guidance or active ways to reduce exposures of concern including possible prescription interventions for mother or child.

Peri-pubertal Given some recent studies showing that interventions in late adolescent/early pubertal children for risk of metabolic syndrome may have greater benefit than somewhat later interventions, the peri-pubertal interval may be a strong opportunity to show that environmental health impacts can be actively treated/mitigated/managed. A key concept is that the intrauterine fetal environment has effects that manifest in the late adolescent or pubertal interval.

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***In utero* endocrinology and effects in adolescence** There is evidence in animals and humans that metabolic syndrome, insulin resistance, obesity etc. as well as female reproduction can be impacted by endogenous intrauterine endocrine or metabolic exposures. Since a number of animal studies had shown that exposure to intrauterine hyperglycemia appeared to increase risk factors for cardiovascular disease, Clausen et al. [34] studied the risk of overweight and metabolic syndrome in adult offspring of women who had diet-treated gestational diabetes or type I diabetes. These investigators assessed over 1000 women and were able to show that adult offspring of mothers with diet-treated gestational diabetes or type I diabetes have an increased risk of overweight and metabolic syndrome. These findings raise the question as to whether caloric restriction during gestation might reduce the risk of these adverse metabolic outcomes for the offspring. A recent study in rats [35], in which moderate caloric restriction of lactating rats was shown to protect from obesity development in the offspring as well as other related metabolic alterations, suggests that such a protective effect in humans is at least plausible.

While there has been extensive consideration of potential effects of exogenous sex hormone-like compounds on later reproductive potential

of female offspring, less attention has been given to the potential effects of endogenous sex steroids during the intrauterine interval. Hart et al. [36] have addressed this question by assessing the impact of maternal circulating androgen levels during normal pregnancy on ovarian function, as determined by early follicular phase antimullerian hormone (AMH) levels, inhibin B levels, and antral follicle count (AFC) in 244 female offspring in adolescence. These investigators found that maternal circulating total testosterone levels at 18 weeks gestation were statistically significantly correlated with early follicular-phase circulating AMH levels in female adolescent offspring, but no other statistically significant correlations were determined among the maternal androgens and the markers of adolescent ovarian function at 18 or 34 weeks of gestation. Clearly such potential relationships between the intrauterine endocrine environment and the subsequent markers of reproductive competency in the peri-pubertal interval deserve further inquiry.

Menopausal transition The decade approximating age 40 to 50 years involves dynamic changes in reproductive endocrine functions and is temporally something of a «last chance» to preempt a number of risk profiles that influence diseases that become progressively more prominent in women after age 50–60. There is a moderate amount of attention now to endocrine, nutrition and lifestyle aspects of this life-stage, but virtually no attention is being given to the potential interaction of environmental chemical exposures with those themes. The menopausal transition results in changes in hormonal profile and changes in FSH, Inhibin B and AMH hormone have been noted; disruption of this intricate process may lead to abnormalities that could generate into disease [27, 37].

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Documented human exposures from biomonitoring programs: what compounds/classes might be implicated?

Chemical exposures of pregnant women: drugs, prescription and OTC

The Food and Drug Administration (FDA) classifies drugs according to the degree of risk they pose for the fetus if they are used during pregnancy [38] (see Table 2).

Table 2: Categories of risk for drugs during pregnancy [38]

Category	Description
A	These drugs are the safest. Well-designed studies in people show no risks to the fetus.

B	Studies in animals show no risk to the fetus, and no well-designed studies in people have been done.
	or
	Studies in animals show a risk to the fetus, but well-designed studies in people do not.
C	No adequate studies in animals or people have been done.
D	Evidence shows a risk to the human fetus, but benefits of the drug may outweigh risks in certain situations. For example, the mother may have a life-threatening disorder or a serious disorder that cannot be treated with safer drugs.
X	Risk to the fetus has been proved to outweigh any possible benefit.

As a general matter, obstetrical care of pregnant women is precautionary and gravidas are discouraged from using prescription pharmaceuticals and over-the-counter (OTC) drugs to minimize exposure of the embryo or fetus to these classes of exogenous chemicals. Reality dictates that most women do in fact use one or more drugs during a pregnancy. More than 90% of pregnant women take prescription or nonprescription (over-the-counter) drugs or use social drugs (such as tobacco and alcohol) or illicit drugs at some time during pregnancy [38]. For the most part regarding this multitude of exposures, data are inadequate to support either our fearful assumption of fetal risk due to such exposures or to tacitly or explicitly endorse selective usage of drugs by gravidas. Given that common and presumptively safe drugs are also the ones frequently used by gravidas, some publications do document some of these *in utero* exposures and their potential fetal effects.

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Acetaminophen (APAP) is one of the most frequently used drugs among pregnant women and is also the most common drug overdose in pregnancy [39]. Nonetheless, data regarding APAP overdose in pregnancy is limited and consists mainly of case reports or a small series of cases and there is no known threshold for occurrence of toxic symptoms. APAP crosses the placenta and high doses are known to be hepatotoxic. Hepatic metabolites may cause fetal hepatic necrosis. Because N-acetylcysteine (NAC) also crosses the placenta, some data suggest that the majority of morbidity and mortality from APAP overdose can be averted by initiation of NAC within the first 16 hours of ingestion and possibly even later. It appears that NAC does not itself pose any demonstrated risk to the fetus, so the standard-of-care would be to administer as early as possible after APAP overdosage in pregnancy. Since the vast majority of usages of APAP are not overdosages, it has been important to determine if usage in proper dosages is or is not associated with an increased prevalence of congenital abnormalities and to

determine a threshold for toxicity. Rebordosa et al. [40] assessed >88,000 pregnant women and their liveborn singletons from the Danish National Birth Cohort who had information on APAP use during the first trimester of pregnancy and 4.3% of children from the cohort were diagnosed with congenital abnormalities. Children exposed to APAP during the first trimester of pregnancy (n > 26,000) did not have an increased prevalence of congenital abnormalities compared with nonexposed children (n > 61,000).

In another study, Snijder et al. [41] examined the influence of maternal exposure to mild analgesics during pregnancy on the occurrence of cryptorchidism and hypospadia in >3000 offspring in the Netherlands Generation R [birth cohort] Study. Cryptorchidism and hypospadia were identified during routine screening assessments performed in child health care centers by trained physicians. The use of mild analgesics was assessed in three prenatal questionnaires in pregnancy, resulting in four periods of use, namely, periconception period, first 14 weeks of gestation, 14–22 weeks of gestation and 20–32 weeks of gestation. The prevalence was 2.1% for cryptorchidism and 0.7% for hypospadia. Use of mild analgesics in the second period of pregnancy (14–22 weeks) increased the risk of congenital cryptorchidism [with adjusted odds ratios (OR) of approximately 2] primarily due to the use of APAP. The results suggest that intrauterine exposure to mild analgesics, primarily APAP, during the period in pregnancy when male sexual differentiation takes place, increases the risk of cryptorchidism.

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In summary, even for this extraordinarily common drug (APAP), we have data that makes advice to a pregnant woman rather equivocal. Namely, we do not think there is a risk of major congenital abnormalities if she were to use APAP during pregnancy but there is a doubling of risk of at least one congenital abnormality for male infants if she uses APAP in mid-gestation. If APAP is something of a "best case scenario" as regards having known developmental exposures and known clinical outcomes for the infants, the challenge to address all other relevant exposures (drugs, environmental and dietary chemicals, etc.) is indeed immense.

Arguably OTC drug usage could be deemed inessential, but some use of prescription drugs by pregnant women is clinically-compelling or truly unavoidable to assure her life and health. A prime example is the use of antiepileptic drugs (AEDs) [42]. For women with seizure disorders, many questions must be addressed even prior to conception. To avoid injury or death for the mother and the fetus, it may be impossible to stop or change the AED regimen during pregnancy. In some few cases an AED can be

discontinued prior to conception, but this requires attentive and expert medical care. Most women with seizure disorders will require continued use of their AEDs before, during and after pregnancy. In practice, the aim is to prescribe the most effective AED while minimizing risks, and avoiding polytherapy and valproate if possible. Additionally, the dosing regimens will often require changes because drug metabolism of AEDs varies across pregnancy. Finally, the fetus faces risks due to potential effects of the AEDs, but also from the exposure to the concurrent maternal medical condition *per se* (her seizure disorder).

Another category of clinically-compelling prescription drug exposure *in utero*is antipsychotics. Antipsychotics are being used in behavioral medicine well beyond psychotic disorders to include depressive, bipolar, and anxiety disorders. As a result, fetal exposure to this class of compounds must be increasing in frequency, but any neurodevelopmental sequelae of fetal antipsychotic exposure is poorly understood. To examine whether intrauterine antipsychotic exposure is associated with deficits in neuromotor performance and habituation in 6-month-old infants, Johnson et al. [43] studied >300 maternal-infant dyads (N=309) at 6 months postpartum with pregnancy exposure to antipsychotics (n=22), antidepressants (n=202), or no psychotropic agents (n=85). Examiners masked to maternal-infant exposure status administered a standardized neuromotor examination (Infant Neurological International Battery [INFANIB]) that tests posture, tone, reflexes, and motor skills and a visual habituation paradigm using a neutral female face. Infants prenatally exposed to antipsychotics showed significantly lower INFANIB scores than those with antidepressant or no psychotropic exposure. These results indicate that 6-month-old infants who were exposed to antipsychotic drugs *in utero* had lower scores on a standard test of neuromotor performance suggesting that this was a neurodevelopmental effect of fetal antipsychotic exposure.

Finally, a recent study by Daw et al. [44] provides a broad and contemporary perspective of the extent of prescription drug use by pregnant women. The investigators assessed the frequency, timing, and type of medicines used before, during, and after pregnancy in a Canadian population. This retrospective cohort analysis used population-based health care data from all pregnancies ending in live births in hospitals in British Columbia from April 2001 to June 2006 (n=163,082). Data from hospital records were linked to those in outpatient prescription-drug claims. Data from prescriptions filled from 6 months before pregnancy to 6 months postpartum were analyzed. Drugs were classified by therapeutic category

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and FDA pregnancy risk categories. Prescriptions were filled in 63.5% of pregnancies. At least 1 prescription for a category D or X medicine was filled in 7.8% of pregnancies (5.5% category D; 2.5% category X). The most frequently filled prescriptions for category D drugs were benzodiazepines and antidepressants. The most frequently filled prescriptions for category X drugs were oral contraceptives and ovulation stimulants filled in the first trimester. Remarkably, these data show that the majority of pregnant women in British Columbia received at least 1 prescription, and ~1 in 13 received a prescription for a drug categorized as D or X by the FDA.

This remarkable prevalence of maternal prescription drug use emphasizes the need for thorough pharmacovigilance and regulatory processes to assess potential fetal risks before, during and after drug approval and marketing. Additionally if a comprehensive translational approach is to be realized in human developmental toxicology, this category of fetal exposures must be included "in the mix" along with other environmental and dietary exposures.

Despite the widespread use of benzodiazepines during pregnancy and lactation, little information is available about their effect on the developing fetus and on nursing infants. Iqbal et al. [45] reviewed what is currently known about the effects of benzodiazepine therapy on the fetus and on nursing infants. Currently available information is insufficient to determine whether the potential benefits of benzodiazepines to the mother outweigh the risks to the fetus. High peak concentrations should be avoided by dividing the daily dosage into two or three doses. Minimizing the risks of benzodiazepine therapy among pregnant or lactating women involves using drugs that have established safety records at the lowest dosage for the shortest possible duration, avoiding use during the first trimester, and avoiding multidrug regimens.

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Chemical exposures of pregnant women: environmental and natural dietary chemicals

Pregnant women and infants are exposed to multiple chemicals through the diet. These include naturally occurring dietary components such as phytoestrogens [46] as well as numerous synthetic compounds. The online database TEDX (The Endocrine Disruption Exchange 2013) [47] lists chemicals with the potential to affect the endocrine system. To date there are approximately 1,517 endocrine disruptors on the TEDX List.

Quantifying some of these dietary exposures may rely on survey instruments such as food frequency questionnaires as well as assays of

biological specimens. Food frequency questionnaires (FFQ) have been adapted to provide a degree of quantification of soy consumption [48] by adult women. Since soy is the primary source of dietary estrogenic isoflavones in Western countries, the demonstrated correlation of data from the FFQ and plasma isoflavone concentrations indicate that either technique may provide adequate documentation of levels of exposure.

In recent years several different types of biological specimens from pregnant women have been assayed for environmental and natural dietary chemicals that are known to or suspected to have hormonal activity. Engel et al. [49] found detectable levels of three phytoestrogens (enterolactone, daidzein and genistein) and bisphenol A (BPA) in 21 amniotic fluid specimens that were collected before 20 weeks gestation. Samples were obtained by amniocentesis from women who were referred to the Mount Sinai Medical Center because of advanced maternal age. The relative concentration of the chemicals in amniotic fluid were enterolactone > daidzein > genistein >> BPA. Woodruff et al. [50] assessed exposure to environmental chemicals during fetal development by analyzing samples from pregnant women who were participants in the National Health and Nutritional Examination Survey (NHANES), a nationally representative sample of the U.S. population. The investigators analyzed data for 163 chemical analytes in 12 chemical classes for subsamples of 268 pregnant women from NHANES 2003–2004. The percentage of pregnant women with detectable levels of an individual chemical ranged from 0 to 100%. Certain polychlorinated biphenyls, organochlorine pesticides, perfluorinated compounds (PFCs), phenols, polybrominated diphenyl ethers (PBDEs), phthalates, polycyclic aromatic hydrocarbons, and perchlorate were detected in 99–100% of pregnant women. The median number of detected chemicals by chemical class ranged from 4 of 12 PFCs to 9 of 13 phthalates. Across chemical classes, median number ranged from 8 of 17 chemical analytes to 50 of 71 chemical analytes. In general, levels in pregnant women were similar to or lower than levels in nonpregnant women.

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Chemical exposures of pregnant women: herbs and supplements

An additional category of potential chemical exposure of the human fetus that is neither drugs nor diet *per se* is the use of herbal products and dietary supplements. A recent national survey in the United States [51] demonstrates the widespread use of these biologically active products. These investigators assessed population and subpopulation changes in rates of herb and supplement use and rates of disclosure of herb and supplement

use to conventional medical providers and compared data from the 2002 (n=30,427) and 2007 (n=22,657) Adult Complementary and Alternative Medicine File to the National Health Interview Survey (NHIS). The number of adults in the United States that ever used herbs or supplements was 55.1 million in 2007. The proportion of adults who reported use of herbs or supplements in the past 12 months was 17.9% in 2007. The proportion of respondents that disclosed herb or supplement use to their physician or another conventional medical professional was 45.4% in 2007. However, <1% of recent herb and supplement users disclosed this use to their pharmacist.

This study did not specifically address the question of usage of herbal products and dietary supplements by pregnant women and we are unaware of any reports documenting whether pregnant women are comparable to other adults in this regard.

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Chemical exposures of pregnant women: smoking, ethanol and drugs of abuse

While animal studies have shown that early life exposure to cigarette smoke can have adverse effects on specific body systems such as the CNS [52], the adverse impact of tobacco (cigarette) smoking on the human fetus had been documented by a number of studies over the last three decades with most outcomes documented in terms of birthweights and/or other general measures of *in utero* growth. Wang et al. [53] reported on the relationship of birth outcomes to the timing and intensity of maternal active and passive smoking estimated both from self-reports and from cotinine concentration in maternal urine during early, middle, and late gestation. Their study included 740 white and Hispanic women in Boston, MA between 1986 and 1992. At each antenatal visit, information on maternal active and passive smoking was obtained by a detailed questionnaire, and by measurement of urine cotinine concentrations. Infant birth outcomes were obtained from hospital records. The percentage of mothers who ever smoked cigarettes during pregnancy was 55.5% for white and 10.2% for Hispanic women. A significant inverse exposure-response relationship between cotinine concentration in maternal urine and infant size (birthweight, length and head circumference) at birth was demonstrated. The relationship was less clear between maternal self-reported smoking status (both active and passive) and these fetal outcomes.

Additional studies have used various biological specimens for assessment of fetal exposure to tobacco smoking by measuring cotinine as the biomarker

of exposure. Maternal salivary cotinine levels (SCLs) were compared to pregnancy outcomes among >700 pregnant women including 126 active smokers [54]. In linear regression analyses adjusting for sociodemographic and medical factors, SCLs of >20 ng/mL were associated with a reduction in birth weight of 88 g when SCLs were measured at baseline (P = .042) and 205 g when SCLs were measured immediately before delivery (P < .001). These investigators concluded that at any cutoff level, birth weight reduction was more significant for the same SCL measured in late pregnancy. Dietz et al. [55] analyzed data from the 1999–2006 National Health and Nutrition Examination Survey and estimated the percentage of 994 pregnant and 3,203 nonpregnant women 20–44 years of age who did not report smoking but had serum cotinine levels that exceeded the defined cut point for active smoking (nondisclosure). Active smoking was defined as self-reporting smoking or having a serum cotinine concentration that exceeded the cut point for active smoking. Overall, 13.0% of pregnant women and 29.7% of nonpregnant women were active smokers. Nondisclosure was higher among pregnant active smokers (22.9%) than among nonpregnant smokers (9.2%). The investigators concluded that studies and surveillance systems that rely on self-reported smoking status are subject to underestimation of smoking prevalence, especially among pregnant women, and underreporting may vary by demographic characteristics. In another study of fetal umbilical cord drug levels and maternal self-report [56] in >100 specimens, there was only fair agreement between self-reported smoking and cotinine levels as well as slight agreement with current drug use and positive drug levels. Compared with maternal self-report, sensitivity of cotinine levels was 27% and specificity was 98%. Sensitivity of positive cord illicit drug levels was 32% and specificity was 85%.A clear blunt summary of the adverse impact of tobacco smoking on women and their infants can be viewed on the Centers for Diseases Control website [57].

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In terms of fetal exposure to ethanol, there is absolute certainty that pregnant women should not consume alcoholic beverages. This is a clear and certain case for fetal protection by avoidance of exposure and is an ongoing critically important public health message from the American College of Obstetrics and Gynecology [58]. A recent review and commentary by Tracy [59], succinctly and powerfully documents the history of fetal alcohol syndrome, the animal and human data indicating that there is no safe level of exposure and that obstetricians and other healthcare providers must be more assertive in identifying and counseling reproductive age women and especially those who are pregnant regarding fetal risk. This is a clear case

where the toxicology, social norms and delivery of healthcare all make the course of appropriate action clear; the human fetus should not be exposed to ethanol.

Use of cocaine, marijuana, and other illicit drugs during pregnancy has been associated with a variety of adverse effects even though causality is not firmly proven. Effects include low birth weight, neurodevelopmental deficits and impairments affecting behavior, cognition, attention, language, and learning skills plus other behavioral problems in children exposed to cocaine, marijuana or heroin *in utero*. Methamphetamine exposure has been associated with fetal growth restriction, decreased arousal, and alterations in motor control in infants [60]. Neurotoxicity and psychopathology associated with methamphetamines and amphetamine exposure has been shown in both animal and epidemiologic studies, with more severe effects in women [61]. Given the high recreational use in young adults, the effects *in-utero* on the developing brains needs further investigation.

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Endocrine disruption as a functional category of toxicants

The spectrum of possibly non-mutagenic but highly specific modes of action of chemical toxicants is implied by the molecular targets through which pharmaceuticals have been shown to act. The notion that some environmental and dietary compounds can act via highly specific signaling pathways to elicit reproductive and/or multifaceted developmental effects has led to appreciation of more subtle effects of exposures in animal models and raised suspicion that important developmental effects may be similarly occurring in humans that have gone unrecognized in the past. The full range of potential modes of action is captured in the "Guide to Receptors and Channels" [62]. This compilation of the major pharmacological targets is divided into seven sections: G protein-coupled receptors, ligand-gated ion channels, ion channels, catalytic receptors, nuclear receptors, transporters and enzymes. Would all of these actions be considered "endocrine disruption" or do we simply need to consider "developmental toxicology" to be the broader designation and limit "EDC" to those compounds that bind to or affect hormone signaling pathways *per se*? Endocrine-disrupting chemicals (EDCs) can have effects at low doses that are not predicted by effects at higher doses [63]. Low-dose effects are those that occur within the range of human exposures or those observed at doses below the range used in traditional toxicological studies. These effects may depend upon nonmonotonic dose–response curves where a nonlinear relationship between dose and effect where the slope of the curve changes sign somewhere within

the range of doses examined. It is noteworthy that nonmonotonic responses and low-dose effects are remarkably common in studies of natural hormones and EDCs. On this basis, Birnbaum [64] has urged collaborations between research scientists in academia, government, and industry to allow for development of more sophisticated study designs to facilitate regulatory decisions. Our core proposal is as follows:

In order to impact human health, the developmental toxicology concept of endocrine disruption must be "put to the test" in any instance where a scientifically valid health concern is raised in which:

- human exposure(s) occur,
- there is a plausible mode of action such that the exposure might elicit developmental effects,
- human health outcomes are assessed and
- both passive [reduce exposures] and active [nutritional, sociobehavioral or pharmaceutical interventions] health risk mitigation/intervention strategies can be developed for exposed individuals.

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What evidence and modes of action link exposure to endocrine disruptors to endocrine or other human diseases? An essential missing link between endocrine disruptor testing and screening and epidemiology/population-based exposure assessments is individualized assessments of exposures and effects that are plausibly linked via known modes-of-action. Because metabolomic methods allow a broad look at multiple potentially perturbed metabolic pathways (and implictly different modes-of-action), and multiple biospecimens can be assayed [some of which are quite non-invasively obtained; urine], this may prove to be the preferred core analytical method for defining these individualized exposure-effect relationships.

What modes of action are known or are plausible from animal, *in vitro*, and human clinical data?

Numerous biological processes and multiple levels of biological organization could be adversely impacted by exposures to exogenous chemicals and thereby affect fetal-neonatal development and measurable endpoints (see Table 3). These modes or sites of action include stem cell/progenitor cell numbers or lineages mutation, epigenetics, higher level effects on target tissue or body system(s) organization, stressors that affect CNS, inflammatory or immune pathways, etc.

Table 3: Developmental toxicology endpoints

Endpoints	Methods of measurement
General growth, morphology of body regions, organs and tissues	Classical teratology
Organization of organs & tissues	Microscopic veterinary or human pathology
Cellular composition of tissues	Relative composition of major cell types; histopathology, histochemistry, histomorphometry
Finer cellular and tissue profiles	Tissue content of stem cells and progenitor cells; inflammatory/migratory cell populations; extracellular matrix/cell matrix interactions; assess cell cycle kinetics, critical cellular process (e.g., apoptosis and autophagy)
Molecular changes within cells	DNA, RNA, proteins (including processing, turnover, etc.), lipids and glycosylation; genomics, epigenomics, transcriptomics, microRNAs/siRNAs
"Accessible" or "translatable" (can be used in lab models and in humans) biomarkers	- Imaging (ultrasound, MRI, CT, etc.)
	- Functional assessments (behavior, treadmill, learning, cognition, renal function studies, lung function studies, grip strength, etc.)
	- Tissue biopsies, male and female gametes/follicular fluid, amniotic fluid; blood, urine
	- Routine chemistries, hematology,
	- Circulating cells (e.g., bone marrow stem cells, leukocytes, other stem/progenitor cells)
Biomolecules in blood plasma, urine including macromolecules, small molecules	- Specific assays for individual molecules
	- "Broad Net" approaches of proteomics, metabolomics, siRNAs, etc.
Integrative Physiology/Systems Biology	Multiple opportunities to link in vitro and animal models to humans and to use mathematical models to dynamically integrate multiple biological parameters

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Stem cell mediated effects

One plausible target of toxic action in almost any target tissue during development is the stem or progenitor cell. Any exposure that might alter the population size or characteristics of stem or progenitor cells could have

profound and lasting effects on the structural and functional integrity of any target tissue, organ or system. Additionally, since tools have been developed to study stem cells, the questions about potential actions of toxicants can now be investigated in relatively direct ways. In particular relationship to developmental effects of fetal exposures, stem cell populations from umbilical cord blood present a unique and well appreciated opportunity for study [65]. Umbilical cord blood contains at least three populations of stem cells, each with unique features and properties.

Hematopoietic stem and progenitor cells continuously egress out of the bone marrow (BM) and into the circulation under homeostatic conditions. This process is dramatically enhanced during stress situations and induced mobilization regimens [66]. Recent studies have clearly identified that hematopoietic stem cells (HSC) are part of a hierarchical organization defined by cells able to initiate long-term repopulation (LT-HSC) of injured BM followed by populations with transient repopulation ability [67]. As HSCs are able to supply blood cells for the lifespan of the individual, LT-HSCs undergo an aging process. The consequences of aging are an increase in the stem cell pool size, increased self-renewal and production of cells with expression of myeloid-specific genes and genes commonly expressed in myeloid leukemia. The age-related increased incidence of myeloproliferative disorders is therefore not surprising. Marrow cells circulate and migrate to a number of different organs. Previously assumed tissue and organ specificities appear to be less stringent and primitive cells residing in one organ may contribute to the structural and functional regeneration of other organs. It is intriguing to speculate that cells and cytokines used in appropriate concentrations may be able to repair damaged organs by using the defective organ as a scaffold, thus reducing the need for the transplantation of solid organs.

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Evidence suggests haematopoietic stem cells (HSCs) can migrate to injured liver and influence tissue repair. However, Kavanaugh et al. [68] found that administered HSCs homed predominantly to lungs rather than liver, highlighting a potential therapeutic hurdle. Stem cells are potential targets of intervention as well as possibly targets of toxic agents.

Mutagenesis and alteration of gene expression

As a result of extensive collection of genome-wide transcriptional expression data from cultured human cells treated with bioactive small molecules, "The Connectivity Map (also known as cmap) has been assembled by use of

simple pattern-matching algorithms [69]. This system enables the discovery of functional connections between drugs, genes and diseases through the transitory feature of common gene expression changes. At present the interface provides access to the current version (build 02) of Connectivity Map which contains more than 7,000 expression profiles representing 1,309 compounds. While not directly focused on toxicology or development, this broad and comprehensive approach toward characterizing the actions of chemicals on cells will surely be the type of valuable tool that will facilitate understanding the impact of toxicant exposures and potential mitigation strategies.

The types of advances being made are illustrated by recent work in hepatocellular carcinoma (HCC), the fifth most common cancer worldwide. HCC is more prevalent in men than women and recent genetic studies have revealed a causal role for androgen receptor (AR) in hepatocarcinogenesis, but the underlying molecular mechanism remains unclear. Feng et al. [70] used genome wide location and functional analyses to identify a critical mediator of AR signaling via cell cycle-related kinase (CCRK). Their results reveal a direct AR transcriptional target, CCRK, which promotes hepatocarcinogenesis through the upregulation of beta-catenin/TCF signaling.

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Epigenetic changes

The field of toxicogenomics is undergoing remarkable expansion due to advances in genomic technologies as well as an expanding set of concepts about how environmental agents may impact gene regulation and heritability of traits. The epigenetic bases for heritable changes in gene expression (without accompanying alterations in the DNA sequence) are becoming more certain as mechanisms such as DNA methylation, histone modifications, non-coding RNAs, and changes in expression or activity of histone acetylases (HAT) and histone deacetylases (HDAC) in the regulation of gene expression patterns are elaborated [71]. It is becoming clear that epigenetic mechanisms play a critical role in normal development and differentiation and if disordered, may lead to diseases such as cancers. The growing body of evidence that environmental exposures especially in early development can induce epigenetic changes implies that these mechanisms will help explain toxicological effects of environmental exposures. Additionally, epigenetic changes are likely to explain a number of later-in-life effects of early-in-life exposures as well as effects in one or more subsequent generations.

The global prevalence of obesity is increasing across most ages in both sexes. Ng et al. [72] found that paternal high-fat-diet (HFD) exposure programs B-cell 'dysfunction' in rat F1 female offspring. This report demonstrates that non-genetic, intergenerational transmission of the metabolic sequelae of a HFD from the father to his offspring occurs in mammals. Thus paternal as well as maternal exposures can bring about epigenetic alterations that influence the metabolic competency of their offspring.

Mathers et al. [73] summarized current knowledge of the impact of dietary, lifestyle, and environmental determinants of cancer risk and proposes that effects of these exposures might be mediated, at least in part, via epigenetic mechanisms. Evidence is presented to support the hypothesis that all recognized epigenetic marks (including DNA methylation, histone modification, and microRNA (miRNA) expression) are influenced by environmental exposures, including diet, tobacco, alcohol, physical activity, stress, environmental carcinogens, genetic factors, and infectious agents which play important roles in the etiology of cancer. Given the plasticity of epigenetic marks in response to cancer-related exposures, such epigenetic marks are attractive candidates for the development of surrogate endpoints which could be used in dietary or lifestyle intervention studies for cancer prevention. The authors argue that research should focus on identifying epigenetic marks which are (i) validated as biomarkers for the cancer under study; (ii) readily measured in easily accessible tissues (e.g., blood, buccal cells, or stool); and (iii) altered in response to dietary or lifestyle interventions for which there is convincing evidence for a relationship with cancer risk.

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Transient environmental influences, such as perinatal nutritional stress, may induce deleterious metabolic symptoms that last for the entire life of individuals, implying that epigenetic modifications play an important role in this process. Jousse et al. [74] investigated, in mice, the consequences of maternal undernutrition during gestation and lactation on DNA methylation and expression of the leptin gene, which plays a major regulatory role in coordinating nutritional state with many aspects of mammalian biology. The investigators found that animals born to mothers fed a low-protein-diet (F1-LPD group) have a lower body weight/adiposity and exhibit a higher food intake than animals born to mothers fed a control diet (F1-CD group). These modifications persisted throughout life and were associated with lower levels of leptin mRNA and protein in starved F1-LPD mice, emphasizing that maternal protein-undernutrition affects the balance between food intake and energy expenditure in adults. Moreover, this nutritional stress resulted in the removal of methyl groups at CpGs located in the promoter

of leptin, causing a permanent specific modification in the dynamics of the expression of leptin, which exhibits a stronger induction in the F1-LPD than in F1-CD mice in response to a meal. This study is an example of a molecular rationale linking transient environmental influences to permanent phenotypic consequences.

Histone modifications are thought to regulate chromatin structure, transcription and other nuclear processes. Histone methylation was originally believed to be an irreversible modification that could only be removed by histone eviction or by dilution during DNA replication. However, the isolation of two families of enzymes that can demethylate histones has changed this notion [75]. The biochemical activities of these histone demethylases towards specific Lys residues on histones, and in some cases non-histone substrates, have highlighted their importance in developmental control, cell-fate decisions and disease. Their ability to be regulated through protein-targeting complexes and post-translational modifications is also beginning to shed light on how they provide dynamic control during transcription.

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O-GlcNAcylation, which is a nutrient-sensitive sugar modification, participates in the epigenetic regulation of gene expression. Thus, O-GlcNAc cycling may serve as a homeostatic mechanism linking nutrient availability to higher-order chromatin organization [76]. The wide range of physiological functions regulated by O-GlcNAc cycling suggests an unexplored nexus between epigenetic regulation in disease and nutrient availability.

To elucidate the role of age-related epigenetic changes in healthy ageing and potential longevity, Bell et al. [77] studied the association between whole-blood DNA methylation patterns in 172 female twins aged 32 to 80 with age and age-related phenotypes. Genome-wide analyses assessed differentially methylated regions (DMRs) for sixteen age-related phenotypes (ap-DMRs) and chronological age (a-DMRs). The majority of age-related changes in DNA methylation were not associated with phenotypic measures of healthy ageing in later life. Their results suggest that in a small set of genes DNA methylation may be a candidate mechanism of mediating not only environmental, but also genetic effects on age-related phenotypes.

Translational Research: Biomarkers as a Key Component

Translatable biomarkers (e.g., true in animals and humans; such as hemoglobin or pulse rate) or biomarker equivalency/ pairings (organ weights or dimensions in animal models vis-à-vis CT, MRI or ultrasound imaging in humans) must be utilized but prudently. To advance understanding of

whether exposures have effects that may be a risk, various molecular and imaging biomarkers have been developed, proposed and used in both laboratory and epidemiological settings. A critical shortcoming in this tactic is that a biomarker result is interpreted as a validated proxy for a clinical outcome. This assumption or interpretive leap is virtually always wrong. In human health research, the outcomes that truly matter are clinical endpoints (how a patient feels, functions or survives). Short of directly measuring clinical endpoints, surrogate endpoints can be measured if such surrogate endpoints have been validated. At a minimum for biomarkers to be plausibly interpreted as indicators of clinical endpoints, such biomarkers must be explicitly validated as predictors of surrogate endpoints. There are numerous such sets of endpoints and biomarkers that are used in medical practice with varying degrees of reliability. To illustrate, a few common examples would be as follows:

- Clinical endpoint: sepsis
- Surrogate endpoint: bacteremia
- Biomarker: fever
- Clinical endpoint: prostate cancer
- Surrogate endpoint: microscopic pathology
- Biomarker: prostate specific antigen (PSA)
- Clinical endpoint: bone fractures
- Surrogate endpoint: bone mineral density
- Biomarker: osteocalcin (OC), trimeric carboxyterminal telopeptide (ICTP).

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In environmental health research, we cannot have control over several key components in the "usual" human health research setting. Key characteristics of both the exposure (the agent, dose, duration, route, etc.) and the exposed subject (person's age, sex, developmental lifestage, intercurrent illnesses, nutritional status, etc.) are not controlled by the investigator. So, for environmental health research where the aim is to assess possible human health effects of exogenous exposures, how can we approximate a thorough validation process of linking biomarker(s) to surrogate endpoint(s) to clinical endpoint(s)? We must transform the usual logic of "translational" health research into an approach that "works" to move toxicology research into actionable environmental health discoveries.

The aim of translational toxicology is to advance environmental health sciences in order to:

- Transform Research & Development programs, especially endocrine disrupter programs, to link them overtly to disease outcomes;
- Link target tissue/organ/system outcomes in animal models to animal biomarkers (mostly molecular) to human biomarkers to human target organ outcomes (pathology, imaging and functional)
- Enhance predictive use of animal and human biomarker data sets by development of circumscribed computational (mathematical, statistical, knowledge-base, even simple schematic) model development.

Timing of Interventions for Prevention or Mitigation

We do need to consider that there may need to be sustained, continuing interventions for certain adverse effects/disease predispositions that are the result of environmental exposures. Nevertheless, key windows for interventions are likely to be the same as those in which adverse effects are elicited. Thus, in utero/neonatal/early childhood, adolescent/peri-pubertal and then menopausal transition in women may be particularly responsive since organizational/re-organizational processes are biologically dynamic in those intervals.

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What are the prospects for screening pregnant women for exposures of concern and then implementation of mitigation strategies? Perhaps the current compliance of obstetrical practices in terms of testing in prenatal care is a guide. Siddique et al. [78] examined whether the frequency of four screening tests during prenatal care conforms to evidence of effectiveness. The investigators estimated rates of urine culture, anemia screening, oral glucose tolerance test (OGTT), and urinalysis during prenatal care by use of national probability samples of office visits to physicians (National Ambulatory Medical Care Survey) and to hospital outpatient departments (National Hospital Ambulatory Medical Care Survey) from 2001 to 2006. These data were compared to recommendations from the U.S. Preventive Services Task Force (USPSTF). On average, women received a urine culture in less than half of pregnancies. Women received just over one anemia screening on average per pregnancy. From 2001–2003, women received an average of 5.6 urinalyses per pregnancy; the average dropped to 4.3 urinalyses per pregnancy in 2004–2006. On average, women received just under one OGTT per pregnancy. Compared to younger white women, minorities and older women tend to get more anemia screenings, urine

cultures, and OGTTs. Compared to USPSTF recommendations, too few women are receiving a urine culture during prenatal care. In contrast, women receive far too many urinalyses, but the rate appears to be falling. Anemia screening conforms closely to recommendations. The USPSTF does not recommend for or against universal diabetes screening using OGTT. Women appear to receive OGTT routinely.

Obstetrical Examples of Interventions for Fetal/Neonatal/Life-long Well-being

"Fetal therapy is broadly defined as any intervention administered to or via the mother with a primary indication to improve perinatal or long-term outcomes for the fetus or newborn." [79]. Due to concern about unknown consequences of fetal exposures, many leaders of public and private research entities, scientists, regulators and non-obstetrical physicians believe that no pharmaceutical interventions should be given to pregnant women for the purpose of enhancing lifelong well-being of the mother's offspring. Beyond immunizations, care of maternal acute and chronic illnesses and specific obstetrical conditions (e.g., pre-eclampsia, gestational diabetes, etc.) including antenatal corticosteroids to enhance fetal lung maturity when premature delivery is anticipated, there are at least five different treatments that are commonly given to pregnant women where a primary aim is to effect fetal benefit that will obviously or likely manifest across the lifespan (see Table 4).

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Table 4: Treatments commonly given to pregnant women for fetal benefit across the lifespan

Disease/Indication	Treatment	Outcome	Selected references
Neural tube defects	Folate	Reduction in risk of neural tube defects	American Academy of Pediatrics Committee on Genetics [80]
			U.S. Preventive Services Task Force [81]
Vision and cognitive function	Docosahexaenoic acid (DHA)	Support optimal visual and cognitive development	Koletzko et al. [82]
Smoking	Nicotine and/or N-acetylcysteine (NAC)	Smoking cessation and/or chemoprevention of DNA (and other) damage	Coleman et al. [83]
			De Flora et al. [84]
			Van Schooten et al. [85]

Maternal (and fetal) hypothyroidism	Thyroid hormone therapy	Avoid potential damage to neural development	Abalovich et al. [86]
			Rivkees & Mandel [87]
			Shields et al. [88]
			Kuppens et al. [89]
			Lazarus et al. [90]
Cerebral palsy associated with preterm delivery	Magnesium sulfate (MgSO4)	Reduction in risk of cerebral palsy/ motor disorders in childhood	Nelson & Grether [91]
			Rouse et al. [92]
			Conde-Agudelo & Romero [93]
			Rouse [94]
			Doyle et al. [95]

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Two studies by Ibanez et al. [96, 97] suggest that the peri-pubertal interval may be an important developmental window in which therapeutic interventions can be safely and effectively administered to mitigate the adverse health impact of earlier exposure to developmental stressors. Low-birthweight (LBW) girls with precocious pubarche (PP) are at risk for an early menarche (<12 years), an adult stature below target level, and PCOS. Hyperinsulinemic insulin resistance is thought to be a key factor. Therefore Ibanez et al. [96] studied the effects of early metformin treatment on menarche, height, and polycystic ovary syndrome (PCOS) markers as an open-label, randomized study in thirty-eight LBW-PP girls. At age 8 years, girls were randomly assigned to remain untreated or to receive metformin for 4 years; subsequently, both subgroups were followed without treatment until each girl was postmenarcheal. At last assessment, girls in each subgroup were on average 2 years beyond menarche; the mean growth velocity was below 2 cm/years. Age at menarche was 11.4±0.1 years in untreated girls and 12.5±0.2 years in metformin-treated girls; the latter girls were taller and much leaner (with less visceral and hepatic fat) and had more favorable levels of circulating insulin, androgens, and lipids. The conclusions were that early metformin therapy (age ~ 8–12 years) delayed menarche; augmented postmenarcheal height; reduced total, visceral, and hepatic adiposity; and curbed the endocrine-metabolic course of LBW-PP girls away from adolescent PCOS. In their second study, Ibanez et al. [97] compared the capacity of early vs. late metformin treatment to prevent adolescent PCOS. This was a randomized, open-label study over 7 yr in the thirty-eight LBW-PP girls who were followed up from the mean age 8 until age 15 yr. Early metformin was study yr 1–4; age 8–12 yr while late metformin was yr 6; age 13–14 yr. None of the girls dropped out of

the study. At age 15 yr, early-metformin girls were taller (4 cm), were in a less proinflammatory state, and had less central fat due to reductions in visceral and hepatic fat. Hirsutism, androgen excess, oligomenorrhea, and PCOS were between 2- and 8-fold more prevalent in late- than early-treated girls. Abdominal adiposity was the first variable to diverge (at age 8–10 yr) between girls without vs. with PCOS at age 15 yr. The investigators conclude that in LBW-PP girls, early metformin therapy was found to prevent or delay the development of hirsutism, androgen excess, oligomenorrhea, and PCOS more effectively than late metformin. They suggest that the time window of late childhood and early puberty may be more critical for the development, and thus for the prevention, of adolescent PCOS than the first years beyond menarche.

CONCLUDING REMARKS

We need to broadly reorient perspectives to make toxicology and/or environmental health an active human health-promoting process. We cannot continue to rely on the existing dichotomy: on one hand, we have laboratory-based molecular, cellular and animal models data while on the other hand we have epidemiological and exposure assessment data. We are convinced that linking these types of data and scientific insights can be done and this is the crux of moving toxicology into an active translational research and interventional science.

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Hormone signaling pathways may serve as mediators of reproductive and developmental effects and earliest indication of exposure. These early exposures may have later effects which may differ across the lifespan depending on other critical hormonal activities in the body.

Exposures are inevitable and the body either builds or has resilience or resistance to the adverse effects, or due to earlier exposures losses its ability to resist and succumbs to disease. The earlier exposures that cause the body to lose its ability to avoid disease in response to environmental factors needs to be evaluated. Stem/progenitor cell populations must be a key component to recovery/resilience to insults and provide a capacity to recover/respond/heal either from the exposure of interest or to multiple future or concurrent insults later in development or across the lifespan. Lab animals, ecological (wildlife) exposures, companion animals, and livestock can serve as bio-monitors and formal ways of getting observations from these populations into the knowledge base on human relevance need to be adopted.

A number of contemporary analytical techniques are available to detect and quantify exposures and effects. These methodological tools are widely called "biomarkers" and cover a rather wide range of techniques/technologies from DNA sequences of individual genes, to FACS of cell subpopulations, to 3-D reconstruction of tissues and organs from pathology slides, to whole body imaging with CT and/or fMRI, etc. Techniques such as "shotgun" proteomics and metabolomics offer further broad scope approaches for assessing responses of cells, tissues or organisms to external signals or stressors. Appropriate recognition of how biomarkers relate to disease outcomes in humans is critical to moving environmental toxicology from its current state to producing effective intervention strategies.

How do we best develop and gain insight by comparisons of critical windows in animal/cellular models to understand the human consequences for effects and the opportunities for protective interventions?

Because exposures occur to many individual compounds, classes of compounds and combinations (mixtures) of compounds and raise legitimate concern, translational links need to rely on various types of biomarkers that address two types of causal toxicopathologic pathways.

- The one (or ones) that are known modes of action of the compound(s) such as most of the EPA Endocrine Disruptor Screening Program Tier 1 Screening Assays [98].
- The possibility of unknown modes of action such as most of the EPA Endocrine Disruptor Screening Program Tier 2 Testing Assays [99] or use of metabolomics [99, 100].

Number 1 is the "silver bullet" or "searching for the lost keys under the lamp-post" while number 2 is "casting the broad net upon the waters" or "searching in the dark." Both need to be done. Number 1 because it relies on using all that we know to trace a straight line between exposure, effect and prospects for mitigation; number 2 because chemicals (even those highly selected to have a very specific action via even a single molecular process, e.g., drugs) have actions in multiple molecular/cellular sites, and biological organisms and components are rife with pleiotropic and redundant systems. So, techniques that broadly ask what effects might result from exposures are essential for translational toxicology. The sorts of techniques that can "cast a broad net" to possibly let us see (previously) unknown modes of action include metabolomics, some forms of proteomics, assays of mutagenic and epigenetic effects, stem cell/progenitor cell/cell fate/cell sorting methods, histomorphometry and advanced microscopy techniques, circulating

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molecules (hormones, microRNAs, etc.), anatomical and functional morphology imaging methods. These approaches must be core components of the research and development strategy if translational environmental health is to advance and favorably impact public health.

SUMMARY

1. The pregnant woman and her fetus are exposed to many chemicals. These chemicals include environmental chemicals, dietary components, over-the-counter drugs, prescription drugs, substances of abuse (tobacco, alcohol and drugs of abuse) and herbal and related products.
2. Any of these may affect the fetus via many potential modes of action such as epigenetic, mutational, endocrine, stem cell, and a multitude of other signaling pathways (G protein receptors, ion channels, etc.).
3. We will depend upon use of biomarkers to assess actions of those compounds and to establish meaning for clinical outcomes. Readily measured biomarkers will have to be linked to surrogate outcomes which have to be representative of clinical outcomes.
4. In order to give substance to the term "translational", we must develop ethical and effective interventions to complement exposure reduction so that the adverse health effects of exposures can be prevented, reversed or mitigated.

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AUTHORS' CONTRIBUTIONS

All authors researched and wrote the debate. All authors provided critical reviews and revisions of the manuscript. All authors read and approved the final manuscript.

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SECTION II: NEUROTOXICITY

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Chapter 3

Genotoxic and Cytotoxic Effects on the Immune Cells of the Freshwater Bivalve Dreissena Polymorpha Exposed to the Environmental Neurotoxin BMAA

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ABSTRACT

The environmental neurotoxin β-*N*-Methylamino-l-alanine (BMAA) has been pointed out to be involved in human neurodegenerative diseases. This molecule is known to be bioaccumulated by bivalves. However, little data about its toxic effects on freshwater mussels is available, particularly on the hemolymphatic compartment and its hemocyte cells involved in various physiological processes such as immune defenses, digestion and excretion, tissue repair, and shell production. Here we exposed *Dreissena polymorpha* to dissolved BMAA, at the environmental concentration of 7.5 µg of /mussel/3 days, during 21 days followed by 14 days of depuration in clear water, with the objective of assessing the BMAA presence in the hemolymphatic compartment, as well as the impact of the hemocyte cells in terms of potential cytotoxicity, immunotoxicity and genotoxiciy. Data showed that hemocytes were in contact with BMAA. The presence of BMAA in hemolymph did not induce significant effect on hemocytes phagocytosis activity. However, significant DNA damage on hemocytes occurred during the first week (days 3 and 8) of BMAA exposure, followed by an increase of hemocyte mortality after 2 weeks of exposure. Those effects might be an indirect consequence of the BMAA-induced oxidative stress in cells. However, DNA strand breaks and mortality did not persist during the entire exposure, despite the BMAA persistence in the hemolymph, suggesting potential induction of some DNA-repair mechanisms.

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Keywords

BMAA, hemocytes, genotoxicity, cytotoxicity, immunotoxicity, freshwater bivalves

Key Contribution: Through a 21-day in vivo exposure of the freshwater bivalve *Dreissena polymorpha* to dissolved β-*N*-Methylamino-l-alanine (BMAA), followed by 14 days of depuration, this study reveals the presence of BMAA in the hemolymphatic compartment, as well as DNA stand breaks and cytotoxicity on the immune cells.

INTRODUCTION

Harmful algal blooms (HAB) are increased by human activities [1] and, because of their ability to produce secondary metabolites that can be harmful to other species, they are known to have an impact on ecosystems and human

health [2]. Among those toxins, the neurotoxin β-*N*-Methylamino-l-alanine (BMAA), a hydrophilic non-protein amino acid, is a subject of growing concern. Indeed, BMAA could be potentially involved in the development of human neuro-degenerative pathologies such as the Amyotrophic Lateral Sclerosis-Parkinsonism-Dementia complex (ALS-PDC) [3]. Historically, the etiological link between BMAA and ALS has been pointed out in a study conducted in Guam in the 50 s, where the incidence of ALS was 50 to 100 times higher than in the rest of the world [3]. As BMAA has been reported in human brains of people who suffer from ALS-PDC [4], it has been alleged that the contamination came from chronic exposure through a biomagnification process. Indeed, the endosymbiotic cyanobacteria of the genera *Nostoc*, present in cycads roots, has been shown to produce BMAA [3,5]. The cycads seeds are then ingested by fruits bats *Pteropus mariannus* while foraging. As bats were traditional food items on Guam, human contamination is thought to have occurred during the consumption of whole individuals [3,5]. However, this hypothesis is subject to controversy [6]. The BMAA toxic mechanism has not been fully determined, but current research shows that the neurotoxin may (i) form carbamate adducts when conjugated with bicarbonate ions at physiological concentrations, inducing a motoneuron excitotoxicity via the L-glutamate receptors [7,8,9], which can lead to an oxidative stress an cell death [8,9]; (ii) inhibit the synthesis and/or the stimulation of glutamine degradation [10]; (iii) inhibit cells cysteine/glutamate antiporter, leading to a depletion of glutathione (GSH) which increases oxidative stress [8]; and (iv) bind to or incorporate into proteins during their synthesis [11], although this hypothesis has been criticized [12]. Little data about animal toxicology or *in situ* occurrence are available for this emerging environmental neurotoxin, which is associated with controversies in relation to analytical challenges for its detection and quantification in various matrices [13].

BMAA was historically thought to be produced ubiquitously by cyanobacteria; recent upgrading of analytical methods has narrowed the list of cyanobacterial BMAA producers and shown that it is also produced by some diatoms and dinoflagellate species [14,15,16,17,18]. The neurotoxin has also been detected in food webs outside of Guam, in animals destined for human consumption (fish, mussel, and oyster) in China, United States, and Europe [15,16,19,20,21,22,23,24]. Moreover, a possible cluster of ALS has been pointed out in the Thau Lagoon (France) that could be potentially linked with the consumption of BMAA-contaminated seafood [25]. Up to now, BMAA bioaccumulation following laboratory exposure

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of invertebrates to dissolved BMAA or to BMAA-producers has been demonstrated in gammarids [26,27,28] and bivalves [29,30], including the freshwater mussel *Dreissena polymorpha* [29] investigated in the present study. The freshwater sessile bivalve *D. polymorpha*, is native from the Ponto-Caspian seas and is now a widespread species [31]. Bivalves are commonly used as bioindicators and biomonitors in relation to their direct contact with medium, their abundance, broad distribution, limited mobility, lifespan, and ease of handling [32]. This mussel can filter particles from 0.4 µm [33] to 200 µm [34] or even 750 µm after 16 h of starvation [35], including phytoplankton species, and is also known to uptake efficiently amino acid from its environment [36]. This wide food size range combined with filtering capacities ranging from 5 to 400 mL/mussel/h [37] allows zebra mussels to be potentially intoxicated by BMAA through three main pathways: (i) the gill absorption of dissolved BMAA; (ii) the ingestion of food particles in which BMAA may be adsorbed; and (iii) the ingestion of BMAA-producers. Those contamination pathways, and more particularly the gill diffusion, may induce a BMAA distribution in the hemolymphatic compartment.

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Bivalves have an open circulatory system composed of a heart and vessels in which the hemolymph circulates. Hemolymph is compossed of cells named hemocytes and dissolved proteins. Those cells are primarily in charge of the immune defense and pathogens elimination [38], but also take part in various physiological processes including digestion, tissue repair, shell production, and excretion [38,39,40]. Because of their involvement in mussels defense and homeostasy, hemocytes have a key role in bivalves physiology. The aim of this study was to assess the impact of the BMAA on hemocytes of *D. polymorpha*, in terms of cell mortality, phagocytosis, and DNA integrity, through a 3-week discontinuous exposure of *D. polymorpha* to dissolved BMAA, followed by 2 weeks of depuration. Due to the lack of a known organism that could produce BMAA steadily, zebra mussels were exposed to dissolved BMAA to a concentration 7.5 µg of dissolved BMAA/mussel/3 days. Few data of environmental BMAA concentrations acquired with a reliable analytical method and reported per liter of water are available to date [41]. One study reports BMAA concentrations from 0.01 to 0.3 µg/L in lake samples (Canada) [42]. Based on the filtering capacity of *D. polymorpha*, ranging from 0.12 to 9.6 L/mussel/day according to food concentrations [43], one zebra mussel could theoretically filtrate from 1.2 ng BMAA/day to 9.6 µg BMAA/day. This broad range of BMAA environmental concentrations in water includes the one used in this study. As the BMAA

pathway into mussels is still unknown, the hemolymphatic concentration of BMAA was followed throughout the study to assess hemocytes exposure. We discuss the BMAA presence in the hemolymph and its impacts on hemocytes, in terms of potent cell penetration and alteration of DNA integrity, as well as subsequent potent DNA reparation.

RESULTS

In this study, freshwater mussels *D.polymorpha* have been exposed to 7.5 µg BMAA/3 days for 21 days, followed by 21 days of depuration in clear water.

BMAA Accumulation in Hemolymph

The total BMAA fraction was evaluated here, comprising the free BMAA, soluble in sample supernatant, and the BMAA associated/bound to proteins or peptides of low molecular weight that requires tissue hydrolysis to be extracted [44]. Total BMAA was detected in all hemolymph samples. However, a strong suppression of the internal standard (D_3BMAA) signal was observed in some samples (2 samples from day 8 of exposure, 2 from day 1 of depuration, and all other depuration samples), which made it impossible to accurately estimate the BMAA concentration in these samples.

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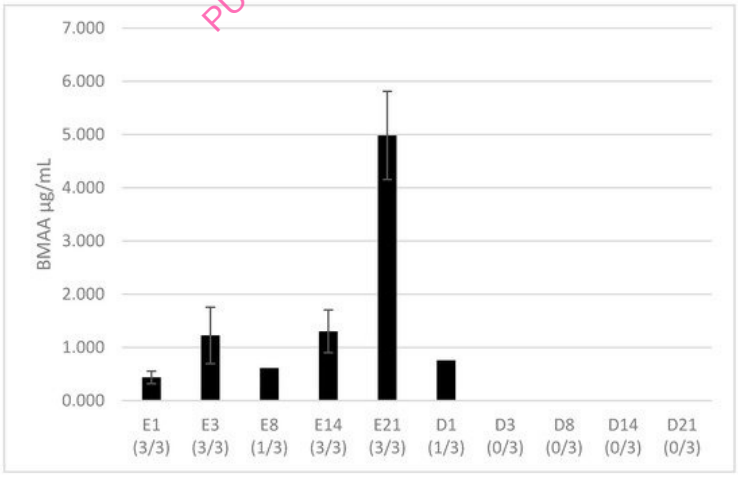


Figure 1: Concentration of BMAA (µg/mL) in hemolymph samples during the exposure (E) and depuration (D) periods; numbers in brackets represent the number of samples in which BMAA quantification has been performed compared to the three samples analyzed. Technical difficulties prevented BMAA

quantification in the other samples, although BMAA was detected. No significant difference between samples appeared with a Mann-Whitney test, $p < 0.01$.

Therefore, only the data that are quantified based on the internal standard response are shown. After 24 h of exposure, total BMAA has been found in all hemolymph samples with a mean of 0.438 ± 0.117 µg/mL (Figure 1). Then, the concentration in samples remained constant until day 21 of exposure, in which the concentration increased ($p > 0.01$) and reached 4.984 ± 0.829 µg/mL. The day after, representing the first day of depuration, BMAA could only be quantified in one sample among three at a concentration of 0.755 µg/mL.

Hemocytes Mortality

The percentage of dead hemocytes has been measured throughout time (Figure 2).

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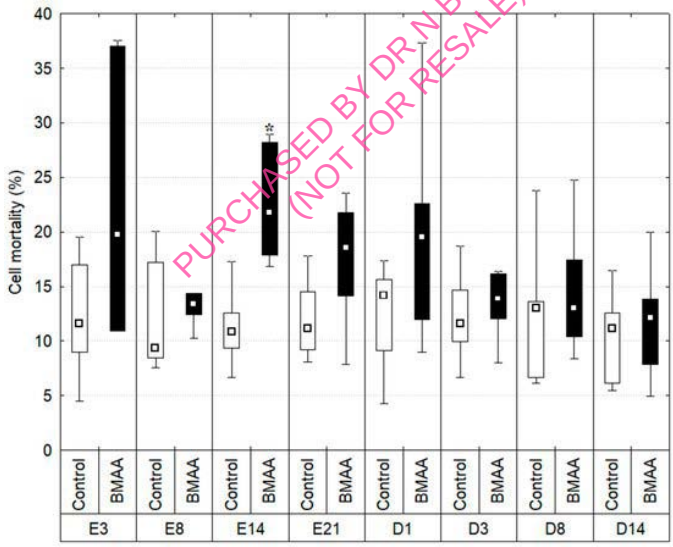


Figure 2: Hemocyte mortality (%) measured during the exposure (E) and depuration (D) period. Boxplots indicate first and third quartile of the observations, whiskers indicate minimum and maximum values, and the median is indicated by a square. Dark bars indicate the BMAA treatment (7.5 µg of dissolved BMAA/mussel/3 days) and the white bars the controls. * Indicates a significant difference between the exposed individual and the control of the same sampling date with a Mann-Whitney test, $p < 0.01$, n between 6 and 10. Due to a technical issue, data from E1 are not shown.

The percentage of mortality in controls remained stable during the experiment: a minimum of 11.513% ± 1.118 of dead cells observed on day 14 of the exposure period, and a maximum of 12.702% ± 1.806 on day 8 of the depuration period (Kruskal-Wallis test: $p > 0.01$). The percentage of mortality from individuals exposed to BMAA did vary during the experiment. On day 3 and 8 of exposure the mortality remained stable (22.672% ± 12.719 on day 3 and 13.540% ± 2.600 on day 8) and did not vary significantly from controls ($p > 0.01$; U values respectively 11 and 9). Then, the percentage of dead hemocytes on day 14 of exposure is significantly higher than the one observed in controls ($p = 0.002$, $U = 1$) and reached 22.582% ± 5.464. Data also shows greater inter-individual variations in individuals exposed to BMAA compared to controls at all sampling times, except at day 8 of exposure.

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Hemocytes Phagocytosis

During the exposure, the phagocytosis efficiency was evaluated through the measure of the percentage of hemocytes that had engulfed 3 beads ore more (Figure 3). The data did not show significant differences or trends between controls and mussels exposed to BMAA during the exposure and the depuration periods (Kruskal-Wallis test: $p < 0.01$).

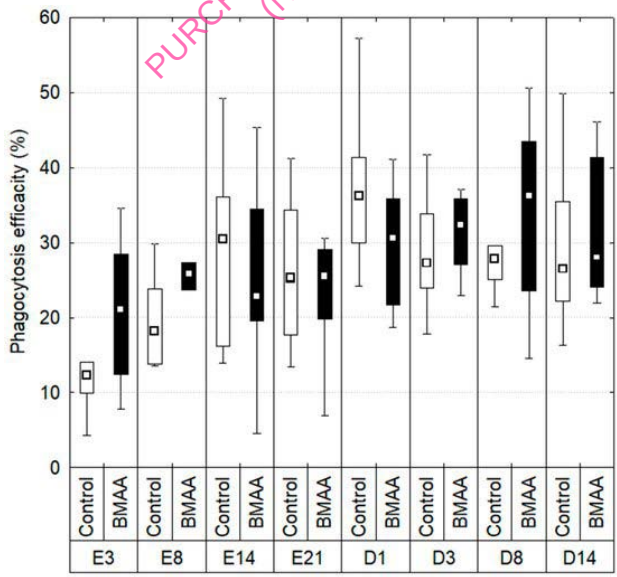
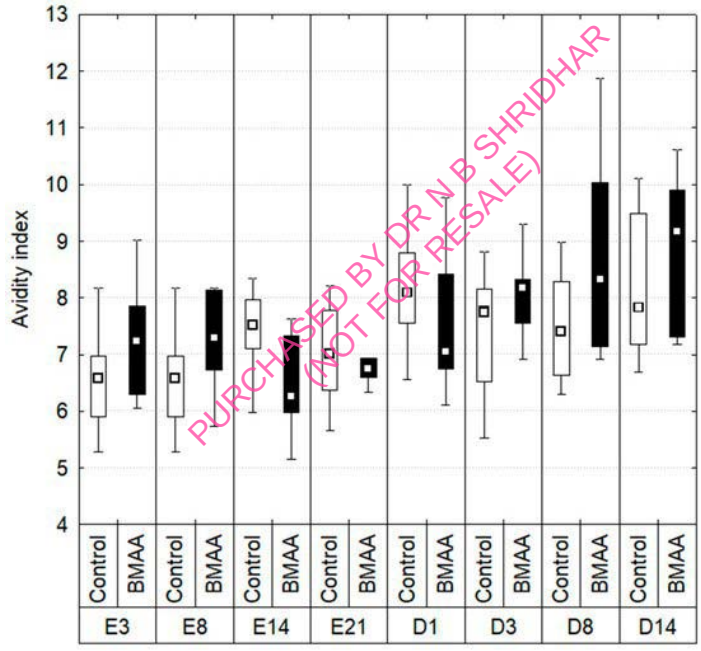


Figure 3: Phagocytosis efficiency measured during the exposure (E) and depuration (D) periods. Boxplots indicate first and third quartile of the

observations, whiskers indicate minimum and maximum values, and the median is indicated by a square. Dark bars indicate the BMAA treatment (7.5 µg of dissolved BMAA/mussel/3 days) and the white bars the controls. No difference between exposed individual and controls of the same time with a Mann-Whitney test, $p < 0.01$, n between 6 and 10.

The mean number of beads engulfed per cell, representing the avidity index (Figure 4), was also measured. As for the phagocytosis efficiency, no significant differences appeared between controls and individuals exposed to BMAA during the exposure and depuration periods (Kruskal-Wallis test: $p > 0.01$).

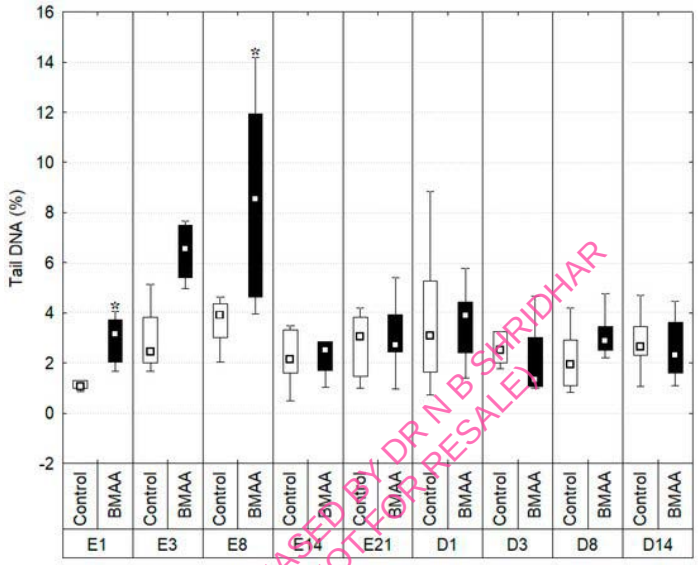


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Figure 4: Avidity index measured during the experiment. (%) measured during the exposure (E) and depuration (D) periods. Boxplots indicate first and third quartile of the observations, whiskers indicate minimum and maximum values, and the median is indicated by a square. Dark bars indicate the BMAA treatment (7.5 µg of dissolved BMAA/mussel/3 days) and the white bars the controls. No difference between exposed individual and controls of the same time with a Mann-Whitney test, $p < 0.01$, n between 6 and 10.

DNA Integrity

The percentage of DNA present in the tail of comets (Tail DNA), reflecting the degradation of DNA, has been studied throughout this experiment (Figure 5).



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Figure 5: Mean tail DNA (%) measured during the exposure (E) and depuration (D) periods. Boxplots indicate first and third quartile of the observations, whiskers indicate minimum and maximum values, and the median is indicated by a square. Dark bars indicate the BMAA treatment (7.5 µg of dissolved BMAA/mussel/3 days) and the white bars the controls. * Indicates a significant difference between the exposed individuals and the controls of the same sampling date with a Mann-Whitney test, $p < 0.01$, $n = 8$.

DNA strand breaks in controls remained stable over the experiment (Kruskal-Wallis test: $p > 0.01$) with a minimum of 1.212 ± 0.379% of tail DNA at the first day of exposure and a maximum of 4.018% ± 4.369 after 21 days of exposure. On the other hand, greater variations have been observed in individuals exposed to BMAA. At the first day of exposure, the percentage of tail DNA (% tDNA) in individuals exposed to BMAA reached 2.942% ± 0.927 and was significantly higher than the one of controls ($p = 0.003$, $U = 1$). However, it should be notice than the % tDNA in controls tended to be lower on the first day of the experiment compared to other sampling dates, although the difference was not significant ($p > 0.01$). This low control value on day 1 revealed significant DNA damage in BMAA-

exposed individuals, while their % tDNA values were comparable to those of controls at following times. Therefore, we do not put emphasis about the significant DNA damages observed at this first day of exposure. Then, DNA integrity of exposed individuals decreased throughout time, reaching a maximum of DNA strand breaks at the eighth day of exposure, with significantly higher damages, 9.748% ± 6.617, than in controls ($p = 0.0087$, $U = 7$). It should be noticed that, even if not significant ($p = 0.012$, $U = 8$), a trend of DNA damages in exposed organism has been observed at the third day of exposure in a higher level (7.290% ± 3.034) compared to controls (3.801% ± 3.554).

From day 14 of exposure, the level of DNA strand breaks in hemocytes of exposed mussels remained low and similar to controls during the rest of the exposure period, as well as during the depuration period (Kruskal-Wallis test: $p > 0.01$).

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DISCUSSION

Through a 3-week discontinuous exposure of the freshwater bivalve *D. polymorpha* to 7.5 µg BMAA/individual/3 days, the objectives of the experiment consisted of analyzing the presence of BMAA (total fraction) in the hemolymphatic compartment and studying potent subsequent impact on hemocytes in terms of (i) cytotoxicity; (ii) immunotoxiciy; and (iii) genotoxicity. As no steady laboratory BMAA-producer has been identified to date, zebra mussels were exposed to dissolved purified BMAA.

The potent immunotoxicity of BMAA has never been studied so far. It is interesting here to notice that BMAA was detected in the hemolymph from the first day of exposure until the last day of depuration, confirming the BMAA penetration in this compartment during in-vivo exposure. Another point is the chronology of the impact of BMAA on the hemocytes of *D. polymorpha*. Indeed, on the third and eight days of exposure, BMAA induced significant DNA damages, while cell mortality remained similar to controls, suggesting the genotoxicity of this molecule on hemocytes. Then, on the fourteenth day of exposure, significant hemocyte mortality was observed in BMAA-exposed cells, compared to controls, while no difference in the BMAA content in hemolymph was noticed compared to previous times of exposure, and no significant individual mortality was observed. This observed hemocyte mortality could potentially be a result of previous DNA damages. At the last day of exposure, BMAA concentration in hemolymph

seemed to increase compared to previous days, but no genotoxicity or cytotoxicity were observed anymore.

Mussels have already been shown to accumulate BMAA during laboratory exposures to dissolved BMAA, but the transfer of BMAA from the medium to the hemolymphatic compartment remains unknown. To date, only the presence of BMAA in the entire mussel body has been demonstrated during an exposure of the marine mussel *Mytilus galloprovincialis* to dissolved BMAA (1, 2.5 and 5 mg/L) for 24 and 48 h and to two BMAA-producing cyanobacterial strains (*Synechocysti salina* and *Microcoleus chthonoplastes*) [30]. Moreover, an exposure of *D. polymorpha* to approximately 100 µg/L of isotopically labeled BMAA for 48 h followed by 24 h of depuration has shown that BMAA was taken up by zebra mussels, and that nearly all of the exogenous-labelled BMAA available has been removed from the medium [29]. In the present study, BMAA dissolved in the medium was later quantified in the hemolymphatic compartment, suggesting two entrance pathways: (i) a gill diffusion directly from the medium to the hemolymph; or (ii) an ingestion through a BMAA adsorption on food particles (*Chlorella* sp. and *Scenedesmus* sp.). However, because mussels feeding and BMAA addition in the medium has been done at different days, this hypothesis seems unlikely. Under such hypotheses, hemocytes may have been in contact with BMAA during the digestion and/or nutrient transport, through the digestive system. However, no information is available concerning either BMAA bioaccessibility (i.e., the BMAA fraction extracted from food after digestion that is available for absorption by the hemolymphatic compartment) or its biodisponibility (i.e., the BMAA fraction that indeed reaches the hemolymphatic compartment). This study, however, confirmed the BMAA transfer from the medium to the hemolymph of mussels. The hemolymph sampled in the posterior adductor muscle represents one fraction of the whole hemolymphatic compartment (the rest being in contact with organs), and the data provided from this analysis only allows the assessment of the presence of total BMAA in this tissue and the subsequent exposure of immune cells contained in it. As the BMAA content in hemolymph remained stable and did not increase in a time-dependent manner from the first day of exposure to day 14, we may hypothesize that hemolymph only transports BMAA into mussel's organs and therefore does not accumulate this toxin, which remains to be investigated. The increase of the total BMAA content (free or bound to peptides or to proteins) in the hemolymph on day 21 of exposure also required further investigations. The total BMAA content measured on day 21 might have been metabolized

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and emitted by some other organs as a degradation product or conjugated molecule for elimination. DNA damages and hemocytes mortality observed in this study suggest that the hydrophilic molecule BMAA may cross the cytoplasmic membrane. As amino-acids diffusion into cells is a minor pathway at physiological concentrations [45], it could be hypothesized that some transporters may allow this toxin to cross membranes. So far, as described in vertebrates' nervous system, pathways that could potentially explain BMAA entrance into cells are (i) the cerebrovascular large neutral amino acid carrier, which allows BMAA to cross the blood-brain barrier in rats [46]; and (ii) the cystine/glutamate antiporter (system xc-), which allows BMAA to cross cells in rats cortical cells cultures [47]. Hemocytes membranes might therefore also have amino-acid transporters that are able to get BMAA into the cell. So far, except for one study carried out on bacteria [48], toxicological studies about the impact of BMAA have been carried out on vertebrates, and the extrapolation of potential mechanisms of action on invertebrates remains tenuous. As BMAA is an emerging environmental neurotoxin, little evidence of animal toxicology is available to date, and its metabolites are also unknown; therefore, it may be difficult to assess if the observed damages where caused by this molecule or its metabolites. DNA damages by BMAA could have been induced either indirectly on the genome or directly by crossing the nuclear membrane. An Ames test on five *Salmonella typhimurium* strains with five BMAA concentrations ranging from 11 to 900 µg/plate has shown that BMAA did not induce any mutation and was neither cytotoxic nor genotoxic for bacteria [48]. A study carried out on human neuroblastoma cell line SH-SY5Y [49] showed, through a plasmid assay, that BMAA is able to induce DNA strand breaks from 309.2 mg/L, potentially linked with the nitrosation of BMAA, which generates an alkylating form that is also able to induce more DNA strand breaks than BMAA itself. DNA damages and cell mortality observed here could also be explained by the ability of BMAA to induce an oxidative stress in cells and/or its potential insertion in or binding to proteins. The induction of an oxidative stress by BMAA has been shown in murine cortical cell cultures; in presence of bicarbonate at physiological concentration, BMAA could form a carbamate adduct that is a glutamate analogue [7]. Under this form, this molecule may thus be able to activate glutamate receptors in motoneurons of the central nervous system, provoking a cascade of reaction leading to excitotoxicity, oxidative stress, and cell death [8,9]. Through the inhibition of cystine/glutamate antiporter in cortical cultures, BMAA could also inhibit the cystine uptake leading to a depletion of glutathione (GSH), thus

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increasing cellular oxidative stress [8]. Glial cells exposed to 0.5–1–3 mM BMAA [50], with and without bicarbonate, showed a relative generation of reactive oxygen species (ROS) compared to untreated samples. At the individual level, the exposure of *D.polymorpha* to 10–100 and 500 µg/L BMAA during a maximum period of 48 h showed no significant alteration in activities of antioxidant enzymes catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), or of the biotransformation glutathione S-transferases (GST) [29]. However, variations of activities of antioxidants enzymes might have been observed in *D. polymorpha* if the study covered a longer period of time. Nevertheless, in the same study, BMAA induced an oxidative stress (i.e., increase of CAT and GR activities) on other freshwater bivalves (*Unio tumidus* and *Corbicula javanicus*) after 24 h and 48 h of exposure, respectively [51]. Oxidative stress has been shown to induce DNA damages that can lead to apoptosis in eukaryotes [52] and could also stress mitochondria, which could lead to DNA damage and even cell death [50]. This oxidative stress might be the first possible pathway of the genotoxicity observed in this study. The mechanism of DNA damages induced by BMAA would deserve to be elucidated by further investigations. In order to study if an oxidative stress is involved in the DNA damages during a BMAA exposure, it could be interesting to use oxidative probes such as DHR-123 [53] or to analyze markers such as 8-hydroxyguanosine, which is used to measure the rate of oxidative damage to nucleic acids and lipids [54]. Also, it could be interesting to use different versions (standard alkaline, modified-) of the comet assay to highlight DNA strand breaks or oxidative DNA lesions.

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Another mechanism that could explain the observed DNA damages is the potential insertion of BMAA into proteins. Indeed, a BMAA insertion into proteins would lead to their misfolding and a subsequent change of their properties, directly or indirectly inducing stress. Exposure of human cell lines MCR-5 and SH-SY5Y to 31 nM of ^{3}H-BMAA and 200–1000 µM of BMAA showed that BMAA was associated with the protein fraction, and this association was positively correlated with protein synthesis. Moreover, the addition of serine in the medium leads to a decrease in the detection of BMAA in the protein fraction [11]. The authors hypothesized that BMAA was misincorporated in human cells in place of the amino acid serine, a phenomenon that could lead to protein misfolding and therefore to a potential cell dysfunction [11]. However, this hypothesis is still a matter of debate and there is yet no evidence that BMAA could be integrated into proteins rather than chemically associated with them [12]. Moreover, this integration into proteins or adhesion might be limited in time: BMAA was

found in neonatal rats of 9 to 10 days, 24 h after 2 days of subcutaneous injection of a dose corresponding from 50 to 600 mg/kg BMAA HCl, but no BMAA was detected at 28 weeks of age [55]. BMAA misincorporation could elicit a stress of the endoplasmic reticulum which, when the accumulation of misfolded protein is excessive, could lead to organelle dysfunction and trigger cell death [56]. If proteins containing proteomimetic amino acids are efficiently degraded or generate truncated proteins, they can give rise to peptides that can induce autoimmune symptoms due to the recognition as non-self-antigens [57].

In this study, the phagocytosis avidity (mean number of beads phagocyted per hemocytes) and efficiency (mean number of hemocytes that have phagocyted three beads and more) did not differ in mussels exposed to BMAA compared to controls. This could suggest that the immune system of bivalves was not activated by BMAA, because it did not recognize this molecule or its metabolites, which is expected for an amino acid. Indeed, hemocytes commonly take charge of particles of the size range of bacteria [58]. On the other hand, it would suggest that the toxin did not interfere with bead phagocytosis by hemocytes, despite the induced genotoxicity on days three and eight of exposure and subsequent cell mortality observed on day 14 of exposure. The absence of impact on both phagocytosis indexes, despite an increase of cell mortality, could be explained by the fact that analyses of phagocytosis have been performed only on cells that were alive. Indeed, those measurements were done with the use of FITC-labelled beads, without the use of a dye that quantifies dead cells, and were performed on events that had the same size and complexity as hemocytes. Because dead cells become smaller and less complex, their signal might be out of our acquisition area. Also, cell mortality has been measured through the use of propidium iodide (PI), a stain excluded by intact membranes. This dye does not rapidly cross intact cells membranes and, if it manages to penetrate them, the molecule is likely to be pumped outside [59]. When membrane integrity is compromised, PI can bind itself non-selectively to nucleic acids [59]. While propidium iodide is used to distinguish living cells from dead ones [60], this dye, in fact, assesses membranes integrity, which reflects cell mortality. Thus, using this probe is only informative of the percentage of cells that are dead. It has been observed that *Saccharomyces cerevisiae* cells can take up PI while undergoing a stress, but membranes of a subpopulation of cells could be later repaired [61]. Thus, it is possible that the increase of PI fluorescence on day 14 of exposure reflects the mortality of most cells but a reversible transient permeability for others, caused by a BMAA-

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induced stress. The absence of genotoxic or cytotoxic effects on day 21 of exposure, while zebra mussels are still exposed to BMAA, could potentially be explained by the establishment of mechanisms that allows hemocytes to remain viable, and functioning, while undergoing a contamination, and/or a BMAA detoxification/metabolization. A mechanism that could explain DNA reparation in this context could be the base excision repair (BER) pathway [52,62]. First, the abnormal base is detected and excised by DNA glycosylases. Then, the remaining sugar is cut, and an undamaged nucleotide is inserted, correcting the site. The BER pathway exists in bivalves and is known to act fast. Indeed, it has been shown that an *in vivo* exposure of the freshwater mussels *Limnoperna fortunei* to pentachlorophenol (PCP) leads to DNA damages that were repaired after 2 h in clean water [63]. If measured genotoxicity and cell death are triggered by protein misfolding, cells could potentially activate the unfolded protein response (UPR) pathways. UPR is a collection of signaling pathways that leads at first to a transient attenuation in the rate of protein synthesis, then an upregulation of genes encoding proteins involved in protein folding, and lastly to the degradation of endoplasmic reticulum-localized proteins [56].

Moreover, as discussed above, the total BMAA measured in the hemolymph on day 21 could have been metabolized or be a degradation product emitted by other organs, and therefore could have lost its toxicity, explaining the absence of effect. This has to be investigated.

Finally, the high dispersion of boxplots showing results of DNA damages and hemocytes mortality in individuals exposed to BMAA can be explained by inter-individual differences in the ability to (i) uptake BMAA from the medium. Indeed, valves movement of mussels is asynchronous and filtration rate may considerably vary between individuals (0.1 to 0.4 L/mussel/h) [43]; (ii) accumulate, metabolize, and eventually detoxify BMAA; and (iii) cope with potent oxidative stress and DNA damages induced by the neurotoxin.

CONCLUSIONS

Through an in vivo exposure of a freshwater mussel, this study constitutes the first evidence that BMAA is present in the hemolympatic compartment of mussels, and induces transient DNA damages on hemocytes, which may lead to a short time cellular death. However, whether this hydrophilic amino acid has directly induced DNA strand breaks and cell cytotoxicity, or indirectly via pathways such as oxidative stress or protein misfolding,

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remained to be investigated. Also, the kinetics of BMAA accumulation and elimination in zebra mussels remains to be deeply investigated via a follow-up of the BMAA content in several organs.

MATERIALS AND METHODS

Mussels Sampling and Acclimation

Zebra mussels were collected at the same spot, at about 5 m depth in March at the Lac-du-Der-Chantecoq (48°36′07.7″ N; 4°44′37.0″ E). In the laboratory, mussels were kept 1 day at 4 °C and in the dark in tanks aerated by an air pump (Zolux, Clisson, France) containing 50% of lake water and 50% of Cristalline® (Saint Yorre, France) source water. Mussels measuring 2 ± 0.3 cm were kept acclimating in the dark only in aerated Cristalline® water at 16 ± 2 °C. They were fed with 2×10^6 cells/individual/day of a 50:50 mix of *Chlorella vulgaris* and *Scenedesmus obliquus* (Greensea, Mèze, France). Algal density was measured with an optic microscope (Primovert, Zeiss, Oberkochen, Germany) and KOVA® slides (Kova slide, VWR, Fontenay-sous-Bois, France)

During the 2 week long acclimation, mussels were randomly divided in two groups in duplicate tanks of 275 individuals that contained 3 L of water and aerated with an air pump. Tanks were put at 16 ± 2 °C with a cycle of 12 h of light and 12 h of darkness. The water was changed every 3 days in order to have the same rhythm as during the exposure.

Exposure

Mussels were exposed for 21 days to dissolved BMAA (L-BMAA hydrochloride B-107, Sigma–Aldrich®, Saint-Louis, MO, USA) at a concentration equivalent to 7.5 µg BMAA/mussel/3 days in two tanks aerated by an air pump (Zolux, Clisson, France) and containing 275 individuals at the beginning of the experiment. Two 3 L control tanks that contained the same number of mussels put in clear water were added. The water was renewed at 3 days of interval in control and exposure treatments, and mussels were fed similarly to during the acclimation period (with 50:50 mix of *Chlorella vulgaris* and *Scenedesmus obliquus*). BMAA spiking was performed at each water change, at 3 days of interval, but mussels were fed two days after it (i.e., one day before water changing) in order to avoid adsorption of BMAA to the algal food. Mussels were randomly collected

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and sacrificed at 0, 1, 3, 8, 14, and 21 days of the exposure period and at 1, 3, 8, 14, and 21 days of the depuration period to perform flow cytometry tests ($n = 10$), COMET assay ($n = 8$) and measurement of the BMAA content in hemolymph ($n = 3$ pools of 2 individuals). During the exposure period, the total amount of BMAA spiked at each water change was adjusted for the number of remaining individuals in order to maintain the exposure concentration of 7.5 µg BMAA/mussel/3 days.

BMAA in Hemolymph

Mussels used for the determination of the BMAA content in hemolymph were placed 24 h in clear water before sampling. This has been done to ensure an evaluation of the BMAA fraction remaining in the hemolymph and not the fraction that would have been early metabolized by individuals or possibly released into the environment. The hemolymph was withdrawn from the posterior adductor muscle using 300 µL-Myjector® (Terumo, Tokyo, Japan) syringes, transferred into Eppendorf® tubes (Eppendorf, Hamburg, Germany), and then put in liquid nitrogen and stored at −80 °C. After being freeze-dried, tissues of 2 individuals were pooled. Total BMAA extraction was done according to previous studies [44]. Briefly, after the addition of D_3BMAA as an internal standard, samples were dried under vacuum then hydrolyzed for 20 h at 105 °C after the addition of 6 M HCl. After a drying step, samples were resuspended in 67% ACN/33% water/0.1% formic acid and analyzed by UHPLC-MS/MS. UHPLC-MS/MS analysis was performed as described in [64] on the same type of equipment but using a 150 mm column instead of a 100 mm column with a 5 mm precolumn. BMAA was well separated from DAB and AEG; the retention time of BMAA and D3BMAA was 9.4 min, the retention time of DAB was 10.8 min, and the retention time of AEG was 11.8 min. For BMAA, the ion ratios were as follows: *m*/*z* 102:88, 27%; *m*/*z* 102:76, 27%; *m*/*z* 102:73, 20%; and *m*/*z* 102:44, 52%. For D3BMAA, the ratios were *m*/*z* 105:88, 31%; and *m*/*z* 105:76, 23%. A 20% relative deviation from these ratios was allowed in the samples. Chromatograms of an analytical standards mixture (Figure S1) and a hemolymph sample (Figure S2) are provided as supplementary information.

Retention times and ion ratios were comparable to those reported in [64]. BMAA concentrations were corrected for the signal intensity of the internal standard. The concentration of BMAA in the hemolymph (in µg/µL fresh tissue) was calculated from the concentration in freeze-dried tissue using a previously established relation between hemolymph fresh weight

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and weight after freeze-drying. As gradation indicated by syringe is not considered precise enough for an exact quantification of the volume sampled, the weight of the hemolymph sample was evaluated. For this, an evaluation of the FW/DW ratio of the hemolymph was performed beforehand. This was done while taking hemolymph from 5 individuals and weighing it. The FW/DW ratio is 275.63 ± 21.93.

Preparation of Samples for Flow Cytometry and COMET Assay Analysis

Hemolymph was withdrawn as described previously, but syringes were coated with L15-15% medium. The medium was prepared as described in [65].

Samples were kept individually in DNase RNase Eppendorf® tubes that were resting on ice to prevent the formation of aggregates. Cell numeration was performed with an optic microscope (Primovert, Zeiss, Oberkochen, Germany) and KOVA® slides (Kova slide, VWR, Fontenay-sous-Bois, France) using 10 µL of hemolymph. During this step, samples were sorted to discard samples that contained other cellular types than hemocytes. Then, each hemolymph sample was divided in two in order to perform the COMET assay and the flow cytometry analysis on the same individual.

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Flow Cytometry

Analyses were performed with a BD Accuri® C6 (Biosciences, Le Pont de Claix, France). A volume corresponding to 5×10^4 hemocytes was used to study the phagocytosis, and an aliquot of the same volume was used to study the mortality. Hemolymph samples were put individually into a 96 well plate. To each sample was added L15-15% medium. Then, the plate was incubated 4 h at 16 °C. 15 min before the end of the incubation, cells were detached with a 1 × trypsin/EDTA mix and incubated 1 min in the dark at room temperature.

Mortality assay

To study the cell mortality, 1% of propidium iodide (IP, Sigma-Aldrich®, Saint-Louis, MO, USA) was added. Propidium iodide is excluded by live cells but enters permeabilized cells and binds DNA by intercalating between the bases; it produces fluorescence that was measured at 670 nm.

Phagocytosis efficacity and avidity index

Cells samples were put individually into a 96 well plate. To each sample was added L15-15% medium, and FITC-labelled beads of 2 µm diameter were added to have a ratio of 50 beads per hemocyte. The plate was then incubated for 4 h in the dark at 16 °C. Fifteen minutes before the end of the incubation, cells were detached with a trypsin solution as described before. The fluorescence was measured at 530 nm. The phagocytosis efficacity is represented by the percentage of cells that have phagocytosed 3 beads or more; the avidity index is represented by the number of beads phagocyted on average per individual.

DNA Integrity Measurements

Due to technical constraint, the measure of DNA strand breaks has been performed on 8 of the 10 sampled individuals. Thus, those containing gametes have been set aside.

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DNA strand breaks were quantified by the alkaline version (pH > 13) of the comet assay, as described [66] with minor modifications. All steps described below were performed under an inactinic light and at 4 °C to prevent any additional DNA damage. The experimental controlconsisted of a THP-1 cell line, provided by ATCC® (Manassas, VA, USA), maintained in lab, and exposed or not to UV rays (λ= 254 nm) for 1 min. Results show us that DNA damages were 19.05 ± 9.59% for negative controls and 42.35 ± 9.43 for positive controls. An ANOVA test showed that DNA damages of negative controls were significantly significant compared to positive controls ($p < 0.01$).

The optimal concentration of hemocytes for the comet assay was between 600,000/mL and 1,000,000 cells/mL to avoid overlapping comets and ensure the fast scoring of results. First, 100 µL of cell suspension was mixed with an equal volume of 1% (*w/v*) Low Melting Point (LMP, type VII) agarose in PBS Ca^{2+} and Mg^{2+} free (10 mM, pH 7.4, 37 °C). Two 30 µL-drops (gel replicates) of the previous mixture were deposited on a superfrosted microscopic slide, previously precoated with 0.8% (*w/v*) Normal Melting Point (NMP, Type I) agarose, which were then covered with a coverslip. Slides were cooled for 10 min until the agarose layer harden. After agarose solidification, the coverslip was removed and microscopic slides were placed in a Coplin jar filled with a cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, with 1% Triton X100 and 10% DMSO added immediately before use) for at least 1 h at 4 °C in the dark. After cell

lysis, slides were rinsed with chilled buffer and then were gently placed in a horizontal electrophoresis chamber (24 cm × 27 cm × 7 cm; W:L:H) filled with a freshly prepared chilled buffer (i.e., 300 mM NaOH, 1 mM Na_2EDTA, pH > 13). Electrophoresis was performed at 0.83 V/cm for 24 min to unwind DNA. After electrophoresis, slides were washed for 20 min in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) before being dehydrated in absolute ethanol (5 min). Each slide was then stained by adding a 30 µL-drop on each gel with a 1X SYBR Green I dye solution according to manufacturer's recommendation and then observed under an epifluorescence microscope (Laborlux S, Leica®, Wetzlar, Germany) equipped with the appropriate dichroic filter. For each slide, a minimum of 50–100 comets was blindly and randomly scored on the central part of the gel. The Tail intensity parameter given by the Comet assay IV software (Perceptive Instruments®, Bury Saint Edmunds, UK), corresponding to the percentage of DNA in the tail of the comet (% tDNA), was chosen for expressing DNA damage. According to the non-normal distribution of comet assay data (% tailDNA), we have considered the median value for each replicate or gel for a mussel. Finally, the mean of the two medians for a mussel (2 replicates or gels by mussel) has been considered in the statistical analysis.

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Statistics

Statistical analysis was performed with Statistica (Version 8.0.360.0, Statsoft, Tulsa, OK, USA, 2007). The normality has been studied with a Shapiro-Wilk test, and the homogeneity of variances was studied with a Levene test. To compare controls and exposed individuals of a same sampling time, a Kruskall-Wallis test has been done. Then, when the p value obtained was significant ($p < 0.01$), a Mann-Whitney test was done. To compare multiples independent conditions, a Kruskall-Wallis test was performed. Here, only data with $p < 0.01$ were considered as significant.

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AUTHOR CONTRIBUTIONS

A.L. and E.L. conceived and designed the experiments; A.L., I.B., F.B. and N.M. performed the experiments; A.L. analyzed the data; A.L. wrote the paper and E.L., E.F., M.B., D.R., M.P.-L. and A.G. contributed to the analysis and revised the paper critically.

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Chapter 4

Vitamin E Attenuates Neurotoxicity Induced by Deltamethrin in Rats

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ABSTRACT

Background

The safety of Deltamethrin (DM) has been raised as a point of concern. The current investigation was envisaged to explore the responsiveness of oxidative stress parameters, DNA fragmentation and expression levels of

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TP53, cycloxygenase 2 (COX2) and cytochrome p4502E1 (CYP2E1) as toxicological endpoint in rats treated with DM. as well as attention was provided to the neuroprotective effect of vitamin E (VE).

Methods

Four different groups of rats were used in this study, group I served as control, group II received DM (0.6 mg/kg BW), group III received both DM plus VE and finally group IV received VE only (200 mg/kg BW). The treatment regimen was extending for one month for all groups and the brain tissues were collected for further analysis.

Results

The obtained results showed a highly statistically significant increase in lipid peroxidation (LPO) content, nitric oxide concentration, and DNA fragmentation percentage and expression level of CYP2E1, TP53 and COX2 genes, in addition statistical significant reduction in total antioxidant capacity in DM treated group as compared to control were detected. Oral administration of VE attenuated the neurotoxic effects of DM through improvement of oxidative status, DNA fragmentation percentage and suppressing the expression level of CYP2E1, TP53 and COX2 genes.

Conclusion

From this study we concluded that VE supplementation has beneficial impacts on DM neurotoxicity in rats through its antioxidant and antiapoptotic properties.

Keywords: Deltamethrin, Brain, Apoptosis, Oxidative stress, Vitamin E

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BACKGROUND

Although pesticides become instrumental in achieving a significant elevation in crop productivity, they cause serious ecological hazards to the non-target organism [1]. According to WHO [2] roughly three million cases of pesticide poisonings occur annually and an excess of 250,000 deaths worldwide had been reported. The human exposures to pyrethroids were increased since they had been replaced the organophosphorus insecticides [3]. Owing to its low toxicity and its high potency in eradication, DM had become an insecticide of choice in most countries [4]. Although initially

thought to be the safest available insecticide, a number of recent reports have been published on its toxicity effect on human, domestics and experimental animals [5, 6]. The direct exposure to DM vapours or consumption of polluted food and water are the most common routes of intoxication [7]. DM induced several pathological changes including inhibition of mitotic index, chromosomal aberrations [8], and induction of histological alterations in several important organs [9]. Recently, attention has been focused on the potential relationship between DM exposure and neurodegenerative diseases [10, 11]. DM creates serious problem because its accumulation in fatty tissue like brain [12]. Whereas the exact mechanism of DM induced neurotoxicity is still poorly understood. Accumulation of DM in body systems increases the reactive oxygen species (ROS) production leading to oxidative stress and apoptotic cell death [5]. The deleterious effects of free radicals accumulation included damage to all macromolecules including proteins, lipids, and nucleic acids. This is believed to be involved in the etiology of many neurodegenerative diseases [12, 13]. Under normal circumstances, the body is endowed with effective antioxidant systems to combat the menace of oxidative stress. Though, in extreme oxidative challenge, such as those observed in pesticide poisoning [14], the body's antioxidants machineries are overwhelmed. Vitamin E is considered as the most important lipid-soluble antioxidant that protects the brain from oxidative hazard. Many authors reported the neuroprotective influence of VE [15, 16]. VE acts upon cell membranes and has the ability to neutralize compounds which may potentially disrupt membrane stability [17]. Administration of VE decreasing the rate of LPO [11], decreases the autophagy and neuronal death [18], prevents the cytochrome oxidase dysfunction, decreases high energy phosphate compounds and NO in different brain regions as well as it increases the expression level of antiapoptotic gene *Bcl-2*[19] leading to decreased severity of neuronal damage. Although previous studies have already presented the toxicity induced by DM, data on neurodegenerative toxicity are scarce. Moreover, there are very limited studies evaluating the neuroprotective effect of VE against DM toxicity. Therefore; the current study was carried out to investigate the protective influence of VE against DM neurotoxicity through monitoring its effect on oxidative status, DNA fragmentation percentage and the expression level of CYP2E1, TP53and COX2 genes in brain tissue.

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METHODS

Animals

Forty male albino rats, weighing 150–170 g were maintained under standard conditions with free access to food and water. The animals were reared according to the principles of the "Guide for the care and Use of Laboratory Animals" prepared by Beni-Suef University. The Animal care and Use committee of Beni-Suef University approved the study. All efforts were made to minimize animal suffering.

Chemicals

Deltamethrin (>99% pure) was obtained from KZ pesticide company (Egypt). Vitamin E (α tocopherol) and the rest of chemicals were purchased from Sigma Aldrich chemicals, USA.

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Experimental Protocol

After two weeks of acclimation, rats were randomly divided into four equal groups. The group (I) administered corn oil (1 ml/kg BW) and act as control group. The group (II) received oral dose of DM (0.6 mg/kg BW). The group (III) was orally received DM (0.6 mg/kg BW) in combination with VE at a dose of (200 mg/kg BW). The group (IV) was given VE only (200 mg/kg BW). All treatments were continued for 30 days and taken once daily via oral gavage. DM and VE were prepared by dissolving in corn oil. The selected dose of DM was based on previous studies in which 1/10 LD50 induced biochemical alteration in rat without morbidity [20]. Animals were treated with VE in dose that was proved to have neuroprotective effect [21].

Sampling

All the animals were sacrificed at the end of experiment. Brain was immediately removed and used for further biochemical analysis, DNA fragmentation assay and RNA extraction.

Biochemical Analysis

Brain samples were homogenized in 0.1 M cold phosphate buffer saline (pH 7.4) using Teflon pestle. The homogenates were centrifuged at 14,000 × *g*for 15 min at 4°C. The supernatant was used for measurement of neural LPO expressed as malondialdehyde (MDA) content according to method

described by Placer et al. [22], nitric oxide concentration (NO) [23] and total antioxidant capacity (TAC) using commercial kit (purchased from Bio diagnostic company, Egypt).

DNA Fragmentation Assay

Apoptotic changes in the brain tissue were evaluated colorimetrically by DNA fragmentation percentage using the diphenylamine (DPA) assay and DNA laddering assay using agarose gel electrophoresis according to the method described by Perandones et al. [24]. Brain samples were homogenized in hypotonic lysis buffer and centrifuged for 15 min at 14,000 rpm. The supernatants containing small DNA fragments were separated; one-half of the volume was used for gel electrophoresis and the other half together with the pellet containing large pieces of DNA were used for quantification of fragmented DNA.

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Isolation of Total RNA and Real-time PCR (qPCR)

Total RNA was purified from 100 mg of rat brain tissue using Qiagen Rneasy Mini Kit following the manufacturer's protocol. Purity of total RNA was measured spectrophotometeircally (Thermo Scientific, USA). The isolated RNA was reverse transcribed into cDNA and used for PCR with primers specific for CYP2E1, TP53, COX2 and GAPDH (Table 1). The mRNA expression levels of TP53, COX2 and CYP2E1 genes were assessed using real time PCR standardized by co-amplification with the housekeeping gene GAPDH, which served as an internal control. Real-time PCR was done in (color for research Laboratory. Qiagen. Egypt). cDNA was added to a SYBR Green qPCR Master Mix (Qiagen) containing 30 pg/ml of each primer. The cDNA was amplified by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 45 s. During the first cycle, the 95°C step was extended to 1 min. The GAPDH gene was amplified in the same reaction to serve as the reference gene. Gene expression levels were calculated and determined following the method described by [25].

Table 1: Primers sequences

Gene	Direction	Primer sequence	Product size	Reference
CYP2E1	Forward	TCCAGGTTTGCACCAGACTCT	76 bp	[26]
	Reverse	TCCTCGCTCCTCCTGAGAAG		

TP53 Exon 7	Forward	GTG GTA CCG TAT GAG CCA CC	157 bp	[27]
	Reverse	CAA CCT GGC ACA CAG CTT CC		
COX2	Forward	AAA GCC TCGTCCAGATGCTA	249 bp	[26]
	Reverse	ATGGTGGCTGTCTTGGTAGG		
GAPDH	Forward	ACCACAGTCCATGCCATCAC	460 bp	[28]
	Reverse	TCCACCACCCTGTTG CTGTA		

Statistical Analysis

The data were statistically analyzed by SPSS version 16.0 statistical package. Data expressed as the mean ± SE. Differences between the groups were assessed using one way analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$.

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RESULTS

Oxidative Stress Parameters

The results obtained from Table 2 proved that DM was able to induced oxidative damage. Administration of DM provoked a statistical significant elevation in the level of MDA; the late biomarker of oxidative stress and the good indicator of the degree of LPO in comparing to control one. In the same consequence significant increase in NO concentration was detected. On the other hand a significant reduction in TAC was observed. Oral administration of VE in combination with DM caused a significant reduction in the MDA by 47.67% and NO concentration by 41.24%. Whereas; a significant elevation of TAC by 66.6% was clear in the group III when compared to group II (Table 2). No significant changes were detected between control and VE treated group.

Table 2: Effect of DM treatment on oxidative stress parameters and protective influence of VE supplementation

Parameters	Group I	Group II	Group III	Group IV
MDA (U/g tissue)	3.17 ± 0.25^{b}	10.99 ± 1.16^{a}	5.75 ± 0.98^{ab} (47.6%)	2.93 ± 0.84^{b}
NO (mmol/L)	2.6 ± 0.4^{b}	6.98 ± 0.25^{a}	4.1 ± 0.12^{ab} (41.24%)	2.5 ± 0.33^{b}
TAC (μ mole/L)	0.289 ± 0.003^{b}	0.15 ± 0.0057^{a}	0.25 ± 0.007^{ab} (66.6%)	0.29 ± 0.009^{b}

Data are expressed as mean ± SEM for 10 animals per group. [(a)]Represents significant difference from control in the same row while [(b)]rep-

resents significant difference from DM treated group at $p < 0.05$ in the same row. (% Represent the protective percentage of VE against DM toxicity).

DNA Fragmentation Assay

The adverse effect of DM on DNA was evaluated by measuring the level of genomic DNA fragmentation percentage using the DPA assay (Figure 1a) and detecting DNA laddering on agarose gel electrophoresis (Figure 1b). Compared to the control group, DM induced marked increase in DNA fragmentation level. The VE treated group (III) showed a significant reduction in the DNA fragmentation percentage by 41.6%. The results presented in (Figure 1b) showed marked DNA laddering pattern induced by DM. VE administration proved to reduce the DNA laddering pattern. Lacking of DNA laddering was observed in both group I and IV.

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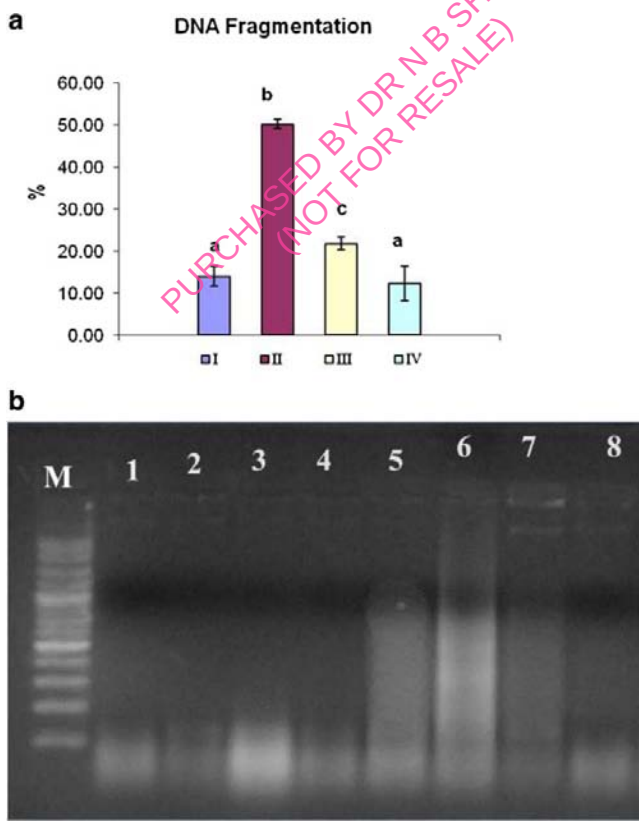
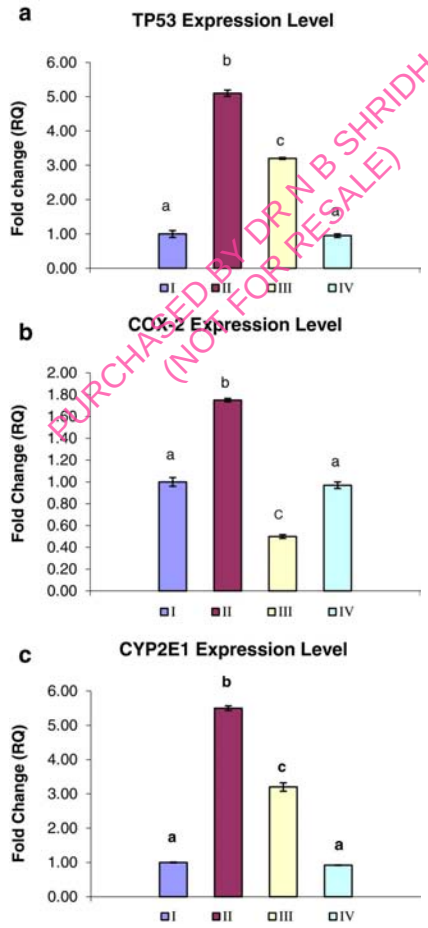


Figure 1: Protective effect of VE against DM-induced DNA damage in rat's brain. (a) DNA fragmentation % by DPA assay; control (I), DM-treated group

(II), DM + VE group (III) and VE group (IV). (b) The electrophoretic pattern of small DNA fragments on 1.5% agarose gel electrophoresis. Control group (lanes 1, 2) vitamin E control group (lanes 3, 4) DM group (lanes 5, 6) and DM + VE treated group (lanes 7 and 8) M 100 bp DNA marker. Values are expressed as mean ± S.E. Different superscripts letters represents significant difference ($p < 0.05$).

Gene Expression

Apoptosis, a form of programmed cell death, is tightly regulated by the expression of several genes.



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Figure 2: Real-time PCR Quantitation of mRNA expression level of TP53 (a), COX-2 (b), and CYP2E1 (c): control (I), DM-treated group (II), DM + VE

group (III) and VE group (IV). Values represent fold increases in mRNA level over the control group. GAPDH was used as an invariant internal control for calculating mRNA-fold changes. Values are expressed as mean ± S.E. Different superscripts letters are significantly different ($p < 0.05$).

In the present study we analyzed the expression of three genes in rat's brain using quantitative real-time RT-PCR. DM upregulated the expression level for TP53 mRNA to 5.1 fold (Figure 2a). The treatment of rats with VE resulted in reduction in the expression level to 3.2 fold. Furthermore the expression level of COX2 showed 1.75 fold increase in the group II, whereas, the VE ameliorate the adverse effect of DM by decreasing the expression level to the half compared to control (Figure 2b). CYP2E1 showed overexpression after DM intoxication reach to 5.5 fold. Oral administration of VE attenuated the expression to 3.2 folds compared to control (Figure 2c).

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DISCUSSION

In the present investigation we employed advanced methods to uncover the molecular mechanisms of neurotoxicity and apoptotic effect of DM in rat's brain. We assessed the effect of DM on oxidative status, DNA fragmentation percentage and the gene expression level of TP53, COX2 and CYP2E1 and also evaluated the ameliorative role of VE; hence the neurodegenerative studies of DM are limited. ROS are continuously produced inside the mammalian body [29]. Oxidative stress occurred as a consequence of imbalance between the production of free radicals and the antioxidative process leading to instabilities in cellular physiology and apoptotic cell death [29]. In general, pesticides intoxication produces oxidative stress through over production of free radicals and induces tissue LPO in mammals and other organisms [30]. The reduced concentration of TAC and elevation of MDA and NO concentration observed in our present study, suggested that DM causes neuronal damage and the pathogenesis may be through the generation of free radicals and oxidative hazard which certainly play a vital role in the pathogenesis of brain injury. Those results are consistent with the literature [17, 31, 32, 33, 34]. The brain tissue contains large amounts of oxidizable substrates such as polyunsaturated fatty acids which are the main target of ROS causing LPO [35]. NO is a potent free radical known to be cytotoxic to neurons and glial cells through its reaction with superoxide and generation of highly reactive radical peroxynitrite [36]. Accumulation of DM in body systems increases the ROS production and leads to depletion

of antioxidant parameters which was monitored by significant reduction in TAC (Table 2). The hydrophobic lipophilic nature of DM may be the main causative agent for its accumulation in body systems; ROS production and DNA damage [34]. DNA damage has been proposed as useful parameter for assessing the genotoxic properties of environmental contaminants [37]. DNA fragmentation is considered as a marker and typical characteristic feature of apoptosis [38]. Oral administration of DM had significantly increased DNA damage percentage (Figure 1a). Those results come in the line with data reported by [11, 39]. the oxidative stress induced by DM causes depletion of mitochondrial energy through inhibition of Na, K-ATPases, induction of proteolytic enzymes leading to DNA fragmentation and apoptosis [40]. Also inhibition of ATPases reduces energy supply in neurons leading to neuronal damage [41]. In the same consequence DM caused significant elevation to neutrophiles which play essential role in free radical mediated injury through the extracellular release of superoxide, which is cytotoxic [42]. Our results reported in (Figure 2) are in accordance with other works [5, 43] which suggested that DM induces neurodegeneration and apoptotic cell death in the brain tissue. The ability of DM to cause apoptosis may be contributed to its potential to initiate a series of cell death signaling events that finally lead to DNA fragmentation [11]. According to the results in our study, oral administration of DM increases the mRNA expression of the pre-apoptotic gene TP53 (Figure 2a). Such result is in harmony with those obtained by [38, 43, 44]. Increased ROS production and subsequent DNA damage might be reasons for this overexpression [45]. In oxidative stress p53 protein is activated through multiple posttranslational events including phosphorylation [46]. Activated p53 triggers a number of signaling pathways that may lead to cell cycle arrest and apoptosis [38]. In the CNS, the apoptotic gene (COX2) is expressed under normal conditions [47]. Moreover, it has been demonstrated that COX2 expression is regulated by TP53 gene [48]. According to our finding we detected that, overexpression of COX2 upon DM toxicity. Kim et al. [49] reported that ROS are considered as mediators for COX2 expression. ROS serve as intracellular signals for activation of gene expression through the involvement of specific redox-sensitive signaling pathways and transcription factors [50]. The pervious findings regarding the metabolism of DM revealed that the vast majority of its metabolism occurs in the liver while the extrahepatic metabolism has been reported in other tissues like brain [51]. In rats, the main reaction involved in DM detoxification is ester cleavage, by cytochrome P450 (CYP) enzymes and carboxyesterase action [52]. CYP2E1 is a widely distributed

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isoform in the brain and is the main inducible CYP against environmental chemicals [53]. The regulation of CYP2E1 expression was occurred through several mechanisms such as protein stabilization and increased translational efficiency which implicated by xenobiotics [54]. Although CYP2E1 plays important role in neuronal detoxification [55], its induction in the brain could increase the risk of neurotoxicity. CYP2E1 bioactivities several cytotoxins to their reactive intermediates and generate ROS and substrate-derived radicals especially when it is induced [56]. Those may be the main cause for CYP2E1 overexpression after DM intoxication (Figure 2c). Those results were in the line with that reported by [53, 57]. In the current study, the oral administration of VE (200 mg/kg) as antioxidant proved to reduce the neurotoxicity induced by DM through statistical significant reduction for MDA, NO concentration and elevation of the TAC (Table 2). Moreover, VE significantly reduced the DNA fragmentation percentage (Figure 1a) and DNA laddering pattern (Figure 1b). In addition VE attenuated the overexpression of TP53 by 37.2%, COX2 by 50% and CYP2E1 by 41.8%, in compared to DM treated group (Figure 2). Oxidative stress plays a major role in DM induced toxicity. Vitamin E is a potent antioxidant which is reported to ameliorate the effect of many known chemotherapeutic agents and pesticides as well. Many previous literatures showed the protective effect of VE against DM intoxication [58, 59, 60]. Vitamin E plays a central role against oxidative damage in neuronal tissue as it is the major lipid-soluble chain breaking antioxidant which effectively protects against neuronal damage [19]. VE maintained the activities of membrane bound enzymes at near normal values and thus preserving mitochondrial membrane integrity and protected enzyme activities from oxidation by free radicals [61]. The protective effect of VE against DNA damage comes in the line with the result reported by [62]. VE play an important role in prevention of neuronal death not only by elimination of ROS through its antioxidant ability, but also through non-antioxidant properties like influencing cellular signaling and transcriptional control [63, 64].

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CONCLUSION

In the current investigation we can concluded that, DM is a potential neurotoxic pesticide, it induced degeneration and apoptotic cell death in rat's brain. On the other hand, VE is proved to be a potent antioxidant which ameliorates the adverse effects triggered by DM.

AUTHORS' CONTRIBUTIONS

Prof Dr. AA participated in the design of the study, Dr. MI carried out the molecular genetic studies, Dr. HA carried out the biochemical parameters helped to draft the manuscript. Dr MK carried out the biochemical parameters and draft the manuscript. All authors read and approved the final manuscript.

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SECTION III: VETERINARY IMMUNOTOXICITY

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Chapter 5

Metallic Nanoparticles: General Research Approaches to Immunological Characterization

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ABSTRACT

Our immunity is guaranteed by a complex system that includes specialized cells and active molecules working in a spatially and temporally coordinated manner. Interaction of nanomaterials with the immune system and their potential immunotoxicity are key aspects for an exhaustive biological characterization. Several assays can be used to unravel the immunological features of nanoparticles, each one giving information on specific pathways leading to immune activation or immune suppression. Size, shape, and surface chemistry determine the surrounding corona, mainly formed by

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soluble proteins, hence, the biological identity of nanoparticles released in cell culture conditions or in a living organism. Here, we review the main laboratory characterization steps and immunological approaches that can be used to understand and predict the responses of the immune system to frequently utilized metallic or metal-containing nanoparticles, in view of their potential uses in diagnostics and selected therapeutic treatments.

Keywords: Nanoparticles, immunology, cellular models, animal models, inflammation

INTRODUCTION

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Nanoparticles (NPs) are extremely attractive for several biomedical applications, due to their capability to interact with molecular or cellular processes and the possibility to influence their functions [1,2,3,4,5]. Specifically functionalized NPs have the potential to overcome some limits of many traditional therapeutics, such as poor water solubility or lack of target specificity [6]. Metal-based NPs possess unique physicochemical properties, offering many advantages and potential usages [7,8] (Table 1). For example, the peculiar optical properties of plasmon-resonant noble metals (i.e., Au, Ag, Pt, and Pd) and luminescent semiconductor nanocrystals (quantum dots (QDs)) make them useful as markers for biological systems imaging [9,10,11,12,13,14,15,16,17,18,19,20,21]. Gold nanoparticles (Au NPs) find a wide spread use in biological applications, such as cancer therapy [22], cell labelling [23], drug delivery [24], and diagnostics [25]. Aluminium (Al) and iron oxide (Fe_2O_3) NPs have been proposed as drug delivery systems [26,27,28]. Fe_2O_3 can be used exploiting their magnetic features to drive loaded NPs to specific target tissues by applied magnetic fields. Furthermore, other metal-based NPs have been deeply investigated as candidates for novel antimicrobial applications. Silver NPs are, indeed, widely used in medicine, and in common household products as additives with antimicrobial activity against more than 650 different types of disease-causing organisms, including viruses [29,30,31,32,33,34,35,36,37,38]. Along with Ag, TiO_2 and Cu NPs also show strong antibacterial activity [39,40]. Different applications of Ag NPs have been explored, such as cancer therapy and wound healing, spreading their exploitation in several fields of biomedicine [41,42,43,44]. Interestingly, the catalytic properties of metal NPs could suggest their potential use in oxidative stress-based disease, as indicated by Reactive Oxygen Species (ROS)-scavenging activity of Pt NPs

[45,46]. However, the same properties may lead to unexpected outcomes when NPs interact with biological tissues. Indeed, the biological effects of materials at the nanoscale cannot be anticipated by the knowledge of the same materials in the bulk form [47,48,49]. The understanding of the cause–effect relationship between the nanomaterial properties and their interference with biological processes would enable the prediction of unforeseen hazards and the synthesis of "safe-by-design" nanomaterials [50]. The safety assessment of NPs must include the consideration of three major topics: an NP's physicochemical characteristics, fate (biological or environmental), and (re)activity [51].

Table 1: Examples of frequently used metal nanoparticles in biomedicine

Application	Nanoparticles	References
Antimicrobial	Ag	Hardman et al., Lansdown, El-echiguerra et al. [30,31,32]
	Cu	Cioffi et al. [40]
	TiO_2	Kubacka et al. [39]
Cancer therapy	Ag	Wieder et al. [44]
	Au	Pissuwan et al., Zharov et al. [22,43]
Diagnostic	Au	Valentini et al. [25]
Drug delivery	Au	Voskerician et al. [24]
	Al	Tyner et al. [26]
Imaging	Ag	Caro et al. [11]
	Au	Sharma et al., Manohar et al., Cole et al., [9,20,21]
	Pt	Deyhimihaghighi et al. [19]
	Pd	Park et al. [12]
	Fe_2O_3	Corot et al., Gupta et al. [27,28]
	QDs	Wang et al., Kim et al., Stroh et al., Michalet et al. [14,15,16,17]
ROS scavenging	Pt	Moglianetti et al., Gatto et al. [45,46]
Wound healing	Ag	Poon et al., Fong et al. [41,42]

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The compatibility with the immune system (IS) represents a crucial issue to comprehend an engineered NP's biological fate, and to approach feasible nanomedical applications [52]. The IS is organized in a complex defense structure, tailored by evolutionary processes. It has a protective

role against foreign bodies, toxic and pathogenic entities considered "non-self" or dangerous, which are safely confined or eliminated [53]. Among the mechanisms aimed at entrapping non-self bodies, the major importance of the so-called "opsonization" phenomenon is recognized. This event involves specific proteins, like antibodies, the complement enzyme-protein system, or membrane receptors with the capability of firmly bind other structures, such as lipoproteins or sugars [54].

Specialized immune cells expressing many of these receptors belong to the innate immune system, the fast responding and non-specific defense against any invading threats for the organism. These cells are characterized by two main activities: phagocyting and/or killing pathogens. In particular, neutrophils, macrophages, and dendritic cells perform phagocytosis driven by those receptors whose cognate agonists "label" the microorganism. On the contrary, NK cells and granulocytes release cytotoxic substances once stimulated or in contact with warning signals.

Two major classes of key membrane proteins mediating immune responses are the antibody-binding fragment crystallizable region receptors (FcRs) and the membrane associated pattern-recognition receptors (PRRs), binding to a variety of conserved pathogen-associated molecular patterns (PAMPs), which distinguish the foreign bodies, such as viruses or bacteria [53]. A set of soluble proteins binding microorganisms is the complement system. Its name reminds the "complementary role" to antibodies, participating together in the several mechanisms to remove intruders and damaged cells [55]. Such enzyme-activated pathway of opsonization is gaining evidence as one of the most important biological effects of nanoparticle delivery [56].

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NPs released in the biological milieu adsorb several of the described molecules on their surface, facilitating the interception and internalization by immune cells. The set of molecules adsorbed onto the NPs is generally called "corona". Most of them are recognized by cell surface receptors which are able to uptake the opsonized particle by different pathways, often depending on the NP–corona complex size [57]. Hence, engineered man-made materials could be considered as non-biological danger signals for the immune system and may, either directly or indirectly, activate phagocytosis. Hence, it is postulated that immune cells could sense nanomaterials, which are designated as nanoparticle-associated molecular patterns (NAMPs) [58]. This may be accomplished via surface-adsorbed biomolecules, or it could result from the recognition of specific structures of the nanomaterial per se.

NPs or NP–protein aggregates may accumulate in the subcellular

compartments, interfering with the physiological immune functions and triggering inflammatory signaling [59]. For example, in macrophages, different metallic NPs or non-degradable particles can accumulate and activate cytosolic inflammasome complexes [60] which regulate the proteolytic enzyme caspase-1 [61]. Caspase-1 induces the maturation of proinflammatory cytokines, in turn, triggering inflammation [62]. On the other hand, the mechanism of NP escape from the cell is not so clear [63], although evidence of the exocytosis mechanisms has been described [64].

Physicochemical characteristics, like size, shape, and surface chemistry of the particles, regulate most of the aspects related to the bio-interaction [65]. Moreover, additional materials used to increase metal NP solubility or as biocompatible coatings, may also change NP interface with the biological environment, including their immunological profile [66]. Although significant improvements in nanotechnology have occurred in many years of research, precise relationships between NP physicochemical features and their biological outcomes still represents an arduous task to be elucidated. Major challenges in immunological studies require meticulous characterization and the choice of suitable experimental models. The intrinsic properties of nanoscale materials often complicate the resolution of several assays and instruments currently used in biochemistry, leading to many inconclusive and contradictory data on NP-induced responses [67].

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The focus of this review is to outline the main characterization steps and the current immunological approaches to understand IS interaction and its inflammatory responses to commonly used metal-based NPs.

IN VITRO

In vitro studies are a fast approach to evaluate NPs' reactivity and induced toxicity, as well as their cellular uptake and inflammatory potential [68]. In order to make a meaningful comparison among different experimental results across different studies, it is imperative to carry out a precise nanomaterial characterization, along with standardization of the experimental protocols.

Currently used *in vitro* methodologies require high accuracy in the assessment of the potential NP-induced immunotoxicity, as several issues may compromise the results. These include the choice of the appropriate cell culture model; NP dose and dose metric; relevant and suitable positive and negative controls; the assay format; the selected endpoint; the NPs' interference with the assays; and, last but not least, the understanding of assay

predictability for the corresponding immunotoxicity *in vivo*. Sometimes, it is undervalued the importance of reliable cell culture models mimicking the actual human immune systems. Different immune cells perform different tasks, and they have diverse protein expression profiles and uptake mechanisms. In addition, frequently used *in vitro*assays involve murine or human cell lines (tumor derived or "artificially" transformed) demonstrating advantageous robustness and facile growing conditions, but may not reflect the natural conditions of the primary cells.

Although the use of cell lines in monoculture systems is recommended for the first stage of safety evaluation, the relevance of specific, more advanced *in vitro* models (i.e, co-cultures or three-dimensional (3D) models) has been highlighted. These models would be preferable for assessing NP interactions and cellular effects, in order to overcome the lack of phenotypic details, physiological functions, and complex cell crosstalk of the traditional monocellular type cultures [69]. For instance, to study gastrointestinal NP exposure, a feasible *in vitro* model could be the simulation of the intestinal barrier by enriching the epithelial cell layer with macrophages, in order to comprehend their functional crosstalk in the generation of inflammatory responses [70]. The cellular model also influences the exposure duration and the relative endpoint assessed, posing limitation of sub- and chronic exposure studies. Major restrictions depend on the fast cell division of most cell lines or the possible de-differentiation during prolonged culture time. In this regard, *in vitro* systems might not be suitable to evaluate any potential dysregulation of immune system long-term responses [71].

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NP Dose

Metal NP dispersion in liquid media represents a critical condition to assure the expected dose to be delivered. The NP suspension preparation must be optimized to avoid aggregation, dissolution, or detachment of functional ligands that could affect the final testing concentration. The cell culture media also alters NP characteristics, mainly because of the presence of serum proteins forming the protein corona and affecting NP agglomeration, sedimentation, as well as their overall biological identity. The serum source (i.e., bovine, calf, horse, human), its treatment and manipulation (i.e., heat-inactivation), as well as the final concentration in culture (e.g., 10% or 1%) used can affect NP interaction with cells, modulating particle uptake and toxicity. Serum interference could be avoided by performing *in vitro* experiments in serum-free conditions, but serum-component deprivation is an unrealistic condition, strongly affecting cell behavior and viability.

The choice of an appropriate dose of exposure could compromise immunotoxicity of a certain NP. Albeit *in vitro*, the assessment of potential immune response should be based on a realistic human exposure to NPs [72,73,74]. Often, unrealistic high concentrations are used for the determination of NP effects. An obstacle is represented by the evaluation of the discrepancy between the administered and the deposited NP amount in the experimental dishes. Dosimetry curves for metal NPs could be defined by elemental analysis of cells treated with metal NPs by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or inductively coupled plasma mass spectrometry (ICP-MS), which are precise methods to evaluate plausible NP concentrations targeting the cells. Unfortunately, these methods do not discriminate between the number of NPs stuck on the cell surface and the ones inside the cell. Many cell washing steps at the end of metal NP administration are helpful, although the procedure of etching metal NPs from the surface would be suggested before NP quantification [75].

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NP Interference

It is worth reminding that specific physicochemical properties of NPs, in particular the metal ones, can interfere with established tests originally developed for biological samples using conventional chemicals [76]. Due to their increased surface to volume ratio, NPs display an increased adsorption capability. The affinity to bind polypeptides may strongly influence the readout of protein concentration or their activity [77]. Due to their optical properties, metal-based NPs present in the reaction mixture or in cell culture medium compromise several assays based on light absorption or fluorescence detection [78,79,80]. The possible NP interferences with the optical reading (absorbance, luminescence, or fluorescence) could lead to false positive results. This imposes several controls, including the NP suspension alone, that may cause optical effects by itself. False results could also arise from peculiar catalytic properties of some metallic NPs.

A comprehensive and reliable understanding of NP effects on the immune cells can be achieved only using specific assays, but, despite global efforts, there are no universal established standards for the abovementioned areas [71,72,81].

Cytotoxicity

The impairment of cell viability is the first parameter to evaluate the NP potential immune response. Many observed inflammatory outcomes are

actually due to cell death and intracellular molecule release in the medium, emerging as consequences of cell or tissue damage.

Since several cell types are commercially available, cells belonging to the presumed target tissue of our nanomaterial should be preferred. Following this rationale, toxicity tests should probe the viability of the immune cells patrolling the specific tissue of interest. For example, NPs designed for the release of drugs in the bloodstream could be monitored for their potential dose-dependent cytotoxicity with endothelial cells and blood resident immune cells. Equally, brain-aimed NPs must be tested with neurons, glia, and microglia cells.

As a result of several cellular processes, the viability could be assessed considering different outcomes: detection of mitochondrial activity (MTT, MTS, WST colorimetric assays) [82], evaluation of necrotic and apoptotic processes (LDH release, annexin V/propidium iodide staining, caspase-3 detection) [83,84,85,86], and the assessment of lysosomal integrity (neutral red uptake) [87]. Complementary to NP impairment of cell viability, proliferation assays could give evidence about the NP-triggered effect on the normal cell cycle in actively replicating cells. On the contrary, interference with the differentiation process could also be considered to define the possible misleading regulation of cell maturation [88].

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As previously mentioned, NP-induced cytotoxicity of fast-proliferating cells can be quite different from its toxic effect on primary cells with a physiological cell cycle. Thus, the use of cell lines is limited to the evaluation of validated biomarkers reflecting the actual response of normal human cells [89]. NPs could also induce an indirect effect by the adsorption of growth factors and nutrients from the cell culture medium [90], interfering with the readout. In toxicity tests using propidium iodide, false positive samples occur by the presence of this dye stuck on metal NP surface, increasing the uptake by viable cells, but finally counted as dead cells [91]. Another example of interference with metabolic tests is the NP interaction with the enzymatic substrate causing the depletion of the free form of the latter (i.e., MTT) and producing false negative results [92]. Metal ions have been clearly shown to interfere with the LDH assay [93,94], and several metal NPs may absorb light, quenching probe fluorescence in different tests [93,95,96,97]. The catalytic activity and the magnetic properties of some metal oxides may also cause erroneous signals in detection methods based on redox reactions [98,99,100,101,102]. For example, metal ions derived by the dissolution of the NPs interfere with the MTT reduction reaction [78].

Along with viability tests, the cellular stress response gives information regarding active cell reaction to exogenous materials. Reactive oxygen species (ROS) are generated by cells as byproducts of normal cellular activity, and are neutralized by cellular antioxidant defenses. If the ROS production exceeds the cell neutralizing capacity, an oxidative stress status occurs. To monitor this oxidative status, the two most common assays are the measurement of ROS generation and the glutathione (GSH) reduction. The generation of ROS is usually investigated using a fluorometric assay based on intracellular oxidation of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) [103], while GSH is measured with assays based on the production of a fluorescent or colored dye upon reaction with GSH [104]. As many fluorescent probes, however, results using DCFH-DA suffer from the interference of metallic NPs, showing plasmon resonance in the visible spectrum and specific absorbance around 490 nm wavelength [105]. Furthermore, the catalytic properties of some metal particles must be taken into account since non-internalized deacetylated DCF may accumulate in the extracellular space, and could react with catalytically active substances outside the cells [106].

Intracellular Ca^{2+} release is another useful indicator of cell activation. Several cellular mechanisms are triggered by an increase in intracellular Ca^{2+} concentration, potentially initiating apoptotic signals or autophagy [107]. Since most of the Ca^{2+} probes are fluorescent, the same metal NP-induced optical interference could be found.

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Inflammatory Potential

An exquisite parameter to assess cellular immune response is the production and release of inflammatory markers, like cytokines and chemokines. The evaluation of this type of response is commonly conducted using enzyme-linked immunosorbent assays (ELISA), that enables simple and accurate quantification of released markers by specific antibodies and enzymatic reactions.

Multiple-parameter analysis can also be performed using multiplex systems [88,108,109]. Cytokine release represents a key activity of immune cell to coordinate either inflammatory or anti-inflammatory responses. Although cytokine release is peculiar for each cell type determining following precise activation fates of the target cell (e.g., lymphocyte polarization in different sub-population depends on specific monocyte/macrophage cytokine released pattern), immune cell lines often show defined

expression patterns commonly shared by several types of NP insults. Similar inflammatory responses obtained *in vitro* do not always mean that all NPs behave in the same way inducing the same response. Specific cell line-gene rearrangement may limit the response to similar expression patterns facing particles made of diverse materials, which probably behave differently in primary cells derived from the same tissue.

Along with these well-known markers, the expression of membrane receptors involved in the cellular immune response can give the researcher important information [88,108]. Membrane proteins, as well as lipids, denote the activity of cells in the different physiological or pathological conditions. Upregulation of CD11b (cluster of differentiation 11b), for instance, can provide an indication of macrophage and microglia activation [110]. Increased expression of a certain protein receptor prepares the cell to respond quickly to its cognate agonist. For example, upregulation of FcRs (immunoglobulin receptors) amplify the macrophage ability to engulf opsonized external bodies, including NPs. Flow cytometry and confocal microscopy are commonly performed to evaluate membrane protein expression. The first represents a suitable analytic method for non-adherent cells (e.g., monocytes) and allows precise and fast quantification of different labelled targets, contemporaneously expressed on the cells [111]. Confocal microscopy, otherwise, is recommended to observe fluorescently probed objects in adherent cells (e.g., macrophages) [112]. Both techniques exploit fluorescence and have been developed for biological samples. As described above for other optical detection techniques, it is suggested to carefully include the proper controls to avoid artifacts created by NPs. Non-specific interactions of protein-labelling antibodies with NPs, or even more likely, with proteins absorbed onto the NPs, could induce variations in the detected fluorescence invalidating the data.

Another key feature of inflammatory responses is the immune cell trafficking and localization in the insulted tissue. Immune cell trafficking in peripheral and lymphoid organs is mediated by specific chemoattractant cytokines (known as "chemokines") and their cognate heptahelical transmembrane receptors [113]. The release of chemokines by inflamed tissues creates a signaling gradient, guiding leukocyte migration to the target site. Persistent metallic NPs endocytosed by immune cells could compromise their ability to reach the inflammation source, impairing their response to chemotactic stimuli. This occurrence is always underestimated. Nevertheless, useful information on the potential reduced, or increased, chemotaxis ability, can be easily tested *in vitro* by Boyden chambers or

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Transwell plate systems [114]. The eventuality of altered NP-induced cytoskeleton rearrangements may have important consequences on the kinetics of the immune response.

Colloidal Suspension Impurities

NP-induced immunological profile imposes the distinction of NP-mediated effects from those triggered by chemical and biological impurities that are not always completely removed during the NP synthesis. These impurities might be responsible for unwanted effects, as suggested by different studies [71,93,115]. Among the possible contaminants, the presence of endotoxins [116] can lead to false positive results [117]. Some nanomaterials, while not inflammatory themselves, are able to potentiate endotoxin-mediated inflammation [118,119,120]. Currently, the FDA-approved method to detect endotoxin is the limulus amoebocyte lysate (LAL) assay for *in vitro* tests.

While many types of NPs have been linked to certain types of immunotoxicity, there are no reference materials to be used to standardize immunotoxicity results. Usually, immunological studies are conducted using traditional controls, such as bacterial lipopolysaccharide (LPS) as an inducer of cytokine production [121].

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GENERAL CONSIDERATIONS ON *IN VIVO* INVESTIGATION

The study of the whole organism provides information on the immune response together with systematic data on NP pharmacokinetics, tissue absorption, distribution, metabolism, accumulation, and excretion [122]. The immune reaction has a dynamic nature and a defined time sequence, turning in a rapid deactivation once the cause has been eliminated. When the defense response is abnormal, by duration or distribution, a pathological state occurs. In this context, the evaluation of a selected biomarker does not always discriminate between pathological inflammation and physiological reaction. Major attention should be paid to the detailed profile of response kinetics [71].

NPs could enter the body through six main routes: intravenous, oral, intraperitoneal, inhalation, dermal, or subcutaneous [123]. The exposure route has enormous importance in the potential response of the immune system. Different antibody molecules are generated and released in the different tissues, so conditioning the eventual opsonization of NPs and their

sequestration by patrolling phagocytes [124]. For example, IgA antibodies dimers intercept NP released by an oral route, and may induce the activation of an IgA-mediated response. On the other hand, injected NPs can be quickly surrounded by pentameric IgM antibodies with a far bigger size than single-molecule memory IgG or complement molecules. The diversity of the corona changes the identity of the particles ruling their biological fate [125,126]. However, the bio-corona generated onto the NP surface, in turn, depends on NP physicochemical characteristics determining what is actually facing the cells. As a consequence, it mediates the uptake and/or activate different signaling pathways [127]. Many of the subset of serum molecules that interact with NPs, such as complement [128] and immunoglobulins, are immune-active, managing the interaction with immune cells in the different tissues.

The physicochemical properties, like NP surface charge, affect the nanomaterial biodistribution, though the current knowledge cannot enable general conclusions. NPs can distribute to various organs, and may retain the same original structure or not, being modified and metabolized [129]. It is worth distinguishing between acute and chronic exposure to NPs, since exposure time determines the optimal technique to monitor the interactions of NPs with the immune system. A high percentage of NPs can normally be sequestered in the liver, or in other organs, including spleen, lymph nodes, and bone marrow. Notwithstanding the importance of finding the threshold dose inducing toxicity, many experiments performed, *in vivo*, use amounts of metal NPs which are unlikely to be reasonable in human exposure through the considered routes [130]. The investigation of very low doses of NPs administered orally or by skin adsorption would be closer to the potential chronic exposure humans may have throughout their life using NP-loaded commercial products. The data obtained, in this way, help in understanding how a few particles can trigger immune reactions without showing massive toxicity or organ failure, "simply" due to the accumulation of metals and release of metal ions [131]. Furthermore, these organs are guarded by specialized macrophages, as part of the mononuclear phagocytes system (MPS) usually dealing with the uptake and metabolism of foreign molecules, which amplifies the retention and increases the ion release by ROS formation and lysosome-dependent metal NPs [132]. NP size is another critical parameter affecting final fate in the organism. For instance, NPs smaller than 5 nm in diameter are excreted in the urine, throughout the capillaries of the renal tubes [133], limiting their persistence into the body, otherwise micron-sized particles. Interestingly, NP size has been shown to

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influence the generation of CD8 or CD4 type I T cell responses [134,135], probably determined by the width of the NP surface in contact with cells, or the amount of absorbed active molecules reacting with membrane receptors. These hints guide us in the choice of the proper methods to reveal the mechanisms of NP-induced immune responses. Nonetheless, immune systems, hence, immune responses, display differences among animal species. The use of animal models in immunology is of indubitable importance. Differences and similarities to the human immune system of the experimental available animal models are still a matter of debate. As a representative example, one of the most used allergy models in mouse, namely ovalbumin as an allergen in BALB/c mice, does not reflect the development of an allergic reaction in humans [136].

As well as for *in vitro* studies, there is a lack of standard controls for NP immunotoxicological studies [137]. While many types of nanomaterials have been linked to certain types of immunotoxicity, standardization of the methods for each material is very difficult. Usually, the immune response to nanomaterials, *in vivo*, relies on traditional tests, with special attention to the innate immune reactions. These include histochemical analysis of the tissue in the site of NP release (e.g., skin or muscle) to monitor of the recruitment of immune cell, as well as the local cell activation and presence of inflammatory mediators, and possible initiation of adaptive immunity in lymph nodes (LNs). Metallic NPs are relatively straightforward to identify by microscopy in cells and tissues, due to their opacity in visible light and electron density in transmission electron microscopy (TEM) (Figure 1) [88]. As previously stated, blood concentration of inflammatory mediators and immunoglobulin over a precise time period is usually performed depending on the experimental model and the administration route. As well as for *in vitro* analysis, specific standardized controls for NPs are missing, and conventional molecules are generally used, such as lipopolysaccharide (LPS) as a positive control for cytokine induction [121]. Although LPS induces reliable immune responses, mimicking the effects of the Gram-negative bacterial wall, it is debated whether this molecule is the appropriate control for metallic NPs. Indeed, the specific LPS cell receptors (e.g., CD14 and beta2 integrins) or the soluble LPS-binding protein LBP, trigger precise signaling cascades which are not tailored for artificial nanomaterials of any kind. Metal NPs, hence, do not stimulate the same pathways of LPS, hiding potential unexpected inflammatory responses.

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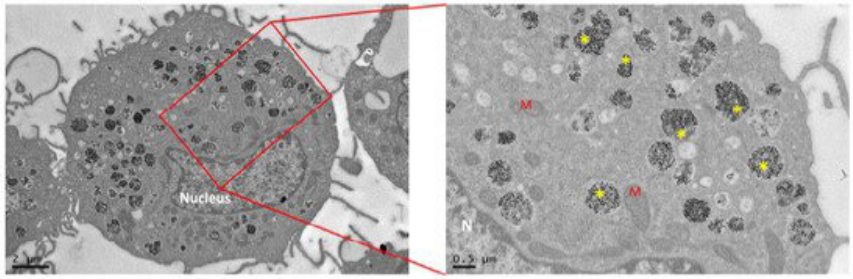


Figure 1: TEM images of human macrophage. Dark electron dense structures represent platinum nanoparticles within intracellular vesicles. M: mitochondria; N: Nucleus; *: NPs.

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CONCLUSIONS

The study of metallic nanomaterials effects on the immune system, their detection, and evaluation, requires cautious and meticulous characterization to avoid erroneous interpretations of the data. The main difficulty is related to the interference that material structures at the nanoscale can introduce into the standard methods currently used for chemicals or biological molecules. Although many studies have demonstrated that metallic NPs can have differential effects on the immune system and differential interaction within the leucocytes, the conclusions may vary using diverse experimental models, including different animal species. Future results will be achieved by a coordinated effort of chemists and immunologists to unravel nanomaterial–immune system interactions, and the appropriate investigation methodology.

AUTHOR CONTRIBUTIONS

F.G. and G.B. conceived and wrote the paper.

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Chapter 6

Zebrafish as a Model to Evaluate Nanoparticle Toxicity

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ABSTRACT

Nanoparticles are increasingly being developed for in vivo use, from targeted drug delivery to diagnostics, where they have enormous potential, while they are also being used for a variety of applications that can result in environmental exposure for humans. Understanding how specific nanoparticles interact with cells and cell systems is essential to gauge their safety with respect to either clinical or environmental exposure. Zebrafish is being increasingly employed as a model to evaluate nanoparticle biocompatibility. This review describes this model and how it can be used to assess nanoparticle toxicity

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at multiple levels, including mortality, teratogenicity, immunotoxicity, genotoxicity, as well as alterations in reproduction, behavior and a range of other physiological readouts. This review also provides an overview of studies using this model to assess the toxicity of metal, metal oxide and carbon-based nanoparticles. It is anticipated that this information will inform research aimed at developing biocompatible nanoparticles for a range of uses.

Keywords: Zebrafish, nanoparticles, nanotoxicity, biocompatibility

INTRODUCTION

Nanoparticles

According to the definitions of the International Organization for Standardization, American Society of Testing and Materials and National Institute of Occupational Safety and Health, particles with a diameter between 1 and 100 nm or fibers spanning the range 1–100 nm are termed as nanoparticles (NPs) [1]. Over the last decade, the synthesis, analysis and application of nanoparticles has grown exponentially, becoming an active area of intense innovation [2]. Recent advances allow for the synthesis of nanosized particles with multiple properties, referred to as multifunctional nanoparticles, which includes metal and metal oxides nanoparticles, fullerenes, carbon nanotubes (CNT), nano polymers and crystalline materials [3,4].

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Applications of Nanoparticles

NPs are being utilized for a wide range of applications. In manufacturing they are employed as chemically-inert additives, from fillers to pigments to anti-caking agents, but increasingly to create functional surfaces/membranes with catalytic, anti-microbial, UV protection, filtration and other diverse properties [5]. They are also fueling a new discipline of nanomedicine, lying at the intersection of chemistry, physics and medicine and focused on a range of biomedical applications. These include use as biosensors for nucleic acids, metabolites, proteins, drugs, pathogens and cancer cells by taking advantage of inherent optical, electrochemical, piezo-electric and photoluminescence properties [5,6]. NPs are also being used in a broad range of bioimaging, drug delivery, tissue engineering and other therapeutic applications, such as photoablation, which are facilitated by the accessibility

of these small molecules throughout the body, and the ability to link with specific targeting modalities as well as carry a functional payload [4,6,7].

Measuring Nanoparticle Toxicity

Biomedical applications explicitly require nanoparticles that are non-toxic. However, toxicity is also an important consideration for nanoparticles used for manufacturing and other applications, since these can result in environmental exposure [2,8]. A number of different platforms are available to assess toxicity, ranging from in vitro cell culture assays to basic model organisms, such as sea urchin and daphnia, to advanced higher vertebrate models, like rodents and primates [9]. Cell lines and simple organisms are useful for cell-level toxicity and genotoxicity studies, but higher vertebrates are essential to detect complex physiological interactions. However, rodent models are high cost, have relatively slow and inaccessible embryo development, require substantial amounts of material for testing due to their relatively large size, and are accompanied by ethical concerns about their use—while primate models have similar issues, but to an even greater extent [9]. Therefore, small, low cost but sophisticated models are very attractive for the evaluation of in vivo nanotoxicity. In this context the zebrafish serves as a compelling, efficient and cost-effective alternative [10].

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ZEBRAFISH AS A MODEL

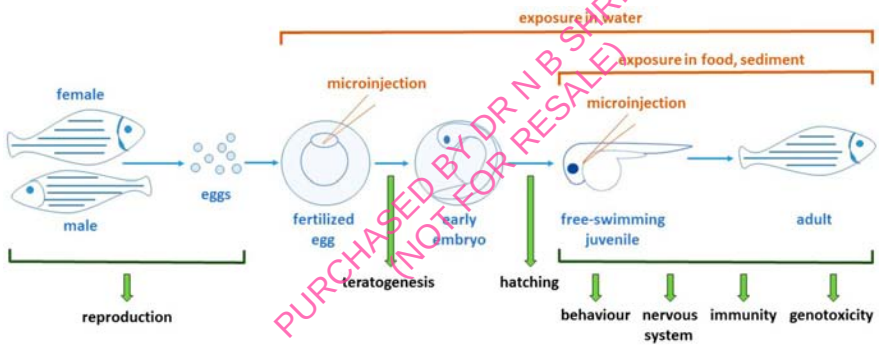
Overview

The zebrafish (*Danio rerio*) is an established vertebrate model for the study of development and disease [11,12,13,14,15,16] and is being increasingly used for both pre-clinical studies and toxicological applications due to a range of favorable traits [17,18]. Zebrafish require relatively inexpensive housing, making them very cost-effective, and are small in size, reducing housing requirements as well as the quantity of agent required for testing [11,16]. They also exhibit a high fecundity rate, with a single female able to produce around 300 eggs, further underpinning their efficiency as a model [11,19]. The zebrafish and human genomes share ~70% similarity [20,21]. There is also very good conservation of major developmental and physiological processes, with key organ systems, such the digestive, nervous and cardiovascular systems, similar to humans [22]. This largely underpins the extensive equivalence in response to pharmacological agents between the

two species [23], with many zebrafish models mimicking human diseases both genetically and phenotypically [16].

Zebrafish Development

Zebrafish eggs are robust and develop externally, making them easy to manipulate and amenable to high-throughput applications. This is further augmented by the optical transparency of the developing zebrafish, which allows exquisite visual analysis, including of fluorescent and other markers [12,16]. Development is also incredibly rapid, with the basic zebrafish body plan well-established by 24 h post fertilization (hpf), with embryogenesis completed by 72 hpf and most organs fully developed by 96 hpf and reaching adulthood in around 3 months [12]. This makes them amenable to a wide variety of toxicological applications throughout their lifespan (Figure 1).



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Figure 1: Toxicological studies performed in zebrafish. Schematic representation of zebrafish development, from spawning of embryos through their rapid development and hatching into free-swimming larvae and further growth and development into adults. Nanoparticles (NPs) can be administered via a variety of routes, including injection into eggs or specific sites on juveniles and adults, or alternatively administered in the water, sediment or food. The key assays used to examine toxicity in this model are indicated.

APPROACHES FOR MEASURING NANOPARTICLE TOXICITY IN ZEBRAFISH

Assessing Teratogenic and Other Developmental Effects

The rapidly developing, transparent and external embryos of zebrafish [24]

are ideally suited for screening for agents that disrupt normal development, from the rapid cell divisions that follow fertilization, to the extensive morphogenesis that occurs during epiboly to the development of the body plan and its key organs and other structures [25,26]. Particularly sensitive to perturbation are the eye, brain, heart, notochord and fin, while visualization of the effects on pigmented cells, including red blood cells, as well as hatching and overall mortality, is very easy. For example, disrupted eye development and pigmentation was observed via simple light microscopy following exposure of zebrafish embryos to functionalized gold nanoparticles [27]. In addition, the dose- and time-dependent toxicity of silica NPs was able to be readily gauged by assessing mortality rates [28] and impacts on the cardiovascular system [29]. Furthermore, the enhanced biocompatibility of chitosan NPs compared to normal chitosan particles was evaluated by quantifying relative hatching [30].

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Immunotoxicity

The immune system has been shown to be very sensitive to a variety of agents, including NPs, particularly the induction of an inflammatory response as well as associated accumulation and activation of neutrophils and macrophages [31]. For example, gold (Au) NPs have been shown to disrupt pathways involved in inflammatory and other immune responses [32], while silver (Ag) NPs caused immunotoxicity in adult zebrafish due to oxidative stress [33].

Genotoxicity

DNA is susceptible to damage following exposure to many chemical entities, which results in gene mutations and larger chromosomal alterations, collectively termed genotoxicity [34]. This can be assessed in embryos, larvae or adult tissues in a number of approaches, including quantitative RAPD-PCR methodology that has been used to demonstrate dose-dependent genotoxicity of TiO_2 NPs [35], and comet assays for examining the impact of ferric oxide (Fe_2O_3) NPs [36].

Reproduction Analysis

The high fecundity rates of zebrafish are well suited to assessing the impact of agents on various aspects of reproduction, from egg production to fertilization rates to subsequent embryo viability. For example, reduced egg production and increased embryo mortality represent a hallmark of

chronic exposure with TiO_2 NPs [37], while in contrast Ag NPs mediated enhanced maturation of zebrafish oocytes due to increased oxidative stress and resultant follicle cell apoptosis [38].

Neurotoxicity and Behavioral Analysis

Zebrafish exhibit a range of complex behaviors that can be used as sensitive parameters to assess toxicity, including color preference, spatial recognition and locomotion [39,40]. The nervous system that underpins these behaviors, including the developing brain, is particularly vulnerable to oxidative stress because of its high energy demand, low level of antioxidants, and high cellular content of lipids and proteins. A range of NPs can trigger free radical activity at their surface, thereby creating oxidative stress at the site of particle deposition and translocation [41,42,43]. Neurotoxicity has been commonly noted for NPs that are able to reach the brain, where they can lead to neurodegeneration [44,45]. Behavioral effects have also been seen that are specific for particular NPs. For example, altered color preferences were found to be caused by silicon dioxide (SiO_2) NPs [46], while locomotor activity was affected by cadmium telluride (CdTe) quantum dots [47]. More detailed analysis has identified enhanced neuron apoptosis and glial cell proliferation, along with altered gene expression, following exposure to titanium dioxide (TiO_2) NPs [48].

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Other Approaches

A range of additional techniques can be used to further understand potential toxicity, including a variety of high-content approaches [49], such as transcriptome analysis [36,50] and plasmonic spectroscopy [51]. Taking advantage of the optical transparency and genetic amenability of zebrafish, transgenic reporter lines have increased the ease and efficiency of toxicology studies [47,52]. Certain agents have inherent luminescent or fluorescent properties that can also be exploited to provide dynamic imaging of zebrafish embryos [53], including studies examining NP distribution in vivo [54,55].

Comparison between Zebrafish and Mammalian Studies

A number of studies have demonstrated that a variety of compounds tested in zebrafish embryos yielded similar results to those observed in rodents. This includes research showing comparable developmental toxicity for a series of 1,2,4-triazoles [56] and organotins [57], as well as correlations between

zebrafish embryo LC50 (lethal concentration 50%) values and rodent LD50 (lethal dose 50%) values for a set of 60 compounds [49], although the latter study showed that the compound class influenced relative toxicity [49]. In a landmark study, a meta-analysis of toxicity testing in zebrafish of over 600 chemicals was performed, which compared aggregated toxicological end-points with outcomes observed in laboratory mammals. This demonstrated that for acute toxicity, there was a good correlation between zebrafish embryo LC50 values and LD50 values obtained across various laboratory mammals when the chemicals were administered via inhalation or injection, but not orally [58], which was consistent with other studies [59,60]. There was also a correlation between effects on zebrafish hatching and pre-natal loss in rabbits [58]. Furthermore, analysis of teratogens using zebrafish correctly ranked known mammalian teratogens [61,62]. Of note, zebrafish embryos were consistently more sensitive than mammalian systems to the agents tested [58,59].

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Standardization of Zebrafish Testing

In light of the effectiveness of using zebrafish and other fish species for toxicity studies, test guidelines have been developed by the Organization for Economic Cooperation and Development (OECD). These include guidelines for the Fish Embryo Toxicity (FET) test [63], Fish: Juvenile Growth Test [64], and the adult-based Fish: Acute Toxicity Test [65]. The FET test has been validated by independent laboratory testing and has been shown to be a robust and reproducible methodology [66]. It has also been shown to have an excellent correlation with the Acute Toxicity Test in adults [67], and has been proposed to be a worthy range-finder prior to more extensive testing [68]. A recent study used the FET test to evaluate toxicity of methylxanthine drugs, which revealed a strong positive correlation between TC50 measurements of mortality, morphological defects and teratogenicity in zebrafish embryos and published mammalian LD50 values [69].

SELECTED NANOTOXICOLOGY STUDIES IN ZEBRAFISH

Zebrafish have been utilized for a myriad of nanotoxicology studies [36,37,38,48,70,71]. A summary of key studies involving the analysis of metal, metal oxide and carbon-based nanoparticles, particularly those related to shape, size and surface charge are discussed below, with relative toxicity summarized (Table 1).

Table 1: LC_{50} toxicity testing in zebrafish embryos and adults of nanoparticles detailed in this review and selected others

Nano Particle	Stage	LC_{50} (mg/L)	Time	Teratogenicity	Reference
Cu	eggs	24.0	48 h	Malformations, delayed hatching	[72]
	adults	4.2	48 h	N/A	[72]
		1.5	48 h	N/A	[73]
Chitosan	eggs	280	96 h	Malformations	[30]
Au	eggs	>200	48 h	None	[72]
	adults	>200	48 h	N/A	[72]
Ag	eggs	2.7	48 h	Malformations	[72]
		1.2	96 h	Malformations	[74]
	adults	2.9	48 h	N/A	[72]
Cd/Te QDs	eggs	186 (nM)	120 h	Malformations, delayed hatching	[75]
TiO_2	eggs	>1600	48 h	Premature hatching	[72]
	adults	>1600	48 h	N/A	[72]
ZnO	eggs	3.5–9.1	120 h	None	[76]
		1.8	96 h	Delayed hatching	[77]
MgO	eggs	>3200	48 h	None	[72]
		428	96 h	Delayed hatching	[78]
	adults	140	48 h	N/A	[72]
Fe_2O_3	eggs	>1600	48 h	None	[72]
	adults	>1600	48 h	N/A	[72]
NiO	eggs	1700	48 h	None	[72]
	adults	760	48 h	N/A	[72]
		45	30 d	N/A	[79]
CuO	eggs	960	48 h	None	[72]
		175	48 h	None	[80]
	adults	400	48 h	N/A	[72]
Fullerene	eggs	>200	48 h	None	[72]
		1.5	96 h	Reduced hatching	[81]
	adults	>200	48 h	N/A	[72]

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CNTs	eggs	>200	48 h	None	[72]
		>360	96 h	None	[82]
	adults	>200	48 h	N/A	[72]

Metal Nanoparticles

Gold

Among metal nanoparticles, Au NPs have been the most widely studied in biomedicine, with key applications as drug carriers [83] and as diagnostic agents [84]. However, Au NPs can cause cytotoxicity in humans [85,86]. As a consequence, zebrafish has become increasingly used as an in vivo model to evaluate the toxicity of Au NPs.

Food containing Au NPs (12 and 50 nm) elicited a variety of cellular dysfunctions as well as genome alterations in adult zebrafish that were dependent on size, concentration and exposure time [87]. Chronic exposure of zebrafish to sediment containing 14 nm Au NPs also resulted in genome modification in various adult tissues, probably related to increased oxidative stress [88]. Au NPs were found to accumulate within tissues, suggesting the potential for higher toxicity compared with ionic Au. This was supported by other work that demonstrated 10–50 nm Au NPs could induce strand breaks in zebrafish ovaries, in addition to other cell types [89].

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Surface charge functionalization has been identified as a critical determinant of toxicity. In one key study, Au NPs functionalized with positively-charged N,N,N-triethylammoniumethanol (TMAT) caused significant mortality, but elicited negligible malformations, whereas those functionalized with negatively-charged 2-mercaptoethanatesulfonate (MES) substantially increased the incidence of malformations, but did not result in significant mortality within the five-day exposure period. Neutral 2,2-mercaptoethoxyethoxyethanol (MEEE) and 2,2-mercaptoethoxyethanol (MEE) functionalized Au NPs did not elicit adverse effects even at higher concentrations of up to 250 ppm. A related study additionally found that exposure to either positively and negatively charged Au NPs resulted in behavioral abnormalities, including hypo-locomotor activity [90]. Kim et al. further showed that 1.3 nm TMAT-functionalized Au NPs caused abnormal eye development, with altered pigmentation and neuronal damage and concomitant behavioral changes [27]. These effects correlated with the ability of charged Au NPs to mediate inflammatory and altered immune effects [32].

Shape represents another important factor mediating the effects of Au NPs. The toxicity and biodistribution of fluorescently-labeled Au NPs of different shapes were examined in adult zebrafish [91]. Rod-shaped Au NPs exhibited enhanced uptake and clearance, while star-shaped Au NPs in contrast displayed slower uptake but longer sequestration in comparison to rod-shaped or spherical particles.

This study also showed that the choice of linker—polyethylene glycol (PEG) or mannose-capped—also had a significant effect [91]. Other research with peptide-capped Au NPs showed that the terminal modification was important, with the presence of terminal histidines being more toxic than tryptophans, with methionine conferring the least toxicity [92]. In addition, co-incubation of zebrafish embryos with Au NPs and a surfactant (polysorbate 20) resulted in increased uptake and toxicity [93]. Together this suggests that the overall shape and surface chemistry of the Au NPs are key determinants of biocompatibility.

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Silver

Ag NPs have also been extensively studied, with broad applications as therapeutic agents [94], antimicrobial agents [95], drug delivery systems [96] and biosensors [96].

Exposure of zebrafish to Ag NPs during early development elicited a range of toxicities, including a reduction in heart rate, damage to neuromast hair cells and more modest but statistically significant increases in both mortality and teratogenicity [97]. Another study demonstrated that low concentrations of 10–20 nm Ag NPs (<5 mg/L) had little impact on normal embryonic development, but higher concentrations resulted in significant effects on the development of mesodermal and ectodermal tissues, possibly due to delayed or inhibited cell division [98]. Exposure of adult zebrafish to Ag NPs resulted in localization in the gills and liver, where they caused oxidative stress and immunotoxicity [33].

The size-dependence of Ag NP-mediated toxicity is still debatable. One study reported similar mortality for zebrafish embryos treated with Ag NPs across a size range of 3–200 nm [99]. In contrast, Lee et al. showed that Ag NPs with a particle size of 30–72 nm were able to selectively enter zebrafish embryos through chorionic pores via random Brownian motion and caused more potent toxic effects [70]. In another study, Ag NPs were found to impact on neural development of zebrafish embryos in a size-dependent manner. In this case, 4 nm Ag NPs were taken up more efficiently than 10 nm-sized

particles, with the head of the exposed zebrafish embryos able to accumulate more Ag NPs than the trunk [100].

The charge characteristics of Ag NPs has also been demonstrated to be an important determinant of toxicity. Investigation of ~12 nm Ag NPs functionalized with peptides of different charge revealed that positively-charged Ag NPs were the most biocompatible, with the extent of deformity and level of mortality greater in those embryos exposed to NPs with a negatively-charged peptide coating and greatest for those with a highly negatively-charged peptide coating [101].

Ag NPs of several different shapes were all shown to induce oxidative stress, but plate shaped Ag NPs were more toxic than spherical and wire-like shaped forms [102,103]. Interestingly, these effects correlated with the presence of surface defects rather than Ag shedding [102]. Coating with cysteine [102] or sulfidation [104] resulted in Ag NPs that elicited reduced oxidative stress in embryos or adults, respectively. Other studies have further demonstrated that embryonic toxicity of Ag NPs was augmented by exposure to simulated solar light [105]. Collectively, this suggests a complex interplay of factors, where a range of physiochemical properties underpin biocompatibility.

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Cadmium-Based Quantum Dots

Quantum dots (QDs) have been extensively employed for biological and medical imaging because of their small size and bright fluorescence, with broad absorption spectra, narrow emission spectra and high photostability [101,106,107,108]. Cadmium selenide (CdSe) and CdTe nanocrystals represent two of the most commonly utilized QDs for biological applications, with their extremely bright fluorescent properties making them powerful labelling agents for diverse in vivo applications. Several studies have shown CdSe QDs to be well tolerated with only modest toxicity even when directly injected into embryos at relatively high concentrations [47,109]. However, exposure of CdTe QDs to zebrafish embryos lead to a range of developmental and behavioral disturbances [75].

Metal Oxides

Metal oxide NPs, and particularly those based on TiO_2, are widely used in a range of products, including paint, food and personal care products [110,111]. Due to concerns over the possible consequences of environmental exposure, their biocompatibility has been extensively studied using zebrafish.

TiO_2

Low dose (1 mg/L) exposure of zebrafish embryos to TiO_2 NPs failed to induce major developmental malformations [112], although several groups have demonstrated that TiO_2 NPs cause premature hatching in a dose-dependent manner [113,114]. In addition, higher doses of TiO_2 NPs can lead to embryonic malformation and death [45]. Another study demonstrated that TiO_2 NPs were able to absorb photons and produce electron-hole pairs that interact with water and oxygen to form reactive oxygen species that were toxic to zebrafish larvae [115]. Chronic exposure of adult zebrafish to TiO_2 NPs at low concentrations (<4 mg/L) for 6 months was also associated with low toxicity, as determined by mortality rate. However, at higher concentration these NPs were found to accumulate in different parts of the fish, including the gill, liver, heart and brain [116]. High level exposure can also lead to genotoxic effects [35].

However, the major effect of TiO_2 NPs is neurotoxicity. Even low levels of TiO_2 NPs showed impacts on embryonic neurogenesis and neuronal differentiation observed [112], while exposure of larvae to TiO_2 NPs significantly affected swimming parameters, including average and maximum velocity [117]. TiO_2 NPs were able to cross the blood-brain barrier to damage the brain [45]. Chronic exposure of adult zebrafish to low dose TiO_2 NPs for 45 days led to reduced levels of neurotransmitters and consequent changes in behavior, as well as histopathological changes in the zebrafish brain, which were associated with dose-dependent increases in nitric oxide levels [48]. Other researchers have compared the effect of bulk TiO_2 and TiO_2 NPs on the zebrafish brain, which revealed that TiO_2 NPs were more toxic than bulk TiO_2 due to enhanced lipid oxidation and degradation [118].

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Zinc Oxide (ZnO)

Zinc oxide (ZnO) NPs (20 nm) have also been shown to cause delayed development and inhibition of hatching in zebrafish embryos but only at higher concentrations (>0.1 mg/L) [77,119]. Again, surface properties and shape appear to be important. Polymer-coated ZnO NPs were shown to be more biocompatible than spherical ZnO, with leaf-shaped ZnO NPs having the greatest impact on hatching [120]. In another study comparing different shapes of ZnO NPs, nanosticks were found to be more toxic than nanospheres and cuboidal submicron particles with respect to hatching and overall mortality [121]. However, similar results were observed between

ZnO NPs and bulk ZnO, suggesting that leaching of the metal oxide may be a key factor in mediating the effects of ZnO NPs [77]. Co-incubation with humic acid was able to suppress the impact of the released zinc [119].

Other Metal Oxides

A number of studies have shown that other metal oxide NPs elicit variable toxicity. For example, magnesium oxide (MgO) NPs (20 nm) decreased hatching rate and survival of zebrafish embryos in a dose-dependent manner, leading to various types of malformations [78]. Fe_2O_3 NPs caused severe deformities in embryos [122] and elicited strong genotoxic effects in adult zebrafish [36]. Others have observed that NPs based on cupric oxide (CuO) and nickel oxide (NiO) also interfered with hatching, whereas those utilizing cobalt oxide (Co_3O_4) [123] or aluminum oxide (Al_2O_3) [77] were relatively inert.

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Carbon Based Nanoparticles

Carbon-based nanomaterials have attracted increasing interest in the field of biomedical research, including as drug delivery systems, tissue scaffold reinforcements and cellular sensors [124]. Carbon NPs are considered particularly promising due to their low toxicity compared to other NPs [125]. In the last few years, the toxicity of different carbon NPs have been evaluated using zebrafish as a model, such as fullerenes, carbon nanoparticles, carbon nanotubes (CNT), graphene QDs and carbon QDs (C-dots).

Fullerenes

Fullerenes are allotropes of carbon discovered in 1985 [126] that have been widely evaluated for biomedical applications, such as drug and gene delivery [127], bioimaging [128] and quenching of reactive oxygen species [128]. Analysis in zebrafish has shown that fullerenes exhibit toxicity that is related to their surface chemistry. Exposure of embryos to C_{60} or C_{70} fullerenes at 200 ppb delayed development and resulted in specific caudal fin malformation and significant pericardial edema, with >200 ppm C_{60} or C_{70} leading to 100% mortality. In contrast, exposure to >2500 ppb $C_{60}(OH)_{24}$ was required to induce fin malformations and pericardial edema, with significant mortality only observed at concentrations of >4000 ppb [129]. Another study of water soluble fullerenes indicated that positively-charged fullerenes showed enhanced toxicity compared to negatively-charged fullerenes with similar structures. Toxicity was shown to vary considerably between the negatively-

charged fullerenes from very low to moderate, depending on structural features [130]. Kuznetsova et al. showed phosphatidylcholine-based phospholipid NPs containing C_{60} elicited low toxicity on zebrafish embryos [131]. Photons appear to exacerbate toxicity, since reducing light levels during exposure to C_{60} (at 200–300 ppb) significantly decreased mortality as well as the incidence of fin malformations and pericardial edema [132]. Other research has suggested that water-soluble fullerenes may protect against apoptotic cell death [130]. This was supported by a study investigating a C_{60}fullerene based derivative (dendrofullerene) containing 18 carboxylic groups, which was able to reduce radiation-induced nerve cell damage through its actions as a free-radical scavenger [133]. Multi-shell fullerene structures, known as nano-onions, demonstrate good biocompatibility, with little toxicity and homogenous biodistribution in zebrafish larvae [134].

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Carbon Nanotubes

Carbon nanotubes (CNTs) have attracted intense interest for various biomedical applications due to their distinctive chemical and physical characteristics [124]. These characteristics make CNTs promising candidates for the delivery of chemotherapeutic agents, including paclitaxel and doxorubicin, small interfering RNAs, genes and antibodies [135,136].

Pristine CNTs have been shown to have high biocompatibility, with single-walled (SW), multi-walled (MW) CNTs or carboxylated MW pristine CNTs having little effect on embryo viability and development, even at high concentrations (200 ug/mL) [137]. In another study SW CNTs functionalized by polyethylene glycol increased mortality, delayed hatching and decreased total larval length only at the highest concentration tested (1 ppm), but with no genotoxicity observed and no evidence of the nanotubes being taken up by tissues [138]. Another group found that the length of CNTs was an important determinant of toxicity with longer CNTs causing distinctive cellular and molecular changes [139].

Exposure of adult zebrafish to MW CNT (diameter 500 nm) caused reversible inflammation in the gills, but again no genotoxicity was seen [140]. Another study showed that MW CNTs were able to accumulate in zebrafish [141], while Li et al. demonstrated that exposure to CNTs can result in alterations in the brain and gonads [142].

Carbon/Graphene Quantum Dots

C-dots are a type of quasi-spherical carbon material with a diameter of <10 nm [143], whereas graphene QDs (GQDs) represent multiple layers of graphene with a size of <30 nm [144,145]. Both C-dots and GQDs have emerged as superior universal fluorophores due to their unique combination of excellent photostability, small size and highly tunable photoluminescence, being effective in photon-harvesting in the short-wavelength region because of π–π* transition of C=C bonds [146], with a variety of imaging applications [147]. C-dots and GQDs are also attractive for drug/gene delivery [148,149,150,151] due to their low toxicity, a consequence of the predominance of inert carbon rather than more reactive hydrogen, nitrogen and oxygen in their make-up [150,152]. However, C-dots and GQDs do contain −OH, $-NH_2$, >C=O and –COOH functional groups on their surface, which increase water solubility [143], as well as assisting in covalent bond formation with antibody, chemotherapeutic agent or other biochemical entity [153,154], which can be combined with their florescence properties (Figure 2) for multi-purpose theranostic applications.

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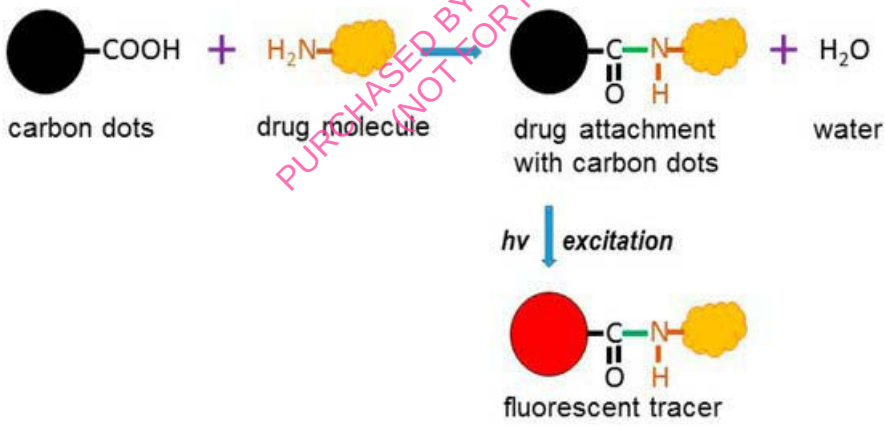


Figure 2: Carbon nanodot-based drug delivery and tracking. Schematic representation showing conjugation of carbon nanodots with drug molecules, and subsequent excitation to generate agents that act simultaneously as fluorescence tracers and drug delivery vehicles.

C-dots have been shown to exhibit higher biocompatibility than other NPs [55]. N-doped C-dots synthesized from BSA were well tolerated by zebrafish embryos immersed in them at concentrations of 6 mg/L and retained fluorescence for up to 2 days, highlighting their low toxicity and stable fluorescence emission [155]. In another study, embryos exposed to

C-dots showed normal development at concentrations of 2 mg/L (soaking) or 1.5 mg/mL (injection). However, at higher concentrations, delayed development, inhibition of pigmentation, pericardial edema, and delayed hatching were observed. GQDs also exhibited high biocompatibility, being readily excreted from adult zebrafish without affecting growth significantly at a concentration lower than 2 mg/mL [54]. The GQDs were found to accumulate in the digestive system, while the blood, muscle and other tissue showed no obvious photoluminescence signal.

CONCLUSIONS

The nanotechnology industry is already large; it was estimated to be worth $39.2 billion in 2016 [156], and is growing rapidly. To facilitate this, efficient and effective testing of new products is essential. Zebrafish represent an excellent in vivo model for testing nanoparticle toxicity and biocompatibility. They are low cost and easy to maintain, and able to test agents efficiently via multiple routes of exposure, including directly in the water, which is especially relevant for environmental toxicology applications. In addition, specific physiological impacts can be assessed at multiple stages of development. In this review, the toxicity of different metal and carbon-based NPs has been described, identifying a range of parameters regarding exposure (concentration, route, length, life-stage, presence of other molecules) and of physiochemical properties of the NPs (size, shape, surface charge/chemistry) that impact on their biocompatibility.

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There is now a growing consensus advocating the greater use of zebrafish models to reduce reliance on rodent testing for financial, ethical and biological reasons [31]. Indeed, toxicity and safety testing in zebrafish has been accepted by the Federal Drug Administration for new drug approval [25]. This will be greatly augmented by standardization of delivery protocols, methods of analysis and strains of zebrafish used. Zebrafish also have great potential for pre-clinical studies and so represent an ideal model to assist the further development of NPs as therapeutic agents, such as testing mitigation strategies when a promising NP agent exhibits toxicity.

AUTHOR CONTRIBUTIONS

Both authors contributed to Writing, including both Original Draft Preparation and Review & Editing.

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SECTION IV: VETERINARY RADIATION AND RADIOACTIVE MATERIALS

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Chapter 7

Whole-Body Counter Evaluation of Internal Radioactive Cesium in Dogs and Cats Exposed to the Fukushima Nuclear Disaster

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ABSTRACT

As a result of the 2011 nuclear incident that occurred at the Fukushima Nuclear Power Plant, a large number of abandoned dogs and cats were left within the disaster zone. A small number of these animals were rescued and cared for at shelters. Prior to the dispersal of these animals to their owners or fosterers, we evaluated the degree of internal radiocesium contamination

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using a specially designed whole-body counter. We conducted 863 non-invasive measurements of gamma rays due to internal radioactive cesium for 68 dogs and 120 cats at one shelter. After plotting graphs of ^{137}Cs density we generated exponential functions of decay from seven dogs and six cats. From the regression formulae, we were able to determine the biological half-lives as 38.2 days for dogs and 30.8 days for cats. We found that in dogs there was a correlation between the biological half-life of radioactive cesium and age. Using our data, we estimated whole-body densities for each cat and dog at the time when they were rescued. We found that there were deviations in the data distributions among the different species, likely due to the timing of rescue, or living habits prior to rescue. A significant correlation was found when extracted feline reproductive organs were analyzed; the coefficients for the estimation of whole-body densities were approximately 7-fold higher than those based on the extracted feline reproductive organs. This may be due to the fact that majority of the radioactive cesium accumulates within muscular tissue with less distribution in other organs. It is possible to plan the appropriate management period in an animal shelter based on the use of the biological half-life of radioactive cesium calculated in this study. We believe that the correlations we uncovered in this work would be of great use for the management of companion animals in the event of a future nuclear accident.

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INTRODUCTION

On 11 March 2011, a major earthquake devastated the Tohoku region of Honshu Island of Japan, ultimately causing a nuclear meltdown in several nuclear reactors at the Fukushima Daiichi Nuclear Power Plant (FNPP). The large amounts of radioactive material that were released caused radioactive contamination throughout the Fukushima prefecture and surrounding areas. On 12 March 2011, evacuation orders were issued within a 20-km radius of FNPP. By 22 April 2011, the 20-km area was designated as a restricted zone, and the movement of animals and goods out of the area was highly restricted.

During this restriction, a ban was imposed on transportation of all livestock animals inside the restricted zone, while all companion animals (dogs and cats) were allowed out of the restricted zone after body surface radioactive contamination checking, as mandated by the Fukushima government.

There have been many analyses of human exposure to the release of radioactive materials into the environment from nuclear tests and atomic accidents. The primary focus of studies into animal exposure has been the transfer coefficients of radioactive materials to meats and milk [1, 2]. No data about domestically raised animals have been published. Prior to the FNPP accident, the 1986 Chernobyl nuclear accident exerted global influence on the handling of radioactive materials. Despite a large number of domestic meat animals being transported out of the restricted zone, all home breeding dogs around nuclear power plant were shot dead with no data on their internal contamination [3]. With regard to other wild animals, there is a large amount of published data on reindeer and roe deer from regions of Ukraine [4], on lynxes from northern Europe [5–7], and on wild boar from Germany [8]. Thus far, there are no reports pertaining to biological half-lives in animals, other than those that refer to the degrees of contamination or biodistribution.

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In general, a multitude of different radioactive materials are released into the environment directly after a nuclear accident. Over time, changes occur within key nuclear species that can have an effect on human exposure. After several years, the nuclear species with the strongest effect are radioactive strontium and cesium. Because the amount of radioactive strontium emitted during the FNPP disaster was comparatively low, radioactive cesium was regarded as the greatest threat to human exposure in Japan [3, 9]. Consequently, radioactive cesium was selected to be the focus of this study. Additionally, the quality of life of domestic animals is taken into serious consideration in modern developed countries, and this is the second reason we focused our work on the biological half-life of radioactive cesium. Currently there are no data on cesium half-life in companion animals, yet this would form an important part in establishing the optimal management period for radioactively contaminated dogs and cats. Indeed, the provision of clean food and water for dogs and cats is a major issue for animal shelters. The longer that an animal stays in the shelter, the greater the probability that the shelter will be unable to provide for them adequately, due to increased numbers.

Although the Fukushima Headquarters for Animal Rescue initially established animal shelters at two locations, one shelter in Iino-machi, Fukushima, had closed by January 2013. The second shelter in Miharu-machi, in the Tamura district of Fukushima, was closed in December 2015 after operating for 4 years and 2 months. The majority of the animals being cared for at the Miharu shelter were dogs and cats that were rescued within

the 20 km restricted area. These animals were treated before they were eventually transferred to owners or people who were willing to adopt them. Prior to the use of our whole-body radiation counter, to prevent exposure to the families who adopted the animals, we measured the radioactive dosage on each animal body surface using a survey meter and lengthened the raising period in the shelter for the attenuation of the internal radioactivity. Because the accurate biological half-life of radioactive cesium in cats and dogs is not known, we used parameters for humans. Although the biological half-life of radioactive cesium obtained from rodent and human data is applicable to other types of animals, there are presumably differences in metabolic rates between species. Additionally, given that many factors affect metabolic rates, it is critical to understand both the representative values and the margins of fluctuation.

To this aim, we installed a whole-body gamma counter at the Miharu shelter to monitor the amount of internal residual radioactivity in dogs and cats. Using this counter, measurements were collected from April 2013, for a period of 2.5 years. The effective half life in dogs and cats was measured in a prospective study and the relationship between the biological half-life and other factors was analyzed in a descriptive study. Data obtained non-invasively from dogs and cats that were exposed to a nuclear accident could be highly valuable. Indeed, our results should be useful for directing rescue activities of animal victims in the event of future nuclear accidents.

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MATERIALS AND METHODS

Animals and Ethics

The Ethics Committee of Fukushima Headquarters for Animal Rescue at the Ministry of the Environment and Fukushima Veterinary Medical Association created the ethical standard manual for animal welfare (approved by an extraordinary meeting of Relief of Animals in Emergencies in May, 2011), studies were conducted in accordance with this manual. We obtained data for this research study at the Miharu shelter, which is a facility established for the humane rescue of dogs and cats affected by the earthquake that caused the Fukushima disaster. After the disaster, 463 dogs and 545 cats were collected from the restricted zone and taken to Miharu shelter, where veterinary care was carried out. The shelter also took responsibility for the acclimation of two to three new generations of animals born in the restricted zone since the

disaster. Routine reports were given to multiple representatives from animal welfare groups and veterinarians about the operation and management of the shelter, the treatment of animals, and the collection of data on health management and exposure. Any necessary improvements were made in response to feedback.

At the Miharu shelter, the animals were housed in clean cages, at a controlled ambient temperature, and given appropriate food and water. To maintain health, animals underwent veterinary health check-ups (including fur checks), bathing, regular exercising, and were given toys to play with. During internal exposure measurements, the animals were housed in plastic cages suitable for their size, and then the measurements were performed non-invasively using a whole-body counter. To prevent breeding and other problematic animal behaviors, a skilled veterinarian castrated or spayed each animal immediately after its rescue. Almost all intact male and female cats underwent ovariohysterectomies and orchiectomies. The extracted reproductive organs were then measured for radioactivity to estimate the amounts of internal exposure.

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Whole-body Counter

A whole-body counter was used to evaluate the internal exposure of rescued and acclimated animals. A total of 863 gamma ray measurements were performed non-invasively on 68 dogs and 120 cats at the Miharu shelter, to quantify the animals' internal radioactive cesium. Measurements were conducted with the animal remaining inside their cage. The external dimensions of the steel detection tank were 880 mm (W), 1143 mm (D), 1225 mm (H) and 5 mm thickness. At the midpoint of the height of the 5-mm-thick steel detection tank, two 3 inch × 3 inch NaI (Tl) scintillation probes (Ohyo Koken Kogyo Co. Ltd., Tokyo, Japan) were installed. 15-mm-thick lead plates shielded all outer faces of the tank. To reduce background counts, the four vertical outer faces (front, back, left and right) were reinforced with a plastic container filled with 18 L of deionized water. They were set side by side and then fixed with cable ties to secure them in position (Fig 1).



Figure 1: A: Front view of the detection tank of the whole-body counter. The entire surface, including the door, is covered with lead plates. The four vertical outer faces (front, back, left, right) were shielded with water. B: Inside the detection tank. The animal cage can be seen on top of polystyrene blocks. The NaI radiation detectors lie on both sides. C: High-voltage power supply, linear amplifier, multi-channel pulse height analyzer and radioactivity analyzer.

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The measuring instruments system consisted of a Nuclear Instrument Modules (NIM) standardized summation inverter, a linear amp, an Analog to Digital Converter (ADC) module, a high voltage power supply, a BIN power supply and a notebook PC with built-in analytical software. Ohyo Koken Kogyo Co. Ltd., Tokyo, Japan, manufactured the whole-body counter instrument. The instrument calibration was performed using a volume source with a ^{137}Cs standard point source, and water contaminated from the nuclear accident as a reference volume source. The peak photoelectric absorption of ^{137}Cs was selected at 661.66 keV energy and 85.1% branching rate of gamma rays, and calculations were performed. It is possible for the measured radioactivity to be affected by the influence of gamma ray emissions from ^{134}Cs (796 keV), resulting in slight overestimations of radioactivity. The degree of the overestimations was confirmed to be less than 5% (at the time of instrument installation) by comparison to measurements of the calibration source by a germanium semiconductor HPGe detector (40% relatively efficient coaxial type; Canberra, Meriden, USA).

The animals were measured in a polypropylene cage that fit each animal's individual physique and restricted their movements during measurements. Animals did not exhibit any violent or large movements during the measurements, meaning anesthetics or tranquilizers were unneeded. Prior to data collection, it was observed that the animals tended to sleep when the cage was darkened, so the decision was made to collect measurements with the lights switched off. In all instances, the measurements were performed in less than 1 h.

Measurement of Radioactivity of Feline Reproductive Organs

The radioactivity of the surgically extracted reproductive organs was calculated via gamma ray spectrometry using a germanium semiconductor HPGe detector (GC3518; Canberra, Meriden, USA). In accordance with the sample radioactivity, the measurement times were between 3,600 and 200,000 s. The gamma ray energy efficiency was determined from measurements of standard point sources: ^{133}Ba, ^{137}Cs, ^{60}Co, and ^{22}Na. To derive the calculation efficiencies of the volumes of the measurement samples, solutions of potassium chloride containing known quantities of potassium were decanted into polyethylene vials (20 ml, 50 ml, and 100 ml) and multiple measurements were conducted on the volume sources. We obtained a calibration curve by evaluation of the photoelectric absorption peak of ^{40}K for each of the 10 types of volume of potassium solution, and the measurement samples were corrected for volume efficiency. Corrections of decaying efficiencies were made on deviations from the ^{40}K volume source. Numerical calculations were performed using the Simpson's rule [10].

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The samples were pretreated uniformly using a homogenizer and sealed in polyethylene vials (20 ml, 50 ml or 100 ml, according to their size); measurements were then performed. The limits of detection of radioactivity were determined from background counts.

Statistical Methods and Analysis of Biological Decay of Radiocesium

The measurements from the whole-body counter were recorded every few days until the internal radioactivity of each animal fell below the limits of detection. The limit of detection was 10–20 Bq/kg in a 1 hour measurement. The values from the first day of continuous measurements were normalized to be 1, and correlations between radioactivity and time were fitted as exponential functions of the logarithmic base. Regression

equations and coefficients of determination were obtained using the least-squares method. For the correlation coefficients, the square roots of the determination coefficients were used, and for tests of no correlation, the Kolmogorov-Smirnov-test and the t-test were performed. The significant difference between ^{137}Cs concentration from the whole-body of dogs and cats, and the genital organs of cats was tested using the Mann–Whitney U test, and for testing if there was no correlation, the Kolmogorov-Sminov test and the t-test were performed. Graphs were created using Microsoft Excel 2013 and statistical analyses were performed using R for Windows v.3.2.3. The effective half-life (Teff) was calculated from the regression equations of exponential decay from each animal, and the biological half-life (Tb) was obtained from the physical half-life of radioactive ^{137}Cs (30.08 years) (Tp) and the effective half-life. Effective half-live was calculated by using the equation below.

$$\frac{1}{\text{Teff}} = \frac{1}{\text{Tp}} + \frac{1}{\text{Tb}}$$

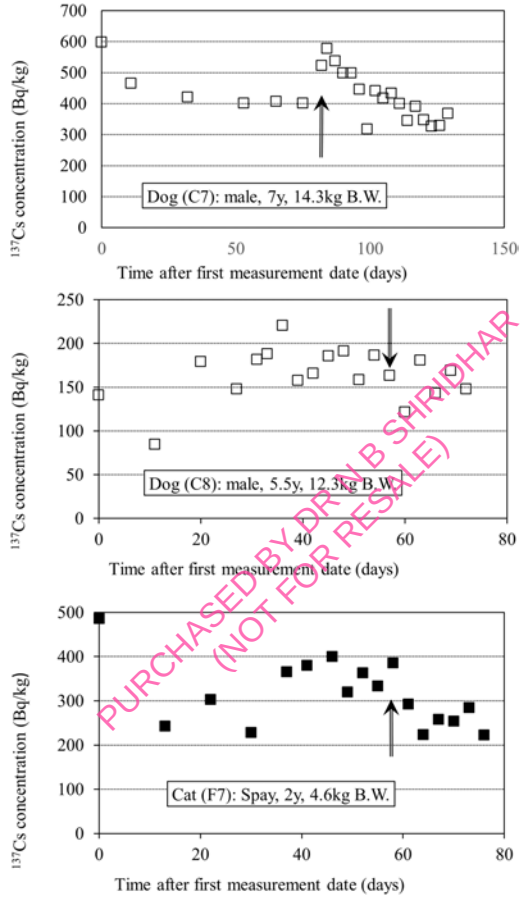
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RESULTS AND DISCUSSION

The Fukushima Headquarters for Animal Rescue have a policy to deliver disaster affected pet animals (rescued in the restricted zone or entrusted from the owner) to either their original owners or fosterers. To this end, a whole-body radiation counter was introduced, as it was essential to confirm the degree of internal exposure at the time when the animals were due to leave the animal rescue center. The use of whole-body counters for the evaluation of human internal exposure had already been widely used for this particular nuclear accident [9]; whereas the use of such instruments for animals had only been reported for evaluating radioactive cesium contamination in living beef cattle [11]. Although there have been examples of whole-body counters for dogs in studies on the effects of plutonium on living bodies [12] and in nuclear medicine [13], the Goiânia accident case was the only report we could find on the evaluation of internal contamination in living dogs [14]. We believe that this study is the first report on measurements of the elimination rates for radioactive cesium in pet dogs and cats using whole-body counters designed for animals.

Because clean food and water were given to the dogs and cats in the shelter, measurements were also taken for animals that had higher ^{137}Cs levels than the limit of detection of the whole-body counter. We obtained

high radiation counts for two dogs and one cat. However, the values did not change with time, so we resumed the measurements after washing the animals with shampoo and warm water.



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Figure 2: Observation of radiocesium decay after intervention.

One of these dogs (C7) initially produced a high radiation measurement; C7 was restless inside the cage during these measurements, so we restricted the animal's movement using a smaller cage and pillow, and observed a significant decline in measurements for C7 after this intervention. This was not seen in two further animals (C8 and F7) (Fig 2). Although we cannot deny the possibility that radioactive cesium was trapped within metabolically slow organs (such as the bones) of these animals, we surmised that because the phenomenon was only observed in those few animals, there was a miniscule amount of radioactivity fixed on their skin and fur. Using the animal data gathered from the decreasing trends over five repetitions,

including the first measurements post-washing, we were able to obtain a significant exponential decay curve from the plotted densities of ^{137}Cs for the 7 dogs and 6 cats.

In these cases it was not observed radiocesium decay, so the animals' bodies were washed again. The arrows indicate the point at which the animals were shampooed. C7 moved around in the cage a lot during the measurement, so its movement was limited by using a smaller cage and pillows.

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The regression equations and decay curves of the dogs and cats internal ^{137}Cs densities are shown in Figs 3 and 4, respectively. The age, body weight, number of measurements, coefficients of determination for the regression formulae, significance levels of correlation, the effective half-lives of ^{137}Cs and their median values, and the biological half-lives and their median values for the 7 dogs in Table 1 and 6 cats in Table 2 are shown. The individual breeds could not be identified since there was an abundance of mixed breeds.

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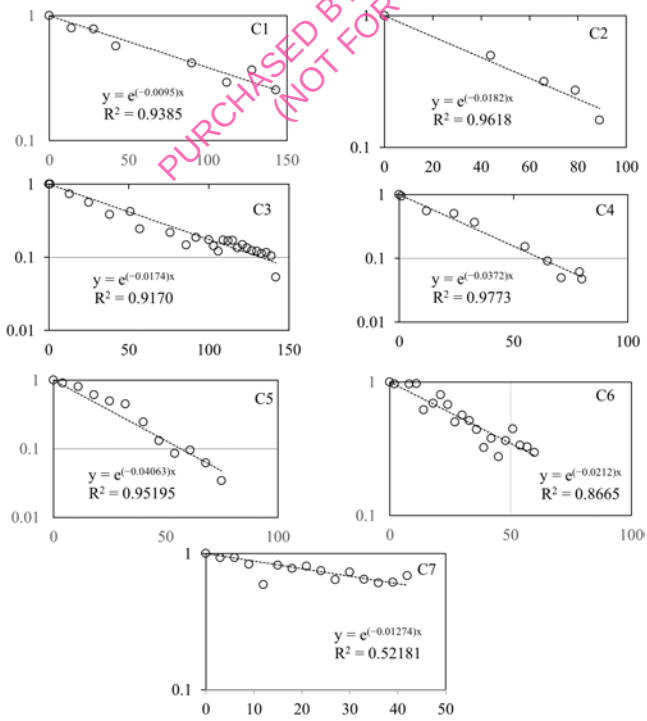
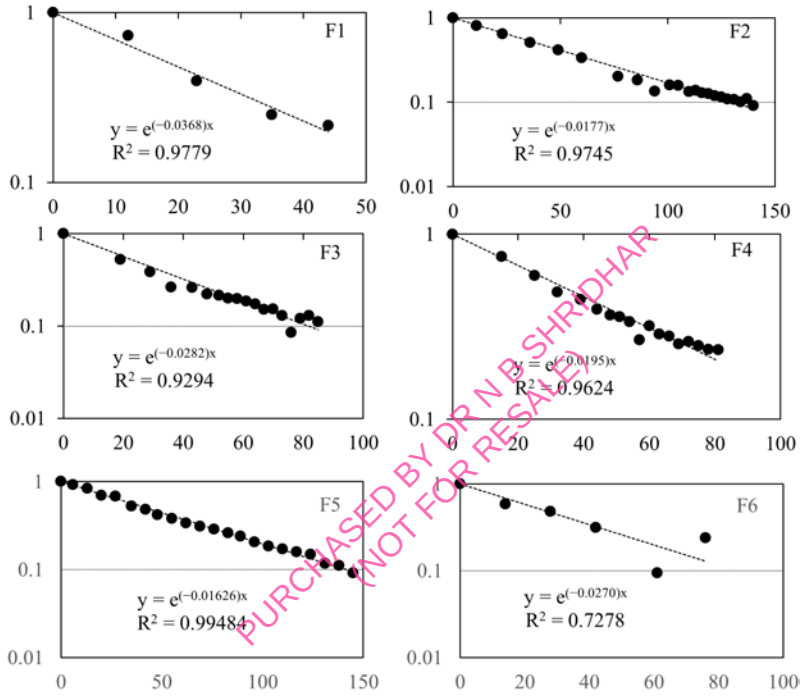


Figure 3: The decay curves of radiocesium (^{137}Cs) in canine bodies.

The graph shows the relative concentration in the whole body versus the number of days elapsed since the first measurement date. Each regression equation and the coefficient of determination are shown.

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Figure 4: The decay curves of radiocesium (^{137}Cs) in feline bodies.

The graph shows the relative concentration in the whole body versus the number of days elapsed since the first measurement date. Each regression equation and the coefficient of determination are shown.

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Table 1: ^{137}Cs biological half-lives and parameters of the decay curves of radiocesium in canine bodies

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Dogs	Sex	Age (years)	Body weight (kg)	Sample size (N)	Coefficient of determination (R^2)	Significant level of correlation	Effective half life (days)	Biological half life (days)
C1	Castrated male	7.5	13.3	8	0.9385	$p < 0.01$	72.7	73.1
C2	Intact male	4.5	17.4	5	0.9618	$p < 0.01$	38.1	38.2
C3	Intact female	5.5	10.2	25	0.9170	$p < 0.01$	39.8	40.0
C4	Intact female	7.5	6.0	10	0.9773	$p < 0.01$	18.6	18.7
C5	Intact male	2	9.1	12	0.9520	$p < 0.01$	17.1	17.1
C6	Intact female	0.67	8.5	20	0.8665	$p < 0.01$	32.7	32.8
C7	Intact male	7	14.3	15	0.5218	$p < 0.01$	54.4	54.7
Median							38.1	38.2
Quartile 25%							25.7	25.7
Quartile 75%							47.1	47.3

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Table 2: ^{137}Cs biological half-lives and parameters of the decay curves of radiocesium in feline bodies

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Cats	Sex	Age (years)	Body weight (kg)	Sample size (N)	Coefficient of determination (R^2)	Significant level of correlation	Effective half life (days)	Biological half life (days)
F1	Castrated male	3	4.76	5	0.9779	$p < 0.01$	18.8	18.9
F2	Spayed female	3	3.94	22	0.9745	$p < 0.01$	39.2	39.3
F3	Castrated male	7.5	4.28	24	0.9294	$p < 0.01$	24.6	24.6
F4	Spayed female	5	4.18	18	0.9624	$p < 0.01$	35.6	35.8
F5	Castrated male	5	4.68	24	0.9957	$p < 0.01$	42.8	43.0
F6	Spayed female	2	4.14	6	0.7278	$p < 0.05$	25.7	25.8
Median							30.7	30.8
Quartile 25%							24.6	24.6
Quartile 75%							38.3	38.4

doi:10.1371/journal.pone.0169365.t002

The effective half-lives shown in Tables 1 and 2 were derived from the regression formulae of exponential decay shown in Figs 3 and 4. The biological half-lives were calculated from these effective half-lives. Given that the physical half-life of the target nuclear species ^{137}Cs is 30.08 years, there were no major differences between the biological half-lives and the effective half-lives. In this study, the calculated biological half-lives of dogs showed a lot of variation, from 17.1 to 73.1 days (Table 1). In comparison, the upper and lower limits for the biological half-lives in cats were narrower, at 18.9 and 43.0 days, respectively (Table 2). However, large individual

differences were still present. There were no significant differences in the median biological half-life values of dogs (38.2 days) and cats (30.8 days) ($p = 0.39$). In humans, the biological half-life of radioactive cesium is age-dependent and is longer for adults than for juveniles [15–17]. In one study, the average half-life of ^{137}Cs in 23 Japanese adults was measured to be 86 days. In humans, the short half-life ^{132}Cs demonstrates biphasic biological half-lives of 1–2 days for the first phase and 50–80 days for the second phase [18]. The existence of a third phase has also been suggested, based on analyses of individuals exposed during the Goiânia accident in Brazil [14]. In our study, however, we were unable to accurately demonstrate the existence of a second phase in dogs and cats. We believe this may be due to the dogs and cats at the Miharu shelter were not exposed to high levels of radioactive cesium.

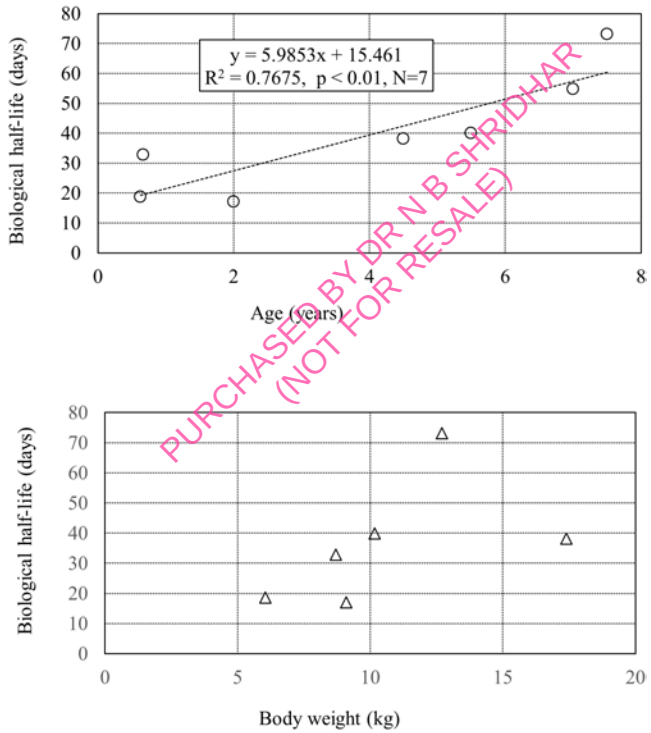
There have been many studies on internal radioactive cesium contamination in wild animals [4–8] and on the transfer coefficients of the meat and milk from animal feed [1, 2]. Despite this, there have been very few studies that have focused on the biological half-lives of radioactive cesium scattered into the environment. Although there are detailed analytical studies on the internal radioactive distribution in cattle during the Fukushima nuclear accident [19, 20], there are few reports pertaining to the detailed metabolism of radioactive cesium [21]. A study was conducted where radioactive cesium-contaminated feed was given to cattle over a 4-month period, after which the contaminated feed was substituted with clean feed [21]. By evaluating the time-dependent biological half-lives in cattle urine, it was reported that the average biological half-life during a 0–9 day period was 7.1 days, while during a 44–128 day period an average of 37.4 days was obtained [21].

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During our study, there was no observation of a biphasic exponential decay. We speculated that the majority of radioactive cesium in dogs and cats resides within the musculature, similarly to cattle [19, 20]. Based on the report of cattle exposure to high levels of cesium [21], it was thought that a bi- or tri-phasic decay pattern would be also observed in dogs and cats during a prolonged period of high level exposure. However, the non-contaminated food given to dogs and cats was provided by volunteer activists. Since this, the pollution level in dogs and cats in the shelter was not as high as that in the cattle.

Because our study obtained the multiple decay curves of radioactive cesium in dogs and cats, we tested for correlations of the biological half-

life of radioactive cesium with body weight or age. The results of the dogs are presented in Fig 5. The biological half-life demonstrates a significant correlation with age, showing that an age dependence is present in dogs. Although we also observe a tendency for the biological half-life to increase with body weight, no significant correlation was observed. Though not depicted in the figure, no relationship was observed between age, body weight, and biological half-life in cats. Because many of the cats were mostly similar in term of population characteristics (i.e., age and body weight) in this study, increasing the sample size may be necessary to probe the relationship between age, body weight and biological half-life.



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Figure 5: Correlation between canine biological half-life with age (top) and body weight (bottom).

Regression equation, the coefficient of determination, and significant level of correlation are shown.

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During the Goiânia accident in Brazil, Prussian blue (an ion-exchange drug) was administered to exposed individuals to encourage the excretion of radioactive cesium from the body. Reduction of the biological half-life has been reported in beagles (as an effect of the Prussian blue), and a correlation between body weight and biological half-life was also demonstrated [15]. Consequently, although it was anticipated that a positive correlation of age and body weight with biological half-life could be demonstrated for dogs—if detailed experiments were conducted on an increased number of individuals—from our data, we can only say that there is a correlation with age.

Using the regression equation, which shows the correlations of biological half-life with age, each animal's full body densities of radioactive cesium internal contamination at their time of capture were estimated for dogs and shown in Fig 6. These data are from 21 dogs and 40 cats, for which radioactive cesium was detected by the whole-body counter. Additionally, using the median biological half-life values, the individual whole-body densities estimated for cats, at their time of capture, are shown in Fig 6. The biological half-life values (C1–C7, F1–F6) used for the calculation in Fig 6 were the data shown in Tables 1 and 2. Furthermore, to curb the problem behaviors accompanied with breeding behavior, both castration and spaying were carried out on the cats. The corrected radioactive cesium densities of the extracted reproductive organs at the time of capture, similarly to the whole-body measurements, are shown in Fig 6.

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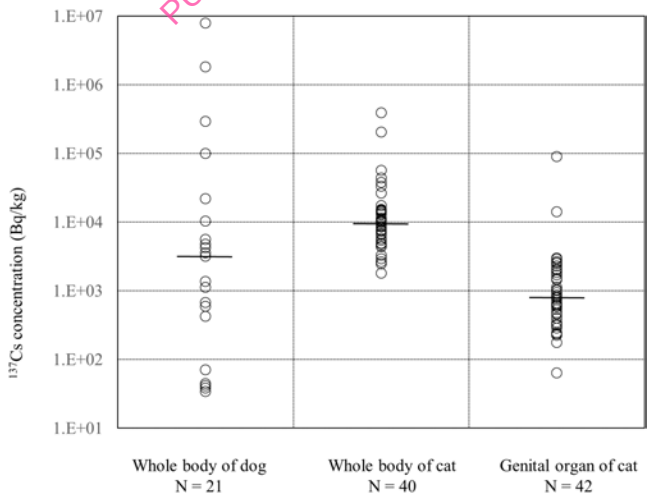


Figure 6: ^{137}Cs concentrations of canine whole-bodies, feline whole-bodies, and feline genital organs.

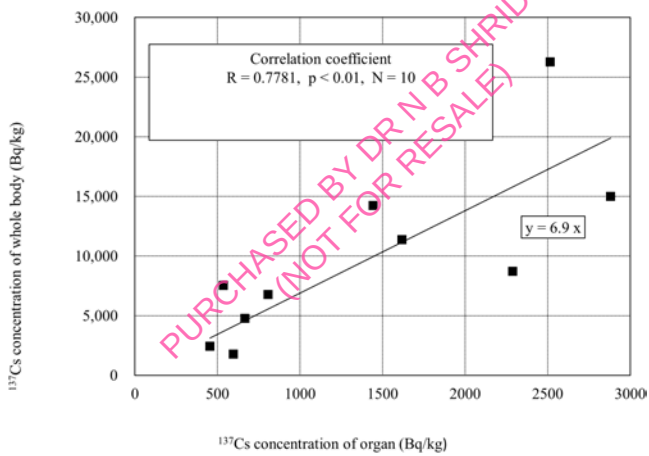
The whole-body concentrations and genital concentrations were corrected to the time caught, using the effective half-life values. Horizontal short bars show the median.

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From Fig 6 the differences in the data distribution of whole-body concentration between the dogs and cats can be clearly observed. The median whole-body value for the cats was higher, at 9,520 Bq/kg, than for the dogs, at 3,160 Bq/kg, and this was a significant difference ($p<0.01$). In addition to this, from the lowest and highest individual densities in dogs, it can be seen that the distribution range was wide, with a 100,000-fold difference. For cats, this distribution range was narrower, with the highest value only 100 times larger than the lowest. The order of the median values for both dogs and cats was on the same power of 10, and while we thought no significant difference in the median values, we believed that there are reasons for the differences in the whole-body data distributions. The first reason is the capture timing and duration of the holding period for the animals' rescue. Among the 40 cats in which radioactive cesium was detected via whole-body counter, the capture timing for 37 cats was concentrated within a 100-day period from 13 September 2012 to 21 December 2012. For the 21 dogs, there was a 3.5-year capturing period, from 5 June 2011 to 12 December 2014. The radiocesium pollution on the soil surface of the restricted zone is expected to decrease with time, in particular the radiocesium pollution of the city environment is expected to decreased faster than that of the forest environment; therefore, the animals that live there are considered to have less internal exposure as time passes. The second reason is due to the presumed differences in behavior between dogs and cats. At the time when the dogs were rescued, all of them roamed wild outdoors. We assumed that since their range of activities was wider and the consumption of water was larger, the internal radioactive concentrations for dogs were a reflection of the contamination in their individually marked territories. Their food was provided by volunteer activists early on in the rescue process, but their drinking water was primarily rain and standing water. In contrast, the cats had lived indoors, only occasionally venturing outside, and so their rescue activities were conducted mainly within human habitations. We surmised that due to this, their habitats might be collectively more similar to human habitats, and thus have a comparatively narrow concentration range. In reality, compared with the dogs, the capture of the cats was more difficult, and because a focused capture of the cats was conducted, it was considered that the distribution of pollution of in cats was not definitively observed.

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The distributions of radioactive cesium densities for the feline reproductive organs are shown in Fig 6. The median value is 790 Bq/kg, which shows a significant difference from the cats' whole-body densities ($p<0.01$). We were able to obtain corresponding data on the whole-body densities and reproductive organ densities for 10 of the cats, and this relationship is plotted in Fig 7. From this, we see that the whole-body densities are higher than the reproductive organ densities, with a significant correlation. Owing to the analysis of internal organs and muscle tissue of cattle contaminated during the FNPP accident, it is already known that the majority of radioactive cesium accumulates in the muscles [19, 20], and very little is distributed to other organs. We assumed that even for cats, the whole-body densities would be high. The whole-body densities of the 10 cats in Fig 7 were roughly 7 times the reproductive organ densities.



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Figure 7: Correlation between whole-body radioactivity and genital organ radioactivity in cats.

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Our whole-body counter, designed specifically for the dogs and cats in this study, was installed at the Miharu shelter 2 years after the FNPP accident; if it had been installed at an earlier stage more precise high quality information could have been acquired. Radioactive cesium taken into the animal body is discharged to the outside of the body by giving them clean food and water. By means of the biological half-life of radioactive cesium in this study, it is possible to plan the appropriate management period in an animal shelter. Optimizations of the animal management period contribute to animal welfare as well as reduction in administration expenses. We were

able to survey the biological half-life of radioactive cesium in dogs and cats contaminated by an actual nuclear accident. We believe that our findings on biological half-life in dogs and cats are valuable. We are confident that this information is useful for the management of domestic animals in the event of a future nuclear accident.

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Chapter 8

Melatonin Protects Rats From Radiotherapy-Induced Small Intestine Toxicity

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ABSTRACT

Radiotherapy-induced gut toxicity is among the most prevalent dose-limiting toxicities following radiotherapy. Prevention of radiation enteropathy requires protection of the small intestine. However, despite the prevalence and burden of this pathology, there are currently no effective treatments for radiotherapy-induced gut toxicity, and this pathology remains unclear. The present study aimed to investigate the changes induced in the rat small intestine after external irradiation of the tongue, and to explore the potential radio-protective effects of melatonin gel. Male Wistar rats were subjected to irradiation of their tongues with an X-Ray YXLON Y.Tu 320-D03 irradiator, receiving a dose of 7.5 Gy/day for 5 days. For 21 days post-irradiation, rats were treated with 45 mg/day melatonin gel or vehicle, by local application into their mouths. Our results showed that mitochondrial oxidative stress, bioenergetic impairment, and subsequent NLRP3 inflammasome activation were involved in the development of radiotherapy-induced gut toxicity. Oral treatment with melatonin gel had a protective effect in the small intestine, which was associated with mitochondrial protection and, consequently, with a reduced inflammatory response, blunting the NF-κB/NLRP3 inflammasome signaling activation. Thus, rats treated with melatonin gel showed reduced intestinal apoptosis, relieving mucosal dysfunction and facilitating intestinal mucosa recovery. Our findings suggest that oral treatment with melatonin gel may be a potential preventive therapy for radiotherapy-induced gut toxicity in cancer patients.

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INTRODUCTION

Mucositis is a debilitating and untreatable condition involving deep mucosal ulcerations [1]. It is among the most common toxicities associated with cytotoxic cancer therapy, imposing a substantial burden on patients undergoing chemotherapy and radiation treatments. Mucositis is caused when cytotoxic cancer treatment damages normal tissue, and can broadly impact various mucosal tissues [2]. Oral mucositis is associated with a cascade of events in multiple tissue regions [3–5], with radiotherapy-induced gut toxicity being a prevalent dose-limiting toxicity that manifests following radiotherapy [6].

In the clinical setting, radiation-induced damage to healthy intestine tissue is a common side-effect caused by out-of-field or scattered radiation. Since it is a rapid turnover system, the small intestine mucosa is a focus for

radiation related side-effects [7]. The severity of intestinal radiation toxicity is determined based on the extent of radiation-induced intestinal crypt cell death. Direct radiation to the small intestine induces changes in cellular function, that can lead to mucosal breakdown, microcirculation damage [8], leukocyte cell adhesion and emigration, and other effects [9,10]. Some of these pathophysiological manifestations in the small intestine have also been described as out-of-field effects of external beam irradiation of other organs [7]. Thus, the prevention of radiation enteropathy requires protection of the small intestine. However, despite the prevalence and burden of radiotherapy-induced gut toxicity, its pathophysiological pathways are yet unclear, and there is currently no effective treatment for this disease.

Melatonin (N-acetyl-5-methoxytryptamine) is a potent free radical scavenger with anti-oxidant and anti-inflammatory properties [11–15]. It reportedly maintains mitochondrial homeostasis under various experimental conditions [16–19], and protects against radiation injury [20–23]. We recently found that melatonin could completely prevent the activation of multiple radiation-induced pathways associated with oral mucositis [24]. In our previous report, we describe the involvement of mitochondrial oxidative stress, bioenergetic impairment, and subsequent NLRP3 (NLR-related protein 3 nucleotide-binding domain leucine-rich repeat containing receptors-related protein 3) inflammasome activation in oral mucositis development following tongue irradiation. Moreover, the application of a melatonin gel prevented mucosal disruption and ulcer formation, protecting the mitochondria from radiation damage and blunting NF-κB/NLRP3 inflammasome signaling activation in the tongue. Phase II clinical trials are now investigating melatonin gel in patients with head and neck cancer who are undergoing chemoradiation, involving cisplatin, carboplatin, or cetuximab plus radiation given in fractionated doses of 2 Gy per day Monday through Friday (10 Gy per week for a total dose of 60–70 Gy).

The presently available data suggest that melatonin is protective against oxidative stress and organ injury, including radiotherapy-induced mucositis. However, the development of effective treatment is hindered by our lack of knowledge regarding the mechanisms involved in radiotherapy-induced gut toxicity. Thus, our present study aimed to investigate the changes induced in rat small intestine following external irradiation of the tongue, and to explore the potential radio-protective effects of a melatonin gel.

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MATERIALS AND METHODS

Animal Irradiation

All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Granada (procedures CEEA-2009-238) and were in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS # 123) and the Spanish law (R.D. 53/2013). This study was conducted using adult (3-month-old) male Wistar rats. The rats used in this study are the same as in reference 24 [24]. They were maintained in the University's facility with a 12-hour light/12-hour dark cycle at 22 ± 2°C, with lights on at 08:00 h (ZT = 0), and they received water and food *ad libitum*. The rats were randomly divided into three groups: control (non-irradiated), irradiated + vehicle (melatonin-free gel), and irradiated + local application of 3% melatonin gel. For this study, we used a gel containing 3% melatonin (Fagron Ibérica SAU, Terrasa, Spain) dissolved in 0.3% ethanol:Pluronic F-127 (Fagron Ibérica SAU, Terrasa, Spain). The gel was made in our laboratory and it is under patent. Previously, we performed a dose-response study and the maximal therapeutic effect was obtained with 3% melatonin gel [24]. Therefore, in the group that received melatonin gel, the total melatonin dose was 45 mg/day for 21 days. A group of irradiated rats was also treated with vehicle, consisting in a 0.3% ethanol:Pluronic F-127 melatonin-free gel. Rats were anesthetized with 1 mL equithesin/kg body weight, intraperitoneal (i.p.), prior to irradiation with a dose of 7.5 Gy/day for five consecutive days (Fig 1A) [24]. Irradiation was always applied in the morning between ZT 1 and ZT 4. Ionizing radiation was delivered using an X-Ray YXLON Y. Tu 320-D03 irradiator (Yxlon, Hamburg, Germany) with a voltage of 207.3 kV, working current of 10.5 mA, 5-mm-diameter irradiation focus, 0.25-mm Cu filter system, 15-cm target distance, irradiation field of 0.78 cm^2, and delivered dosage of 100.2 cGY/min. For irradiation, each animal was placed in the irradiator with a standardized snout positioning, and with a lead shield covering the entire animal except for the mouth. The radiation dose to shielded areas was 2.4×10^{-2} cGy/min (7.5 min per rat), ensuring that the total body dose received by rats during irradiation treatment was less than 9.0×10^{-3} Gy. After radiation, animals that received the same treatment were housed with four or five animals per cage.

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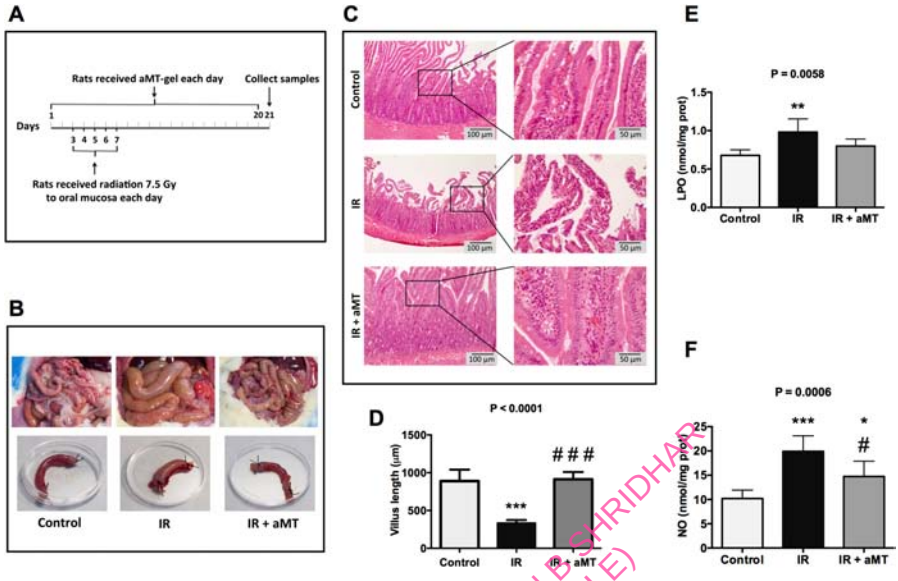


Figure 1: Effects of melatonin gel on small intestine damage induced by external radiation in rats.

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Schedule of fractionated radiation and melatonin treatment (A). Representative pictures of small intestine from non-irradiated rats (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT) (B). H&E staining of the small intestine (C) and villus length (D) from non-irradiated rats (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT). The right panels show details at higher magnification. Scale bar = 100 μm and 50 μm in the left and right panels, respectively. LPO (E) and NO levels (F) in small intestine tissue homogenate from non-irradiated rats (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT); $n = 4$ per group. Data are expressed as mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control, and $^{\#}P < 0.05$ vs. IR.

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Melatonin gel or vehicle was applied in the oral cavity starting 48 hours before the first irradiation dose, until up to 14 days after the last irradiation exposure. Using a plastic Pasteur pipette, the gel was applied in the intraoral regions three times per day (ZT 0, ZT 6, and ZT 12). On days of irradiation, the gel was applied 1 hour before and 1 hour after each radiation dose, with the third dose 6 hours later. Rats were monitored daily, and euthanized under an over dose of anesthesia (>1.5 mL equithesin/kg body weight, i.p.)

followed by cervical dislocation, 14 days after the last radiation dose. All the animals were sacrificed in the morning between ZT 0 and ZT 4. Upon sacrifice, the animals' small intestines were harvested and processed as described below for each analysis/technique.

Histology, Histopathology, and Electron Microscopy

Small intestine samples were fixed in 10% buffered formalin for 48 hours, embedded in paraffin, and sliced into 4-μm-thick sections. Sections were deparaffinized with xylene and stained with hematoxylin and eosin (H&E).

In these same sections, we then performed immunohistochemistry using primary rabbit polyclonal antibody to zonula occludens-1 (ZO-1) (MABT11, 1:50; Millipore, Madrid, Spain) and primary rabbit antibody Ki-67 (Ab16667, 1:100; Abcam, Cambridge, United Kingdom). For rabbit primary antibodies, we used the Vectastain ABC kit (Vector PK6101; Burlington, NC, USA). For qualitative identification of antigens by light microscopy, we used the peroxidase substrate DAB kit (Vector SK-4100; Thermo Scientific, Madrid, Spain). Sections were examined using an OLYMPUS CX41 microscope, and images were scanned under equal light conditions using the CELL A program (Olympus,Hicksville,NY,USA). The epithelia region was selected based on its histological appearance, and the same region was used for all immunohistochemistry studies. Ki-67 activity was quantified using ImageJ 1.46r, with the Cell Counter plugin. The labeling percentage was assessed as the ratio of the number of cells stained with Ki-67 to the total number of cells counted per section. ZO-1 expression was also quantified using ImageJ 1.46r software. Briefly, immunohistochemistry images were acquired using identical parameters, the figures were transformed into 8-bit images, and background was subtracted, and the ZO-1 staining intensity was measured. The reported values are the average of different images from four different rats (at least ten images from each rat).

For electron microscopy, rats were perfused with physiologic saline (0.9% NaCl) and fixation solution (2% paraformaldehyde and 2% glutaraldehyde). Small intestine samples were fixed with 2% osmium, rinsed, dehydrated, and embedded in Durcupan resin (Fluka; Sigma-Aldrich, St. Louis, USA). Ultra-thin sections (0.08 μm) were cut using a diamond knife, and then stained with lead citrate (Reynolds solution). Samples were analyzed using a Leo 906E transmission electron microscope (Carl Zeiss, Jena, Germany) equipped with a Morada digital camera (Silicon Integrated Systems, Hsinchu, Taiwan).

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Isolation of Pure Mitochondria, Nuclei, and Cytosol

After the animals were sacrificed, we immediately isolated the small intestine mitochondria, nuclei, and cytosol by differential centrifugation and Percoll density gradient as described previously [25].

Measurement of LPO and NOx Levels

Tissues were weighed and then homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 800 × *g* for 10 min at 4°C. Some aliquots of the supernatant were stored at −80°C for total protein determination [26], while other aliquots were used for lipid peroxidation (LPO) or nitrite plus nitrate (NOx) measurement, a marker for NO levels. LPO was assessed using the Bioxytech LPO-568 assay kit (OxisResearch, Portland, OR, USA) [27]. LPO levels were expressed as nmol/mg protein. NOx levels were determinate using the Griess reaction [28], and NOx concentrations were expressed as nmol nitrite/mg protein.

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Measurement of GPx, GRd, and Mn-SOD2 Activities

To measure glutathione peroxidase (GPx) activity, small intestine mitochondria were incubated for 4 min at 37°C in a working solution, containing buffer A plus 4 mM sodium azide, 4 mM GSH, 0.2 mM NADPH, and 0.5 U/mL GRd. After incubation, the reaction was started with the addition of cumene hydroperoxide (0.3%). GPx activity was determined by following NADPH oxidation at 340 nm for 3 min in a UV spectrophotometer (Shimadzu Deutschland GmBH, Duisburg, Germany) [29]. To measure glutathione reductase (GRd) activity, samples were incubated for 4 min at 37°C in a working solution containing buffer A plus 2 mM GSSG. Then, the reaction was started by adding 9.5 mM NADPH solution. GRd activity was measured by following NADPH oxidation at 340 nm for 3 min. For both measurements, nonenzymatic NADPH oxidation was subtracted from the overall rates. GPx and GRd activities were expressed as nmol/min/mg protein. Mn superoxide dismutase (SOD2) was assayed in terms of its ability to inhibit the auto-oxidation of adrenaline to adrenochrome at pH 10.2 [30]. SOD2 activity was expressed as U/mg protein, where 1 unit equals 50% inhibition of epinephrine auto-oxidation.

Measurement of GSH and GSSG Levels

Glutathione (GSH) and glutathione disulfide (GSSG) were measured using an established fluorometric method [31]. Small intestine mitochondria were

sonicated in ice-cold 50 mM Tris-HCl buffer (pH 7.4), and then centrifuged at 800 × *g* for 10 min at 4°C. Aliquots of the supernatant were deproteinized using ice-cold 10% trichloroacetic acid, and then centrifuged at 20,000 × g for 15 min. For GSH measurement, supernatant aliquots were incubated for 15 minutes at room temperature with an ortho-phthalaldehyde/ethanol solution (1 mg/mL) and phosphate buffer (100 mM sodium phosphate and 5 mM EDTA-Na2, pH 8.0). Then the sample fluorescence was measured at 340 nm excitation and 420 nm emission wavelengths using a plate-reader spectrofluorometer (Bio-Tek Instruments, Inc., Winooski, VT, USA).

For GSSG measurement, supernatant aliquots were preincubated with N-ethylmaleimide solution (5 mg/mL) at room temperature for 40 min, and then alkalinized with 0.1 N NaOH. Aliquots of this mixture were then incubated for 15 minutes at room temperature with ortho-phthalaldehyde solution (1 mg/mL) and 0.1 N NaOH, and the sample fluorescence was then measured. GSH and GSSG concentrations were expressed as nmol/mg protein.

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Western Blot Analysis

We performed protein extraction and western blot analyses as described previously [32]. The utilized antibodies included rabbit antibody to GPx (sc-30147, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), GRd (sc-32886, 1:200; Santa Cruz Biotechnology), Mn-SOD (sc-30080, 1:500; Santa Cruz Biotechnology), p53 (sc-6243, 1:200; Santa Cruz Biotechnology), Bax (sc-526, 1:200; Santa Cruz Biotechnology), Bcl2 (sc-492, 1:200; Santa Cruz Biotechnology), ASC (AG-25B-0006-C100, 1:1,000; AdipoGen, San Diego, CA, USA), caspase 1 (sc-22165, 1:500; Santa Cruz Biotechnology), IL-1β (sc-7884, 1:100; Santa Cruz Biotechnology), COX-2 (sc-7951, 1:200; Santa Cruz Biotechnology); mouse antibody to OXPHOS (MitoProfile Total OXPHOS Rodent WB Antibody Cocktail, MS604, 1:250; Mitosciences, Eugene, OR, USA), NLRP3 (AG-20B-0014, 1:500; AdipoGen), NFκB (p65) (sc-8008, 1:100; Santa Cruz Biotechnology), TNFα (sc-52746, 1:200; Santa Cruz Biotechnology) and GAPDH (sc-25778, 1/500; Santa Cruz Biotechnology); goat antibody to pro-caspase 1 (sc-622, 1:500; Santa Cruz Biotechnology); goat anti-rabbit IgG (Pierce® Antibody 31460, 1:5,000; Thermo Scientific); HRP goat anti-mouse IgG (554002, 1:1,000; BD PharmigenTM, San Jose, CA, USA); and donkey anti-goat IgG-HRP (sc-2020, 1:1,000; Santa Cruz Biotechnology). The proteins were visualized using a Western Lightning Plus-ECL chemiluminescence kit (PerkinElmer, Billerica, MA, USA) following the manufacturer's protocol. Images were

analyzed using the Kodak Image Station 2000R (Eastman Kodak Company, Rochester, NY, USA). Protein bands intensity were normalized to GAPDH, and the data expressed in terms of percent relative to controls.

Melatonin Determination by HPLC

Melatonin was extracted using trichloromethane, and the organic phase was evaporated to dryness using a SPD 2010 Speed Vac System (Fisher Scientific, Madrid, Spain). Samples were analyzed by HPLC (Shimadzu Europe GmbH, Duisburg, Germany) with a Waters Sunfire C18 column (150 × 4.5 mm, 5 µm). Melatonin fluorescence was measured in a Shimadzu RF-10A XL fluorescence detector (Shimadzu Europe GmbH, Duisburg, Germany), with excitation and emission wavelengths of 285 and 345 nm, respectively [32].

Statistical Analysis

A one-way Analysis of Variance (ANOVA) was performed to assess differences among means and when significant pairwise comparisons were carried out using Tukey´s penalizations. All tests were declared significant at 0.05 error but *P* values were reported in all the cases. STATA 14.1 was used for statistical computations. Data are presented as mean ± s.e.m.

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RESULTS

Melatonin Gel Prevents Deleterious Effects of External Radiation in Small Intestine Epithelia

Although the intestine was outside of the irradiation field, macroscopic changes in the small intestine were apparent at 21 days after tongue irradiation with 5 Gy/day for 5 consecutive days (Fig 1A and 1B). Typical signs of mucositis were detectable in the small intestines of irradiated animals, but not in the rats treated with melatonin gel. H&E staining was performed to assess the tissue damage after irradiation (Fig 1C). IR-treated animals exhibited significant loss of crypt architecture, severe villous epithelial atrophy, villus degeneration and shortening, and marked infiltration of the lamina propia with inflammatory cells (Fig 1C and 1D). Animals treated with melatonin gel showed reduced intestinal morphological changes, including increased villi and reductions of cellular infiltration and areas of hemorrhage (Fig 1C and 1D).

Oral irradiation to rats also resulted in increased small intestinal damage associated with oxidative stress, as indicated by enhanced LPO ($P < 0.01$) and NO ($P < 0.001$) compared to non-irradiated controls. Treatment with melatonin gel decreased oxidative stress, especially NO levels, ($P < 0.05$) (Fig 1E and 1F). Since the intestinal epithelium is one of the fastest proliferating tissues in the body [33], it is particularly vulnerable to reactive oxygen species (ROS) and, therefore, to radiotherapy. Based on the evidence of increased oxidative stress in the small intestine (Fig 1E and 1F), proliferating cells were identified by immunohistochemical staining of Ki-67 (Fig 2A and 2B). Irradiated rats showed decreased numbers of Ki-67-positive cells ($P < 0.01$), and the group treated with melatonin gel showed greater numbers of Ki-67-positive cells compared to the irradiated group ($P < 0.01$) (Fig 2A and 2B).

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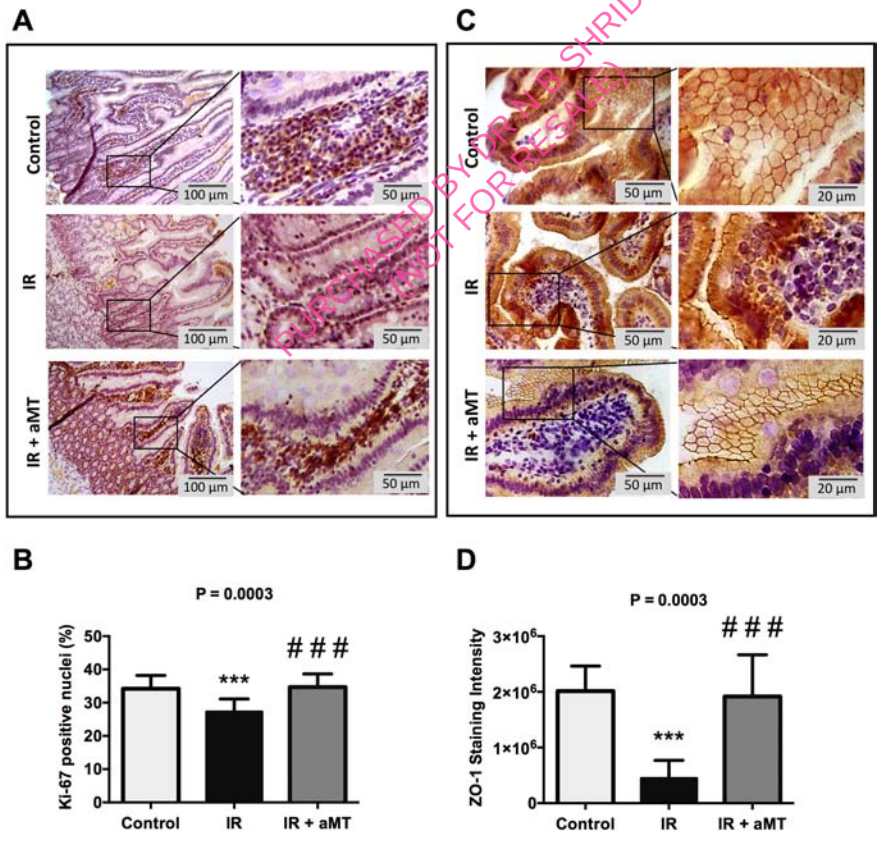


Figure 2: Immunohistochemical staining of Ki-67 and ZO-1.

Immunohistochemical staining of the proliferation marker Ki-67 (A) and of the tight junctions protein zonula occludens 1 (ZO-1) (C) in rat small intestine from non-irradiated rats (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT). The right panels show details at higher magnification. Scale bar = 100 μm and 50 μm in the left and right panels, respectively. (B, D) Quantification of the staining in A and C; n = 4 per group. Data are expressed as mean ± s.e.m. ***$P < 0.001$ vs. control and ###$P < 0.001$ vs. IR.

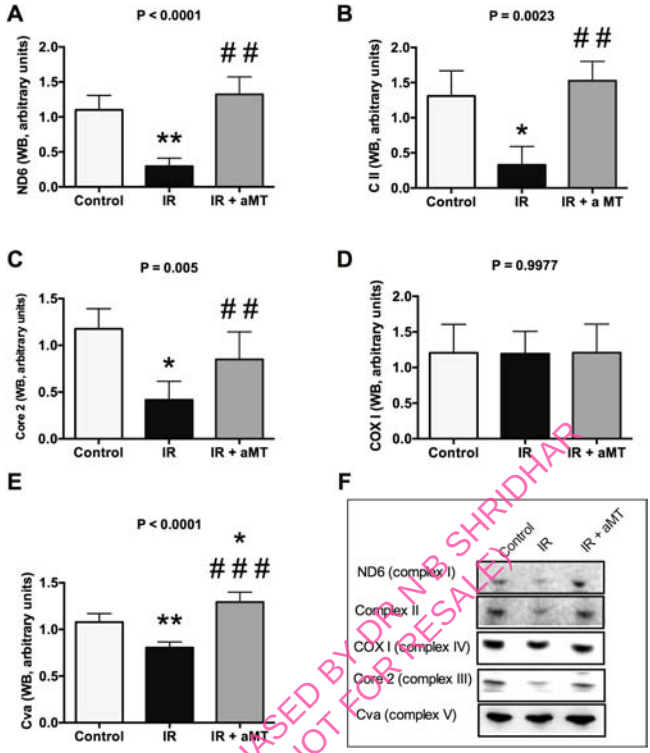
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To confirm the association between ROS, loss of proliferation, and intestinal barrier dysfunction, we performed an immunohistochemical analysis of the tight junction (TJ) protein ZO-1 in rat small intestine tissue (Fig 2C and 2D). We found that tongue irradiation significantly decreased ZO-1 levels ($P < 0.01$) in rat small intestine compared to in the non-irradiated group. Moreover, treatment with melatonin gel completely counteracted this effect ($P < 0.01$).

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Melatonin Gel Prevents External Radiation-induced Mitochondrial Dysfunction in Small Intestine Epithelia

Interestingly, the oxidative stress in the small intestine caused by tongue irradiation was associated with increased mitochondrial damage. Our analysis of bioenergetic capacity revealed that compared to non-irradiated rats, the irradiated rats showed significantly reduced expressions of respiratory complexes I, II, and III, as well as the ATP synthase ($P < 0.001$, $P < 0.5$, $P < 0.5$, and $P < 0.01$, respectively) (Fig 3A–3F). Treatment with melatonin gel completely counteracted this mitochondrial dysfunction, restoring the levels of the complexes I, II, III, and V to above the levels in controls ($P < 0.01$, $P < 0.01$, $P < 0.01$, and $P < 0.001$, respectively) (Fig 3A–3F).



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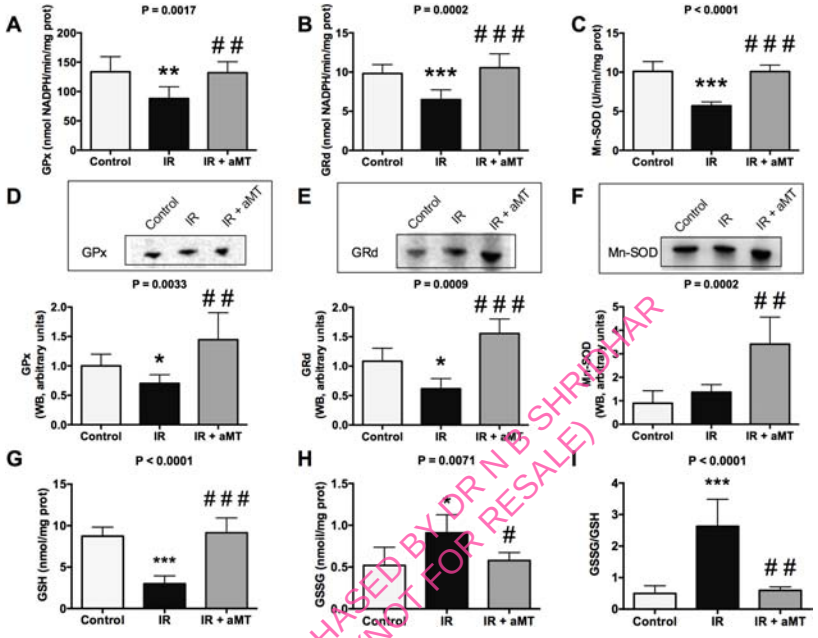
Figure 3: OXPHOS analysis.

Western blot (WB) analysis and densitometric quantification of OXPHOS: ND6 (complex I) (A), complex II (B), core II (complex III) (C), COX I (complex IV) (D), Cva (complex V) and Western blot (WB) analysis of OXPHOS (F) in the mitochondria of small intestine cells from non-irradiated rats (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT); n = 6 per group. Data are expressed as mean ± s.e.m. *$P < 0.05$, **$P < 0.01$ vs. control; and ##$P < 0.01$, ###$P < 0.001$ vs. IR.

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Mitochondrial dysfunction leads to enhanced production of ROS and reactive nitrogen species (as shown in Fig 1E and 1F), inducing cell death. We observed that radiation decreased the activity and protein levels of mitochondrial GPx (Fig 4A and 4D) ($P < 0.01$ and $P < 0.05$) and GRd (Fig 4B and 4E) ($P < 0.001$ and $P < 0.05$). Irradiation also decreased the SOD2 activity (Fig 4C) ($P < 0.001$) without altering the protein levels (Fig 4F). Melatonin gel treatment significantly increased the activity and protein

levels of GPx (Fig 4A and 4D) ($P < 0.01$ and $P < 0.01$), GRd (Fig 4B and 4E) ($P < 0.001$ and $P < 0.001$), and SOD2 (Fig 4C and 4F) ($P < 0.001$ and $P < 0.01$).



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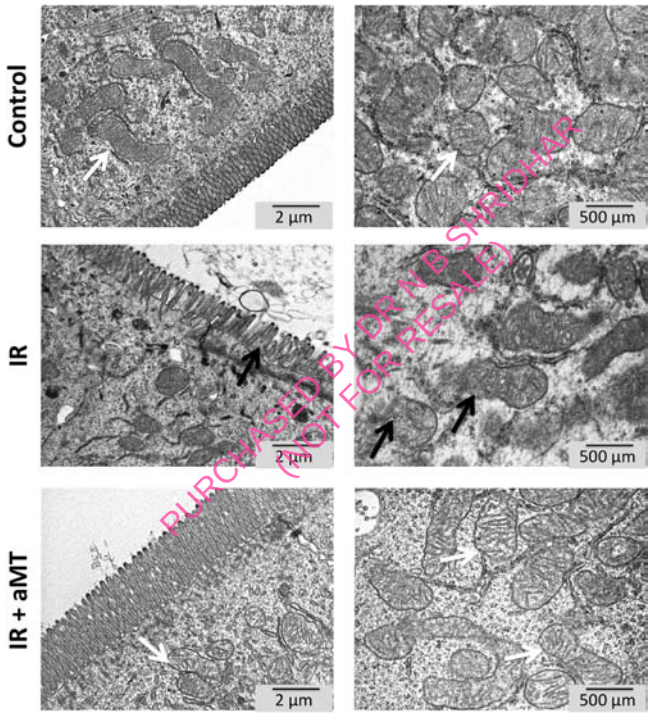
Figure 4. Oxidative stress analysis.

Activity and western blot (WB) analysis of GPx (A and D), GRd (B and E), and Mn-SOD (C and F) in the mitochondria of small intestine cells from non-irradiated rats (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT). GSH (G) and GSSG levels (H), and GSSG/GSH ratio (I) in the mitochondria of small intestine cells. n = 6 per group. Data are expressed as mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. IR.

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Our results suggested that small intestine mitochondria showed an oxidizing response to oral radiation, which was further supported by the increased mitochondrial GSSG/GSH ratio ($P < 0.001$) (Fig 4I). Treatment with melatonin gel decreased the oxidative stress in the small intestine characterized by increased GSH levels (Fig 4G) ($P < 0.001$), restoring a normal GSSG/GSH ratio ($P < 0.001$) (Fig 4I). These findings were in line with our observation that melatonin treatment reduced mitochondrial dysfunction in the small intestine and could thus reduce mitochondrial

damage. To confirm this hypothesis, we analyzed mitochondrial structure using EM (Fig 5). Compared to non-irradiated rats, irradiated rats showed cells with abundant broken and swollen mitochondria, poor cristae, and numerous vacuoles. Melatonin gel administration completely prevented this mitochondrial disintegration induced by external radiation. This suggests that mitochondrial damage could be involved in radiotherapy-induced gut toxicity [24]. We also observed that shortened and degenerated villi completely recovered with melatonin gel treatment.



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Figure 5: Electron microscopy (EM) analysis.

EM images of mitochondria of small intestine cells from non-irradiated rats (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT); n = 4 per group. Swollen and less dense mitochondria are indicated by black arrows. Normal mitochondria are indicated by white arrows. Degenerated and shorted villi are indicated by black arrows. Right panels show details at higher magnification. Scale bar = 2 µm and 500 nm in the left and right panels, respectively.

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Melatonin Gel Prevents External Radiation-induced NLRP3 Inflammasome Pathway Activation in Small Intestine Epithelia

It was recently reported that mitochondrial ROS may promote inflammation by activating the multiprotein cytoplasmic complexes inflammasome NLRP3 [34,35]. NLRP3 inflammasome activation leads to caspase-1 activation and subsequent cleavage of pro-cytokines, such as IL-1β, into their mature forms. This inflammasome is also reportedly involved in irradiation-induced mucositis [24]. In agreement with these prior studies, our present results revealed significantly increased NLRP3 in the small intestine ($P < 0.001$) following tongue irradiation compared to in non-irradiated controls (Fig 6A and 6D). Moreover, irradiation led to decreased protein levels of pro-caspase-1 (Fig 6B and 6D) ($P < 0.001$), reflecting its activation to caspase-1 (Fig 6C and 6D) ($P < 0.001$) by activated NLRP3, and yielding a significant increase in IL-1β ($P < 0.05$) (Fig 7C and 7D). Treatment with melatonin gel completely suppressed inflammasome activation in the small intestine (Fig 6A–6D), decreasing IL-1β levels ($P < 0.05$) (Fig 7C and 7D).

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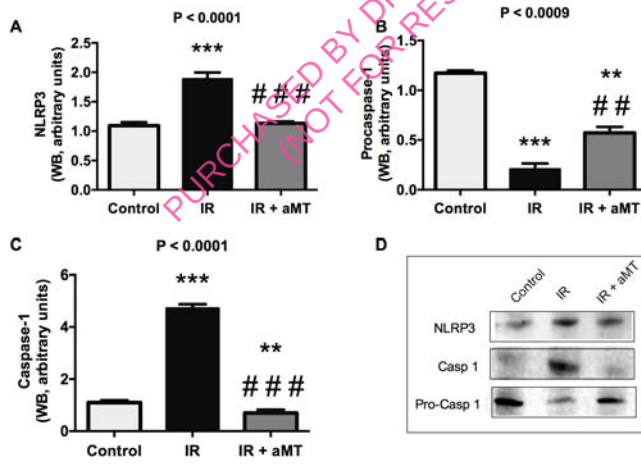
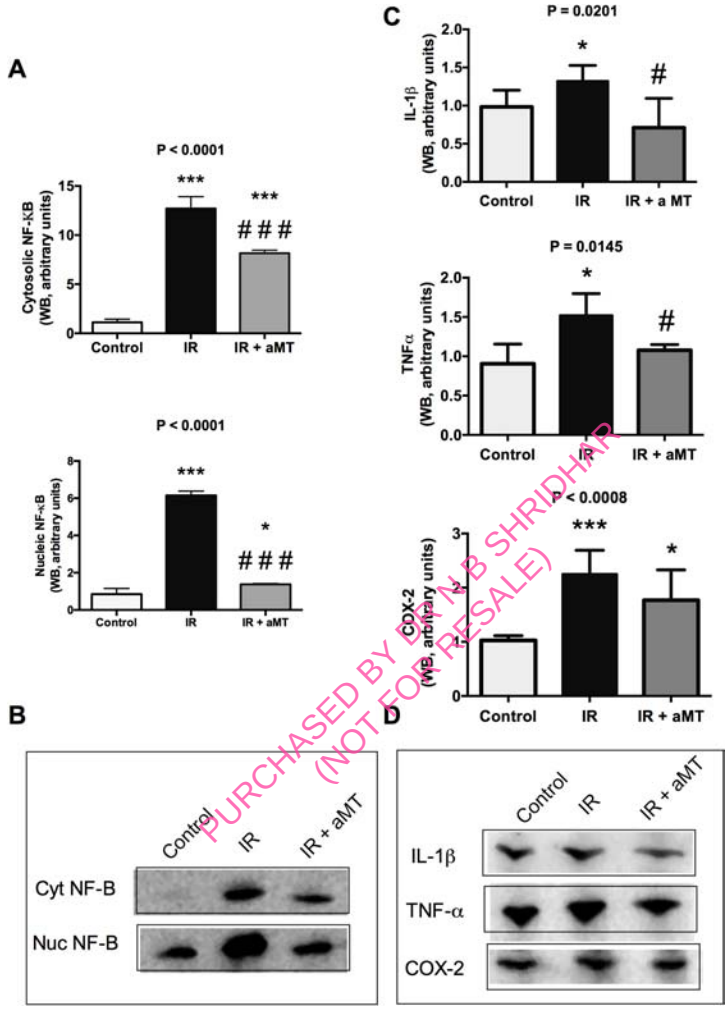


Figure 6: NLRP3 inflammasome pathway.

Western blot (WB) analysis and densitometric quantification of NLRP3 (A, D), pro-caspase 1 (B, D), and caspase 1 (C, D) in small intestine from non-irradiated (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT); n = 6 per group. Data are expressed as mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; and $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. IR.

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Figure 7: NF-**κB** pathway.

Western blot (WB) analysis and densitometric quantification of NF-κB subunit p65 in nuclear and cytosolic fractions (A, B); cellular inflammatory cytokines IL-1β, TNF-α and the enzyme COX-2 (C, D) from small intestine cells from non-irradiated (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT); n = 6 per group. Data are expressed as mean ± s.e.m. *$P < 0.05$, ***$P < 0.001$ vs. control; and #$P < 0.05$, ###$P <$ 0.001 vs. IR.

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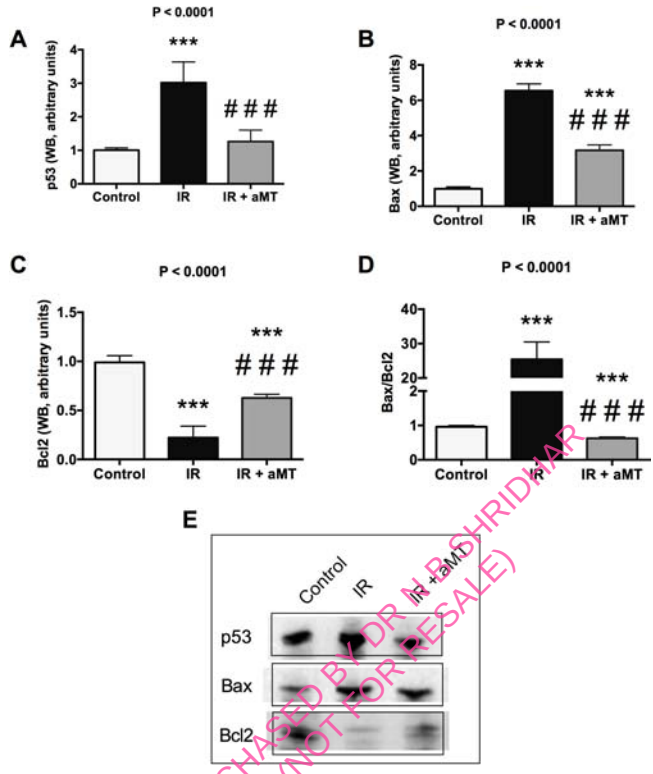
Melatonin Gel Prevents External Radiation-induced NF-kB Pathway Activation in Small Intestine Epithelia

NLRP3-activated caspase-1 requires the substrate pro-IL-1ß, which is produced by the NF-κB pathway. Thus, we evaluated the contribution of the nuclear factor kappa B (NF-κB) pathway to irradiation-induced small intestine toxicity (Fig 7A and 7B). Irradiation significantly increased the protein levels of NF-kB in the cytosol and nuclei ($P < 0.001$ in both) in the small intestine compared to in the non-irradiated group, and treatment with melatonin gel suppressed the irradiation-activated NF-κB pathway. NF-kB activation leads to the expression of up to 200 inflammatory response-related genes. Our present results confirmed that irradiation led to increased protein levels of IL-1β, TNFα, and COX-2 ($P < 0.05$, $P < 0.05$, and $P < 0.001$, respectively) in the small intestine (Fig 7C and 7D). We further observed significant reductions of these inflammatory mediators of the NF-κB response following treatment with melatonin gel (Fig 7C and 7D).

Melatonin Gel Prevents External Radiation-induced Apoptosis in Small Intestine Epithelia

The inflammatory mediators produced by NF-κB pathways substantially increase apoptosis, decrease proliferation, and degrade the extracellular matrix [1,5,36]. Increased apoptosis is also seen in relation to the mitochondrial dysfunction and increased ROS caused by irradiation [24]. Compared to non-irradiated controls, irradiation effectively upregulated p53 (Fig 8A and 8E) ($P < 0.001$), leading to a marked increase of Bax (Fig 8B and 8E) ($P < 0.001$) and decrease of Bcl2 (Fig 8C and 8E) ($P < 0.001$), reflecting significant apoptotic changes and a high degree of cellular injury. Treatment with melatonin gel significantly decreased p53 expression (Fig 8A and 8E) ($P < 0.001$), resulting in a strong reduction of the Bax/Bcl2 ratio compared to irradiated rats ($P < 0.001$) (Fig 8D and 8E).

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Figure 8: Apoptotic pathway.

WB analysis and densitometric quantification of p53 (A), Bax (B), and Bcl2 (C), along with the Bax/Bcl2 ratio (D) in small intestine from non-irradiated rats (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT); n = 6 per group. Data are expressed as mean ± s.e.m. ***$P <$ 0.001 vs. control and ###$P < 0.001$ vs. IR.

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Melatonin Levels in Small Intestine Epithelia

To investigate whether melatonin reached the small intestine to protect it against radiotherapy-induced gut toxicity *in situ*, we assessed the indoleamine content of the rats' small intestines (Fig 9). Compared to rats that were irradiated and not treated with melatonin, the rats treated with melatonin gel showed significantly increased melatonin content in their small intestines ($P < 0.001$), suggesting that melatonin reaches the small intestine in an adequate quantity to protect them.

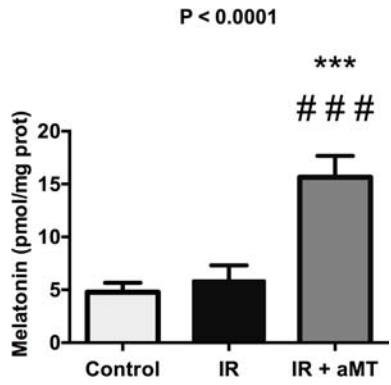


Figure 9: Melatonin levels.

HPLC analysis of the melatonin content of the small intestine from non-irradiated rats (control) and irradiated rats treated with vehicle (IR) or melatonin gel (IR + aMT); n = 6 per group. Data are expressed as mean ± s.e.m. ***$P < 0.001$ vs. control and ###$P < 0.001$ vs. IR.

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DISCUSSION

Mucositis is an important side effect associated with radiotherapy alone or in combination with systemic therapies, and there is presently no definitive prophylaxis or treatment available [1]. Severe mucositis commonly necessitates deescalation of chemotherapy dosing or breaks in radiation treatment, both of which negatively impact a patient's cancer treatment outcome [1].

Although it is well known that irradiation induces intestinal mucositis [37], the present report is the first to demonstrate intestinal damage induced by tongue irradiation. In our study, the small intestine was located outside of the irradiation field, but still exhibited radiation damage, including macroscopic signs of intestinal mucositis and morphological changes associated with substantial changes in intestinal architecture. Rats subjected to tongue irradiation showed signs of mucosal damage, including a significant decrease in villus height. Interestingly, the results were similar to those observed when radiation is applied directly to the intestine. Radiation affects the integrity of the clonogenic compartment, resulting in villi shortening and a loss of epithelial cell lining, severe crypt damage, cell necrosis, increased collagen deposition in the submucosa, edema of

the muscle layer, and incipient serosal fibrosis [8]. We also observed that radiation applied to the tongue led to a reduction of the proliferative marker Ki-67 in the small intestine. Loss of proliferative function affects epithelium development and renders the intestine permeable to luminal bacteria and antigens, potentially exacerbating mucosal inflammation and dysfunction or leading to bacteremia [37]. We also demonstrated that irradiation to the tongue led to reduced ZO-1 in the small intestine. Such damage permits bacterial translocation, potentially contributing to enhancement of the mucositis [38].

On the other hand, severe injury to the mouth mucosa and/or salivary glands after irradiation causes an overall weakening of the animals, which could substantially contribute to the general pathophysiologic manifestations of the body including intestinal toxicity. Such weaken effects might induce small intestine inflammation, an increase of ROS and, consequently, cell death and accumulation of toxic substances within the lumen. Thus, we cannot discard that the gut damage reported in our results might arise secondarily after oral irradiation, promoting epithelial cell breakdown. Other authors have already demonstrated that external radiation of other different organs from the gut, such as the liver, also induces damage in different segments of the rat intestine [7,39]. Therefore, the prevention of severe radiation enteropathy requires protection of the small intestine. Our present results demonstrated significant reductions of these processes in the presence of melatonin. Oral treatment with melatonin gel was associated with improved preservation of small intestinal mucosal architecture. Compare to treatment with vehicle, rats treated with melatonin gel showed significantly greater villus height following tongue irradiation, suggesting that melatonin gel reduced intestinal damage. We cannot exclude an indirect effect of melatonin on this response, by reducing radiation injury in the mouth. However, the gel was topically applied to the rat's mouth, and the rats swallowed the gel, coating all gastrointestinal mucosa as well as the mouth. Moreover, melatonin is depleted in the mouth and in the intestine after irradiation, and treatment with the gel recovered its physiological levels in both tissues. These findings suggest that the melatonin gel is directly protecting all gastrointestinal mucosa from mucositis.

While the severity of intestinal radiation toxicity is typically determined based on the extent of radiation-induced intestinal crypt cell death, radiation also induces changes in cellular function and activation of the innate immune system, which lead to mucosal breakdown [40]. Our immunohistological studies revealed that the rats treated with melatonin gel also showed increased

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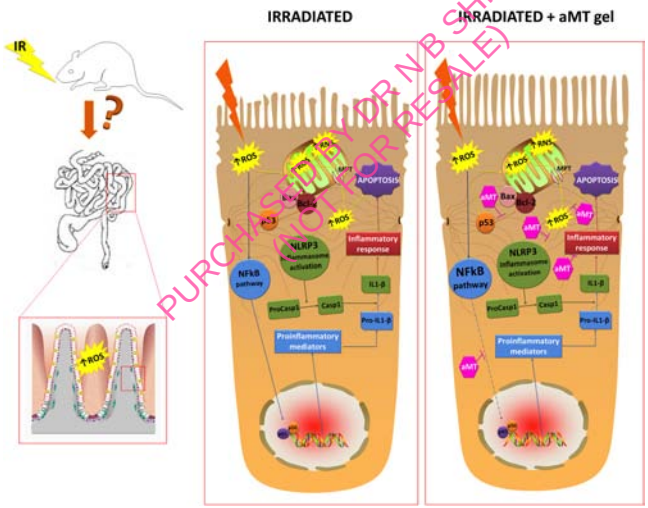
intestinal Ki-67 and ZO-1 levels, potentially indicating the recovery of intestinal damage as an out-of-field effect of radiation. Moreover, IR in the tongue was associated with oxidative stress in the small intestine, as shown by increased LPO and NO levels, which was counteracted by melatonin gel treatment. Levels of ROS typically increase following chemotherapy or radiation, along with a series of events leading to tissue destruction [41]. Our present results suggest that small intestine injury and apoptosis are also impacted by radiation-induced inhibition of mitochondrial respiratory chain complexes and the ensuing bioenergetics failure induced by mitochondrial dysfunction [42]. IR was associated with decreases of mitochondrial antioxidant enzymes, including GPx, GRd, and SOD, and an increase of GSSG/GSH. Treatment with melatonin gel decreased the mitochondrial oxidative stress, restoring normal levels of mitochondrial complexes.

ROS may promote inflammation by activating multiprotein cytoplasmic complexes inflammasomes, such as NLRP3. Assembly of the NLRP3 inflammasome leads to caspase-1 activation and subsequent cleavage of pro-IL-1β cytokines into their mature forms. Importantly, IL-1β is one of the inflammatory mediators that is reportedly released during mucositis. We recently defined the crucial role of ASC/caspase-1 activation and consequent IL-1β production in the establishment of inflammatory responses in irradiation-induced oral mucositis in rats [24]. This innate immune system pathway activates proinflammatory cytokines produced by NF-κB pathway activation [43]. Our present work also demonstrated the importance of proinflammatory cytokines processed by caspase-1 for the development of irradiation-induced mucositis. We observed a significant increase in the tissue levels of IL-1β, TNF-a, and COX-2. These findings support the hypothesis that NF-κB and NLRP3 work together to activate inflammatory pathways of the innate immune response, resulting in its overstimulation [24]. Since melatonin has antagonist effects on both NF-κB and NLRP3 signaling, it could be a more efficient anti-inflammatory molecule than other drugs. Here we evaluated whether mitochondrial dysfunction participated in NLRP3 inflammasome activation in the small intestine following tongue irradiation. Interestingly, the protective effect of melatonin in mitochondria was associated with inhibition of the NLRP3 inflammasome and decreased mucositis signals.

Studies of apoptotic signaling cascades demonstrate that mitochondria play a major role in regulating the apoptotic process by directly influencing energy production, regulating caspase activation, participating in intracellular calcium ion homeostasis, and releasing ROS [44]. Moreover,

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exposure to radiation leads to a reduced activity of mitochondrial complexes, accompanied by decreased concentrations of cytochrome c and Bcl-2. Lower levels of the anti-apoptotic Bcl-2 protein facilitate the release of apoptogenic proteins, such as cytochrome c, from the mitochondria into the cytosol, thereby initiating the apoptotic cascade [24]. Correspondingly, here we showed that the radiation-induced inhibition of mitochondrial respiratory chain complexes, and corresponding mitochondrial dysfunction, was accompanied by an increased Bax/Bcl2 ratio. The antiapoptotic benefit of melatonin gel may be attributed to its ability to protect mitochondria in epithelial cells, as well as stem cells, from radiation. The protective effect of melatonin gel in the small intestine was associated with mitochondrial protection and, consequently, with a reduced inflammatory response, decreased intestinal apoptosis, relief of mucosal dysfunction, and facilitated intestinal mucosa recovery (Fig 10).



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Figure 10: The molecular pathway involved in small intestine damage induced by external radiation, which is inhibited by melatonin gel.

Radiation administered to the tongue induced small intestine damage, activating several molecular pathways. Melatonin gel protected mitochondria and inhibited the NF-κB and NLRP3 inflammasome pathways, reducing inflammation and apoptosis, relieving mucosal dysfunction, and facilitating intestinal mucosa recovery.

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A clinical trial is currently underway to test melatonin gel for the prevention of oral mucositis in head and neck cancer patients. Interestingly, local application of melatonin gel in the mouth leads to the accumulation of effective concentrations of melatonin in the small intestine to prevent radiotherapy-induced gut toxicity. Additionally, numerous studies show that melatonin has remarkable oncostatic properties, and can reduce tumor promotion and/or progression [45–50]. Thus, the application of melatonin represents an innovative adjuvant strategy in cancer treatment, combining oncostatic and cytoprotective traits. Our present results suggested that treatment with melatonin gel both reduced the toxicity of irradiation and prevented treatment side effects.

CONCLUSIONS

Irradiation of the tongue results in damage to the small intestine, which is outside of the irradiation field. This small intestine damage is likely caused by indirect effects, such as the accumulation of toxic substances within the lumen, promoting epithelial cell breakdown and innate immune system activation [40]. Our present study is unique in that the results indicate that melatonin is a candidate drug that may protect small intestinal cells from the side effects of radiation therapy applied in the oral mucosa.

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SECTION V:
VETERINARY TOXICITY METALS

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Chapter 9

Urinary Metals in a Spontaneous Canine Model of Calcium Oxalate Urolithiasis

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ABSTRACT

Calcium oxalate urolithiasis is a common and painful condition in people. The pathogenesis of this disease is complex and poorly understood. Laboratory animal and *in vitro* studies have demonstrated an effect of multiple trace metals in the crystallization process, and studies in humans have reported relationships between urinary metal concentrations and stone risk. Dogs are a spontaneous model of calcium oxalate urolithiasis, and the metal

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content of canine calcium oxalate stones mirrors that of human stones. The aim of this study was to test for a relationship between urinary metals and calcium oxalate urolithiasis in dogs. We hypothesized that urinary metals would differ between dogs with and without calcium oxalate urolithiasis. Urine from 122 dogs (71 cases and 51 stone-free controls) was analyzed for calcium and 12 other metals. The cases had higher urinary calcium, copper, iron, and vanadium and lower urinary cobalt. Higher urinary vanadium in the cases was associated with being fed a therapeutic stone-prevention diet. Urinary calcium had a strong positive correlation with strontium and moderate positive correlations with chromium, nickel, and zinc. The results of this study complement the findings of similar human studies and suggest a potential role of trace metals in calcium oxalate urolithiasis. Further investigation into how trace metals may affect stone formation is warranted.

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INTRODUCTION

Urolithiasis is a significant health problem, with a lifetime incidence of 12% in men and 6% in women [1,2]. Preventative treatments such as increasing water intake and thiazide diuretics can significantly reduce risk [3], but the overall stone recurrence rate is high at 50% by 5 years [4]. Approximately 75% of kidney stones are composed of calcium oxalate (CaOx) [5,6], and idiopathic hypercalciuria (high urinary calcium) is the most common metabolic abnormality in stone patients [7]. The prevalence of CaOx urolithiasis is increasing; however, specific environmental and dietary exposures involved have been difficult to identify [8]. Novel research approaches are needed to uncover etiopathologic factors in stone development.

While most laboratory animals seem relatively resistant to forming CaOx stones, dogs develop them naturally. As with humans, canine CaOx stones are common, recurrent, and associated with hypercalciuria [9,10]. The prevalence of CaOx urolithiasis is also on the rise in dogs, and it is hypothesized that shared environmental risk factors are responsible [11]. Dogs are one of the top sentinel animals for detecting environmental toxins [12] and thus offer a model for studying the role of environmental exposures in stone development.

Several metals have been implicated in lithogenesis in humans and laboratory animals, but the relationships between individual metals and stone risk are not well understood [13]. Zinc, strontium, and iron are the

most abundant metals in kidney stones, totaling close to 1% of the stone composition, and these metals are detected at greater quantities in calcium-containing stones than other stone types [14]. A recent study discovered that the metal content of canine CaOx stones closely mirrors that of human CaOx stones with relatively high zinc, strontium, and iron [15].

Additionally, metals that are not commonly detected within uroliths can still play an important role in the disease. Cadmium is a classic example. It is an environmental toxin that causes renal damage and alters calcium homeostasis. Low level Cd exposure in people is associated with risk for osteoporosis, hypercalciuria and nephrolithiasis, without other overt signs of toxicity [16, 17]. Cadmium concentrations in canine blood can detect environmental cadmium contamination [18].

The objective of this study was to evaluate the relationship between urinary metals, urinary calcium, and CaOx urolithiasis in a large population of dogs from diverse breed groups. We hypothesized that urinary metal concentrations would differ between dogs with and without CaOx urolithiasis. The findings of this study parallel data from human stone formers and stress the need for further investigation into the role of metals in lithogenesis in both people and companion animals.

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MATERIALS AND METHODS

Ethics Statement

Written informed consent was obtained from owners of study participants, and the study protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee (Protocol #1509-33019A).

Animals

The study took place at the Veterinary Medical Center, University of Minnesota (VMC UMN). Dogs were recruited from February 2011 to March 2014. This was a cross-sectional study, and data was collected at a single time point for each dog. Cases were dogs with a history of uroliths composed of CaOx (≥70% of the central core), as determined by standard stone analysis at the Minnesota Urolith Center (polarizing light microscopy and infrared spectroscopy). The majority of the cases were recruited when presenting for stone removal procedures (active stone disease), but a subset of cases was recruited at variable time points after historical stone removal.

Controls were ≥ 8 yo and had no history of CaOx uroliths, no evidence of current radiopaque uroliths on screening abdominal radiographs, and no CaOx crystalluria on urinalysis. Roughly two thirds of the study population comprised a breed- and sex-matched case-control cohort that was also enrolled in another study on urinary calcium in three breeds with the greatest risk for CaOx urolithiasis (Bichons Frise, Miniature Schnauzers, and Shih Tzus) [10]. No breed restrictions were placed on the other dogs recruited.

Dogs were excluded from the study if they had received glucocorticoids within the past week or another drug with known effects on urinary calcium excretion (i.e. furosemide, thiazide diuretics, levothyroxine, theophylline, potassium citrate) within the past 24 hours. Dogs were also excluded if they had a clinical diagnosis of a disease that alters urinary calcium excretion (i.e. hyperparathyroidism, hypercalcemia of malignancy, hyperadrenocorticism, diabetes mellitus, osteolytic disease, granulomatous disease). The current diet was recorded for each dog and classified into one of three groups: the therapeutic stone-prevention diet Hill's Prescription Diet u/d Canine (Hill's Pet Nutrition Inc.), the therapeutic stone-prevention diet Royal Canin Veterinary Diet canine urinary SO (Waltham Centre for Pet Nutrition), or other (diets not formulated for the prevention of stone disease; this group comprised a large variety of diets).

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Urine Measurements

Owners were instructed to withhold food, but not water, for 12–18 hours prior to sample collection. The dogs urinated at least once prior to sample collection such that the research urine sample was never the first micturition of the day. Urine was collected by free catch, cystocentesis, or catheterization. Unfiltered 1–3 ml urine aliquots were immediately analyzed for calcium (spectroscopy and the calcium sensitive dye Arsenazo II) and creatinine (modified Jaffe procedure) [19,20]. A 4 ml urine aliquot was filtered through a 0.22 μm polyethersulfone membrane (MJ Research, Inc.), stored at -80°C, and analyzed for metals within 18 months. Urinary metal analysis was performed using inductively coupled plasma mass spectrometry [21]. Twelve metals were included in the analysis: barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), rubidium (Rb), strontium (Sr), vanadium (V), and zinc (Zn). Spot urinary element-to-creatinine ratios (e.g. Ca/Cre, Ba/Cre, Cd/Cre) were calculated for each dog and are reported in mg/g for urinary Ca/Cre and μg/g for the other metals.

Statistical Analysis

Ages are reported as mean ± standard deviation and were compared between cases and controls with a Student's t-test. Weight did not follow a normal distribution, is reported as median (range), and was compared between cases and controls with a Wilcoxon rank sum test. Sex proportions (males versus females) between cases and controls were compared with a Pearson's chi-squared test. The data distribution for the urinary element-to-creatinine ratios were visually inspected with Q-Q plots. The majority of the measurements did not follow a normal distribution, thus non-parametric tests and transformations were used in the statistical analysis. Wilcoxon rank sum tests were used to compare measurements between case and control groups and for cases with and without uroliths present at the time of urine sampling. The Benjamini-Hochberg procedure was used to determine significance with a false discovery rate of 0.1; those variables that met this level of significance were analyzed further with multiple regression. For regression analyses, data was log-transformed to create a more normal distribution. Zero values were present for some of the urinary metal measurements. To manage this, the minimum non-zero value was added to each dog's measurement for that metal such that the entire group was shifted up by the minimum value prior to log-transformation, and the data set was effectively censured at the minimum value. Regression analyses were performed for the log-transformed urinary element-to-creatinine ratios with stone status (CaOx case versus control), breed (Miniature Schnauzer, Bichon Frise, Shih Tzu, or other [breeds with $n < 10$ were combined]), log-transformed weight (in kg), age at the time of urine sampling (in years), and sex (male versus female) as predictors. Reduced models were generated with stepwise selection of predictors in both forward and backward directions. Akaike Information Criterion (AIC) scores were calculated as a measure of model fit, and the model with the lowest AIC was selected for the reduced model. The significance of each model predictor was assessed using Type II tests (ANCOVA). For each urinary metal found to have a relationship with stone status in the reduced model, multivariate regression were performed with the urinary element-to-creatinine ratios as the outcome and diet (Hill's u/d Canine, Royal Canin SO, and other) and active stone disease (versus historical) as predictors. Correlation coefficients between element-to-creatinine ratios were calculated for all possible pairs. All analyses were performed using the R software for statistical computing (http://www.R-project.org/), and a p-value <0.05 was considered significant.

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RESULTS

Study Participant Characteristics

One hundred and twenty-two dogs (71 cases and 51 controls) were enrolled in the study. The most common breeds were the Miniature Schnauzer (50), Bichon Frise (22), and Shih Tzu (13). See Table 1 for additional breeds, clinical characteristics, and diets of the study participants. The control group was significantly older than the case group ($p = 0.0056$). Weight did not differ significantly between cases and controls ($p = 0.18$). There were more males in the case group (80% of cases versus 53% of controls, $p = 0.0025$), and all except for three dogs (1 male case, 1 female case, and 1 male control) were neutered. Fifty-seven cases (80%) had CaOx cystoliths at the time of sample collection (active stone disease) and had their stones removed later that same day. The remaining 14 cases had a history of prior CaOx urolithiasis. Forty-six of the cases (65%) had suffered from recurrent CaOx uroliths (median 2 stone episodes, range 1–5). Twenty-seven of the cases (38%) were on therapeutic stone-prevention diets; in all cases, these diets were prescribed after one or more episodes of CaOx urolithiasis. A single control was on a therapeutic stone-prevention diet because a household dog had a history of CaOx urolithiasis, and the owner elected to feed both dogs the same diet.

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Table 1: Characteristics of the 122 study dogs

Cases are dogs with confirmed CaOx urolithiasis, and controls are stone-free as determined by abdominal radiography. Age at the time of urine sampling is reported as mean ± standard deviation, and weight is reported as median (range).

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Stone Status	Breed	Weight	Sex	Age	Diet
Cases (71 dogs)	22 Miniature Schnauzers 11 Mixed breed dogs 10 Bichons Frise 7 Shih Tzus 5 Lhasa Apsos 3 Yorkshire Terriers 2 Rat Terriers 1 each: Chihuahua, Cocker Spaniel, Fox Terrier, Havanese, Jack Russell Terrier, Kerry Blue Terrier, Maltese, Pomeranian, Pug, Shetland Sheepdog, Welsh Terrier	8.8 (2.9–23.0) kg	57 M, 14 F	9.0 ± 2.8 yo	17 Hill's u/d 11 Royal Canin SO 43 Other
Controls (51 dogs)	28 Miniature Schnauzers 12 Bichons Frise 6 Shih Tzus 3 Standard Schnauzer 1 each: Mixed breed, Miniature Poodle	8.2 (3.0–18.3) kg	27 M, 24 F	10.2 ± 1.9 yo	1 Royal Canin SO 40 Other

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Urinary Metals and CaOx Stone Status

The median element-to-creatinine ratios for the case and control groups and p-values for the Wilcoxon rank sum test are reported in Table 2. Five urinary variables met the significance criteria and were selected for multivariable regression analysis: Ca/Cre, Co/Cre, Cu/Cre, Fe/Cre, and V/Cre. The results for the reduced models for each of these five urinary outcomes are reported in Table 3; full models are provided in S1 Table. For Ca/Cre, a CaOx case stone status was a strong positive predictor, and male sex was a negative predictor. For Co/Cre, stone status was the sole predictor in the reduced model with CaOx case status having a negative effect.

Table 2: Urinary element-to-creatinine ratios in dogs with CaOx urolithiasis (cases) and stone-free controls

Values are reported in mg/g for urinary Ca/Cre and µg/g for the other urinary element-to-creatinine ratios.

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Variable	Controls, Median (IQR)	Cases, Median (IQR)	Raw p-value
Ca/Cre	**33 (21–32)**	**67 (47–123)**	**1.3E-06***
Cu/Cre	**113 (66–200)**	**155 (107–263)**	**0.011***
Co/Cre	**2.5 (1.5–3.7)**	**1.7 (1.1–2.7)**	**0.013***
V/Cre	**2.9 (1.7–8.2)**	**6.7 (2.8–12.8)**	**0.022***
Fe/Cre	**15 (11–25)**	**22 (13–55)**	**0.030***
Rb/Cre	2035 (1453–2577)	1702 (1199–2378)	0.099
Ni/Cre	9.5 (5.2–13.5)	8.2 (4.3–10.6)	0.13
Sr/Cre	100 (63–134)	113 (74–185)	0.21
Mn/Cre	0.19 (0.09–0.43)	0.31 (0.16–0.52)	0.24
Cd/Cre	0.099 (0.059–0.16)	0.090 (0.042–0.14)	0.36
Cr/Cre	0.75 (0.37–1.00)	0.70 (0.41–1.74)	0.36
Zn/Cre	253 (145–182)	204 (124–332)	0.40
Ba/Cre	8.6 (5.9–12.6)	8.4 (4.5–12.6)	0.43

*Denotes significance using the Benjamini-Hochberg procedure with a 0.1 false discovery rate.
IQR = Interquartile range

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Stone status as a CaOx case, age, and the Miniature Schnauzer breed all had a positive relationship with Cu/Cre, with stone status having the strongest effect. The models for Fe/Cre and V/Cre revealed positive relationships between these metals and being a CaOx case; stone status was the sole significant predictor for both. When the cases were analyzed alone in regressions with diet and active stone disease as predictors, the Hill's u/d diet was found to have a negative association with urinary Fe/Cre (estimate

= -0.90, p = 0.0042) and a positive association with urinary V/Cre (estimate = 0.74, p = 0.032). Diet did not have a significant effect on urinary Ca/Cre, Co/Cre, or Cu/Cre, and active versus historical stone disease did not have a significant relationship with any of the five urinary metals tested. The urinary Ca/Cre, Co/Cre, Cu/Cre, Fe/Cre, and V/Cre measurements in cases and controls are displayed in Fig 1.

Table 3: Reduced multivariable regression models for the effects of CaOx stone status and environmental factors on log-transformed urinary element-to-creatinine ratios (Ca/Cre, Co/Cre, Cu/Cre, Fe/Cre and V/Cre)

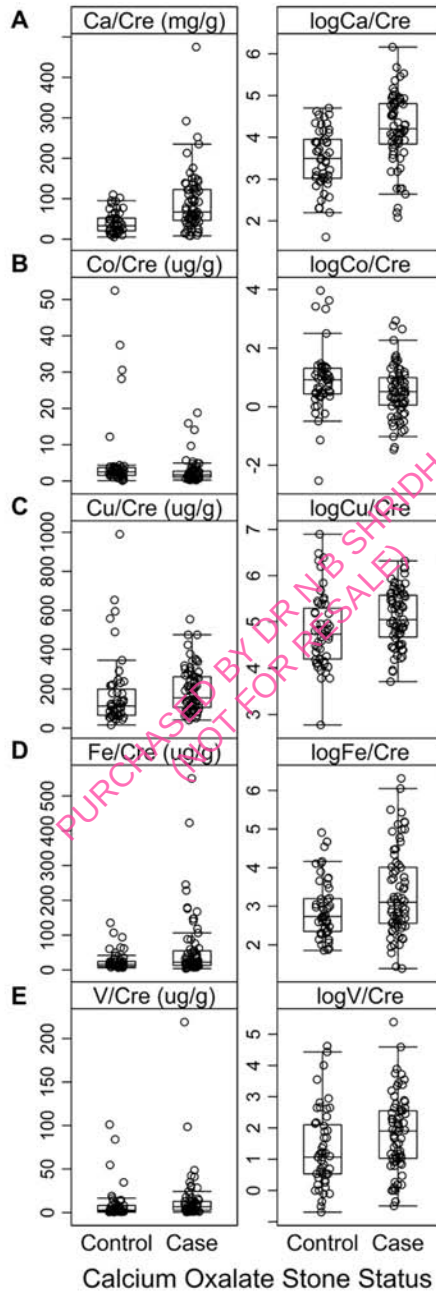
For variables with 1 degree of freedom, the status corresponding to the estimate is in parentheses.

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	Estimate	Standard Error	T value	Degrees of Freedom	P-value
Ca/Cre					
				2	**8.7E-07**
Stone status (case)	0.82	0.15	5.58	1	**1.5E-07**
Sex (male)	-0.33	0.16	-2.12	1	**0.036**
Co/Cre					
Stone status (case)	-0.39	0.18	-2.19	1	**0.030**
Cu/Cre					
				5	**0.0060**
Stone status (case)	0.44	0.13	3.26	1	**0.0015**
Age	0.06	0.03	2.21	1	**0.029**
Breed				3	0.081
Miniature Schnauzer	0.32	0.16	2.09		**0.039**
Bichon Frise	0.18	0.19	0.96		0.34
Shih Tzu	-0.12	0.22	-0.54		0.59
Fe/Cre					
Stone status (case)	0.44	0.18	2.4	1	**0.016**
V/Cre					
				2	**0.038**
Stone status (case)	0.50	0.22	2.23	1	**0.027**
Log(weight)	-0.44	0.31	-1.42	1	**0.16**

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Figure 1: Box and whisker plots of urinary A) calcium-to-creatinine (Ca/Cre, mg/g), B) cobalt-to-creatinine (Co/Cre, **µg**/g), C) copper-to-creatinine (Cu/Cre, **µg**/g), and D) iron-to-creatinine (Fe/Cre, **µg**/g), and E) vanadium-to-creatinine

(V/Cre, **µg**/g) ratios for dogs with a history of CaOx stones (cases) and stone-free dogs (controls).

The boxes represent the interquartile range (25th– 75th percentile), the horizontal line within the boxes represents the median, and the whisker bars extend to 1.5 times the interquartile range. Raw and log-transformed data are shown.

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The medical history for the dogs with the 10 highest values for urinary Ca/Cre, Cu/Cre, Fe/Cre, and V/Cre were examined to determine if there was any overt shared characteristic or exposure history (diet or medication). The 10 highest urinary Ca/Cre values were all from male CaOx cases. Breeds included 5 Miniature Schnauzers, 3 Bichons Frise, 2 Lhasa Apsos, and a mixed breed dog. Three dogs were fed Hill's u/d, two were fed Royal Canin SO, and the others were consuming five diverse diets. Medications included doxycycline (1), tramadol (1), and carprofen (1). The top 10 urinary Cu/Cre values were from 7 male and 3 female dogs with 5 CaOx cases and 5 controls. Breeds included 7 Miniature Schnauzers, 1 Bichon Frise, 1 Yorkshire Terrier, and a mixed breed dog. Two dogs were fed Royal Canin SO, and the others were consuming seven diverse diets. One dog was receiving daily supplements (GNC Pets Ultra Mega Hip & Joint Health for Senior Dogs and Ultra Mega Multivitamin Plus for Adult Dogs). The 10 highest urinary Fe/Cre values came from 9 male and 1 female CaOx cases. Breeds included 4 Miniature Schnauzers, 2 Shih Tzus, 1 Bichon Frise, 1 Fox Terrier, 1 Rat Terrier, and a mixed breed dog. One dog was consuming Royal Canin SO, and the others were consuming nine diverse diets. Medications included doxycycline (1, the dog mentioned above) and amoxicillin/clavulanic acid (1). The 10 highest urinary V/Cre values came from 6 male and 4 female dogs with 6 CaOx cases and 4 controls. Breeds included 5 Miniature Schnauzers, and 1 each of Bichon Frise, Chihuahua, Lhasa Apso, Pomeranian, and Standard Schnauzer. Two dogs were consuming Hill's u/d, one was consuming Royal Canin SO, and the others were consuming seven diverse diets. One dog was being administered cyclosporine ophthalmic drops.

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Correlations between Urinary Metals

Correlation coefficients were calculated for the urinary metals and are presented in Table 4. Urinary Ca/Cre has a strong positive correlation (r = 0.7) with Sr/Cre, moderate positive correlations (r = 0.3) with urinary Cr/

Cre, Ni/Cre, and Zn/Cre, and weak positive correlations with other metals.

Table 4: Heat map of correlation coefficients between urinary metals in dogs with and without CaOx urolithiasis.

Correlation coefficients in bold font had p-values < 0.05

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	Ca/Cre	Sr/Cre	Cr/Cre	Ni/Cre	Zn/Cre	Ba/Cre	Cu/Cre	Mn/Cre	Rb/Cre	Cd/Cre	V/Cre	Fe/Cre	Co/Cre
Ca/Cre													
Sr/Cre	**0.7**												
Cr/Cre	**0.3**	**0.3**											
Ni/Cre	**0.3**	**0.3**	**0.4**										
Zn/Cre	**0.3**	**0.3**	**0.4**	**0.6**									
Ba/Cre	**0.2**	**0.5**	**0.2**	**0.2**	**0.4**								
Cu/Cre	**0.2**	0.1	**0.2**	0.1	**0.3**	0.2							
Mn/Cre	**0.2**	**0.2**	**0.3**	0.2	**0.5**	**0.5**	**0.3**						
Rb/Cre	**0.2**	**0.2**	0.2	**0.3**	**0.3**	0.2	**0.4**	0.2					
Cd/Cre	0.1	**0.2**	**0.5**	**0.4**	**0.5**	**0.3**	**0.2**	**0.4**	**0.3**				
V/Cre	0.1	0	**0.4**	**0.2**	**0.2**	0	0.1	0.1	0	0.1			
Fe/Cre	0.1	0	**0.3**	0.1	**0.4**	0.1	**0.3**	**0.4**	**0.2**	**0.2**	0		
Co/Cre	0.1	**0.3**	**0.2**	**0.4**	**0.2**	0.2	**0.2**	0.1	**0.2**	**0.3**	0	0.1	

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DISCUSSION

The present study describes relationships between urinary metal concentrations and CaOx urolithiasis in a spontaneous canine model. While the design of this study does not permit causality to be evaluated, it provides data to stimulate further research into the role of metals in stone disease. Many of the relationships identified in this study parallel findings in human CaOx stone formers and thus highlight the suitability of the canine model.

Hypercalciuria is the most common metabolic abnormality underlying urolithiasis in people [7] and occurs in canine CaOx stone formers as well [10, 22, 23]. Approximately two thirds of the study population was comprised of three breeds, the Bichon Frise, Miniature Schnauzer, and Shih Tzu, included in a previous study demonstrating idiopathic hypercalciuria in each of these breed [10]. In line with this, urinary Ca/Cre was significantly higher in CaOx cases in this study. Unexpectedly, male sex had a negative association with Ca/Cre in a multivariable regression analysis. Men have higher daily urinary calcium excretion than women, but this difference has been attributed to body weight [24]; weight was included in the multivariate regression analysis for urinary calcium in the present study and did not have a significant effect. The previous canine study that included the

aforementioned breed-matched subset of the current study population found no relationship between sex and urinary Ca/Cre [10]. The finding of lower urinary Ca/Cre in male dogs in this study warrants further investigation.

In this study, urinary Fe/Cre was significantly higher in dogs with CaOx urolithiasis. This finding was not explained by age, sex, or the diet categories evaluated. Hematuria can occur with urolithiasis and would presumably increase urinary iron. Urine sediment was not examined in this study, but median urinary Fe/Cre did not differ between dogs that had stones at the time of urine collection compared to those with historic urolithiasis. Iron is the third most abundant metal in human kidney stones, after zinc and strontium, and is more abundant in calcium-containing stones than other stone types [14]. Iron has also been detected in canine CaOx stones [15]. Iron overload is linked to hypercalciuria in animal models and people and is believed to be due to increased bone turnover [25, 26]. However, urinary Fe/Cre was not correlated with urinary Ca/Cre in this study. An alternative explanation is that Fe has an effect on CaOx urolithiasis via other mechanisms. A study of human idiopathic CaOx kidney stone formers found that the mean urinary Fe was more than 30 times greater in these patients compared with controls, despite lower serum iron concentrations [27]. Ferric ions and citrate are inhibitors of CaOx crystal development *in vitro*, but when present together, their inhibitory effects diminish due to formation of iron-citrate complexes that lack inhibitory activity [28]. Thus, high urinary iron could theoretically increase risk for stone formation via limiting the free citrate available to bind calcium and prevent crystallization.

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It is noteworthy that one of the common therapeutic stone-prevention diets (Hill's u/d) had a negative correlation with urinary Fe/Cre. This diet was only prescribed after stone diagnosis, and the cases on the diet may have previously had a higher urinary Fe/Cre. Thus, the association between urinary iron and stone risk could be greater than estimated by this data. A more in-depth discussion of this limitation is included later.

The CaOx cases also had higher urinary Cu/Cre. Trace concentrations of copper have been reported in human and canine CaOx stones [14, 15, 29], but the relationship between urinary copper and CaOx urolithiasis is unclear with conflicting results across human studies [27, 30, 31]. In this study, though the median Cu/Cre was higher in CaOx cases, the 10 highest measurements were evenly distributed among cases and controls. Urinary Cu/Cre had a weak correlation with urinary Ca/Cre ($r = 0.2$). Copper may inhibit calcium phosphate crystallization but is not believed to have a direct effect on CaOx

crystallization [13]. Urinary V/Cre was higher in CaOx cases compared to controls; however, further analysis revealed a strong positive relationship with the therapeutic stone-prevention diet Hill's u/d and a trend with Royal Canin SO. Neither of these diets report vanadium content, but brewers rice is the top ingredient in both. Rice has a moderate to high vanadium content [32, 33] and may be the explanation for the association of higher urinary V/Cre in dogs eating these diets.

In contrast to the other metals that differed between cases and controls, urinary Co/Cre was lower in CaOx cases than controls. Cobalt has been detected in human CaOx stones, but it is also present in other stone types and had no correlation with stone composition [34]. Cobalt may inhibit CaOx crystallization *in vitro* [13], but limited data is available on the role of this metal in lithogenesis. Urinary Sr/Cre was found to have a strong correlation with Ca/Cre (r = 0.7), though it was significantly different between CaOx cases and controls. This finding matches data from previous literature. Strontium is the second most abundant metal detected in human kidney stones and Randall's plaques [14, 35, 36]. Strontium has a similar size and charge to calcium, and its absorption and excretion follow that of calcium [37]. In fact, oral strontium loading is used to study calcium metabolism in patients with idiopathic hypercalciuria [38, 39]. To the authors' knowledge, a direct role of strontium in lithogenesis has not been reported.

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Unlike the other metals analyzed, cadmium is an environmental toxin with a well-documented effect on urolithiasis risk. It accumulates in renal tubular cells and causes hypercalciuria through inhibition of calcium resorption, effects on calcium uptake by bone, and alterations in vitamin D metabolism [16, 40]. Low level exposure is associated with increased stone risk without other classic signs of toxicity [17]. Renal tissue accumulates cadmium, and it is very slowly eliminated through the urine. Urinary cadmium is therefore considered a biomarker of lifetime cadmium exposure [41]. Toxicokinetic studies of chronic oral cadmium dosing in dogs have estimated the biological half-life at 1–2 years for a two compartment model, and human data suggests that it may take 10–30 years for elimination from the renal cortex and other tissues [42, 43]. Evidence of cadmium toxicity was not present in the urine of the dogs in this study. Future studies involving greater numbers of dogs from diverse regions of the country are needed to determine whether cadmium is a substantial environmental risk factor for canine CaOx urolithiasis. As stated above, one important limitation of this study is that the cross-sectional design does not permit determination of the temporal relationship between metals and CaOx urolithiasis or whether they

are direct relationships or the result of a mediator variable. Urine samples were obtained from the majority of the cases at the time that a stone was present, but some dogs had been diagnosed with stones more than a year prior to study enrollment. The time gap between stone formation and the urine sample complicates the ability to determine the source of the metal exposure and when the exposure occurred relative to stone formation. A metal exposure may have been missed if the metal was eliminated from the body before the urine sample was obtained. This is most concerning for elements that are tightly regulated by the body and have short elimination half-lives, such as calcium, zinc, and iron. Over a third of the cases were on therapeutic stone-prevention diets formulated to reduce the urinary CaOx relative supersaturation that were not prescribed until after one or more stone episodes. As such, diet is an example of an exposure that changed for some dogs between stone formation and study enrollment and could confound the analyses. Diet was included in the regression models for the cases and, as described above, one of the therapeutic stone-prevention diets was associated with urinary iron and vanadium. Detailed diet histories and analyses were not performed beyond the classification described, and we may have missed other relationships between nutrients and urinary metals.

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Other limitations pertain to the population of dogs studied. Most of the dogs in this study were of three breeds. While breed was considered as a covariate in the regression analyses, it was classified into only four categories, the three top breeds and "other." The "other" category comprised 25 dogs of 16 breeds plus 12 mixed breed dogs. There could be breed differences in the pathophysiology of stone formation, and the study was not designed to enable detection of breed-specific associations between urinary metals and CaOx stone disease outside of the three top breeds included. The cases and controls were also not sex- and age-matched, but these variables were included as predictors in the regression models. The study took place at the University of Minnesota, and all dogs were local to Minnesota and its surrounding states. There may be geographical differences in risk factors for CaOx stone disease, and the findings of this study may not be applicable to the general canine population.

There are also some limitations to the methodology. Twenty-four hour urine collection is a considered superior method of screening for hypercalciuria than a fasting spot UCa/Cr in people [44–46], and 24-hour samples are commonly used to quantify other urinary metals as well [27, 30–31, 34]. The authors are not aware of any direct comparisons of spot versus 24-hour urinary element measurements in dogs, and both methods have

been used to demonstrate the presence of hypercalciuria in dogs with CaOx urolithiasis [10, 47–49]. Spot samples were used in this study due to the convenience, technical ease, and lower expense compared with complete urine collection in dogs. Finally, the study used a preset panel for metal analysis. Other metals not included on the panel could have an important relationship to CaOx stones.

CONCLUSIONS

This study used a spontaneous canine model to identify associations between urinary metals and CaOx urolithiasis. The CaOx cases had hypercalciuria, the most common metabolic abnormality in people with CaOx stones. The cases also had higher urinary iron, which again parallels human data. *In vitro* studies suggest that iron can bind citrate and create complexes that diminish the inhibitory activity of citrate on crystallization. However, a direct role of iron in lithogenesis has not been established. Higher copper and vanadium and lower urinary cobalt were also present in the CaOx cases. The higher urinary vanadium was linked to a therapeutic stone-prevention diet. The significance of the other metals in relation to stone formation is unknown. Further investigation into the role of metals in stone formation could shed light on the pathogenesis of CaOx urolithiasis and have implications for nutritional recommendations.

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Chapter 10

Endothelial Function and Serum Concentration of Toxic Metals in Frequent Consumers of Fish

PURCHASED BY BRYNB SHRIDHAR (NOT FOR RESALE)

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ABSTRACT

Background

Endothelial dysfunction is involved in the pathogenesis of atherosclerosis. Consumption of fish is associated with reduced cardiovascular risk, but there is paucity of data concerning its effect on endothelial function. Furthermore, investigation of the effects of fish consumption on health must take into account the ingestion of contaminants, including transition metals and some metalloids, which may have unfavorable effects on health, including those on the cardiovascular system. We investigated the association between fish consumption, endothelial function (flow mediated dilation of the brachial artery), and serum concentration of some toxic metals in apparently healthy people.

Methods

Twenty-nine high fish consumers (at least 3 portions a week) were compared with 25 low fish consumers (less than 1 portion a week). All participants were free of diabetes, cardiovascular or other systemic diseases. Serum metal (antimonium, arsenic, mercury, lead, cobalt, copper, zinc, selenium, strontium) concentrations were measured in subgroups of 24 high fish consumers and 19 low fish consumers.

Results

Both groups exhibited similar habitual dietary patterns, age and anthropometric characteristics. The high fish consumers had higher flow mediated dilation (9.7±1.8 vs. 7.3±1.9%; P<0.001), but also higher serum concentrations of mercury (5.87±2.69 vs. 1.65±1.10 mcg/L; P<0.001) and arsenic (6.04±3.25 vs. 2.30±1.58 mcg/L; P<0.001). The fasting plasma glucose concentrations were significantly correlated with both mercury (r=0.39; P=0.01) and arsenic concentrations (r=0.55; P<0.001).

Conclusions

Habitual consumption of high amounts of fish is associated with better endothelial function despite higher serum concentrations of mercury and arsenic.

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INTRODUCTION

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in the Western world [1]. Both inflammation and endothelial function play a key role in the activation and progression of atherosclerosis [2]. In particular, increased production of reactive oxygen species (ROS), as well as oxidative and inflammatory stresses, are associated with impaired endothelial function. A reduced bioavailability of nitric oxide, a potent vasodilator and inhibitor of platelet adhesion and aggregation, with anti-inflammatory and anti-proliferative properties [3], [4], is the hallmark of endothelial dysfunction. Endothelial function can be measured in vivo by flow mediated dilation (FMD) in the brachial artery, which has proven to be a strong predictor of cardiovascular events [5]–[9]. Endothelial function is influenced by many factors, including insulin resistance, diabetes, dyslipidemia, drugs, diet and some foods [10]–[15]. Data have shown that fish consumption should be part of a healthy diet, and helps reduce the risk of cardiovascular diseases [16]–[18]. We recently found that consuming fish at least 2 times a week is associated with less carotid atherosclerosis in apparently healthy people [19]. Fish consumption influences the pathways leading to atherosclerosis in several ways. n-3 polyunsaturated fatty acids (n-3 PUFAs) have been indicated as the most active component of fish in mediating cardiovascular protection. Fish consumption is associated with decreased circulating biomarkers of endothelial dysfunction and inflammation [20], and FMD values seem to improve after n-3 PUFA intake [21]. However, there is paucity of data concerning the association between levels of habitual fish consumption and FMD, and it is unknown what the level of habitual fish intake that influences the FMD is. Furthermore, the relationship between fish intake and health is even more complex considering that fish contaminants, such as metals and other toxic substances, have been recently associated with adverse effects on the cardiovascular system [22]–[26], diabetes [27]–[29] and tumors [30].

In this study we investigated whether regular fish consumption is associated with improved endothelial function, measured by FMD. We also determined blood concentrations of metal toxic contaminants, such as mercury and arsenic, in relation to the level of fish consumption.

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METHODS

Participants

The participants in this study were recruited among those who took part in a previous study [31], which investigated the association between dietary patterns, carotid atherosclerosis, and insulin resistance in 1,231 participants (465 males and 766 females). In that study, semi-quantitative habitual intakes of different foods during the preceding 12 months were assessed with the Food Frequency Questionnaire (FFQ) [32]. A retrospective, cluster analysis of the same cohort was conducted to identify dietary patterns, a procedure that is based on the intercorrelation among food groups or nutrients. A diet that could be defined as Unhealthy was identified: this was characterized by high consumption of soft drinks, fried foods, seed oils, cured meats, butter, red meat and sweets; a dietary pattern that resembled the Mediterranean diet, defined as healthy, was characterized by high intakes of fruit, milk and cheese, olive oil, vegetables, pasta and bread; a third pattern of dietary habits was defined as Intermediate, and had characteristics that lay between the two other diets. In our study, we examined low fish consumers (LFC), defined as those individuals who typically consumed less than 1 portion of fish per week, and high fish consumers (HFC) who consumed at least 3 portions of fish per week. People with any form of diabetes, cardiovascular or systemic disease, and women who were pregnant or lactating in the past 6 months, were excluded from this analysis. Exclusion criteria were also the use of acetyl-salicylic acid or other antiplatelet drugs, statins or fibrates, oral hypoglycemic drugs, nitrates, non-steroidal anti-inflammatory drugs, corticosteroids, drugs interfering with coagulation, supplementation with vitamins and/or anti-oxidants, and regular sports activity. There was no incentive provided to the participants. Our hospital's Ethics Committee approved the study protocol, and each participant signed an approved informed consent form.

All participants were examined in the morning, and in post-absorptive fasting condition. A fasting blood sample was obtained for biochemical measurements and serum samples were frozen at −80°C for subsequent analysis. Height, body weight and circumferences (waist circumference, obtained at the umbilicus level; hip circumference, obtained at the most prominent buttock level); and systolic and diastolic arterial blood pressure (two measurements at 5-minute intervals in seated position; Omron M6; Omron Healthcare Co., Matsusaka, Mie, Japan) were measured according

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to standardized procedures. Electrocardiogram, carotid echo-Doppler and FMD were obtained for all participants.

Measurements

Carotid Intima-media Thickness

Images of the right and left extracranial carotid artery walls were obtained in several projections with a high-resolution ultrasonographic 10-MHz linear array probe (Sonoline G50; Siemens, Germany). End-diastolic intima-media thickness (c-IMT) of the far wall of both common carotid arteries was measured as described elsewhere [33]; the highest value was considered for calculations (c-IMTmax). A single physician was responsible for carrying out the carotid ultrasonographic examinations.

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Assessment of Endothelial Function

Endothelium-dependent reactivity in the macrocirculation, measured by FMD of the brachial artery, was determined using high-resolution vascular ultrasound (Sonoline G50; Siemens, Germany) with a 10 MHz linear array transducer. The transducer was held at the same position throughout the test by a stereotactic clamp with micrometer adjustment (EDI Progetti e Sviluppo; Pisa, Italy) to ensure image consistency. Reactive hyperemia was produced by inflating a sphygmomanometer cuff 2 cm below the antecubital fossa to occlude the artery for 5 minutes at approximately 220–250 mm Hg, then deflating it. A video processing system computed the brachial artery diameter in real-time by analyzing B-mode ultrasound images (FMD Studio; Institute of Physiology CNR; Pisa, Italy); the device captures the analog video signal from the ultrasound equipment. An edge detection algorithm, based on the localization of gray level discontinuities, automatically locates the two walls of the vessel. The diameter is obtained with subpixel precision and temporal resolution of 25 samples/s. The brachial artery diameters were displayed on a graphic interface over a time scale of 9 minutes. The baseline vessel size was considered the mean of the measures obtained during the first minute. The FMD was calculated as the maximum percentage increase of the brachial artery diameter over baseline. These procedures are described in detail elsewhere [34]–[37]. The FMD test was performed by the same operator, who was blinded to the participant's classification. Ultrasound images were video recorded and analyzed by a trained reader, who was blinded to the participant's treatment classification. In our laboratory, the intra-observer coefficient of variation for FMD is 2.9%.

Laboratory Analysis

Basal lipid measurements, glucose, uric acid, and creatinine were ascertained using standard clinical chemistry methods (Glucosio HK UV; Colesterolo tot. Mod P/D; Colesterolo HDL gen3 mod P/917; Trigliceridi; Acido urico MOD P/917; Creatinina enzimatica; Roche diagnostics, Monza, Italy). Basal insulin concentrations (Elecsys insulina; Roche diagnostics; Monza, Italy) and glycated hemoglobin (HbA_1c; HbA_1c gen3; Roche diagnostics; Monza, Italy) were also measured. Low-density lipoprotein (LDL) cholesterol concentration was calculated with Friedewald's formula [38].

The glomerular filtration rate (GFR) was calculated according to the Modification of Diet in Renal Disease study (MDRD) equation [39]. Insulin resistance was measured in terms of HOMA-IR as described by Matthews et al. [40].

The plasma concentrations of the following metals, which include the transition metals and some metalloids, were measured: mercury, cobalt, lead, copper, zinc, arsenic, selenium, strontium and antimonium. Analysis of metal levels in the blood was done with an inductively coupled plasma mass spectroscopy (ICP-MS Thermo Scientific XSeriesII, Thermo Fisher Scientific, Bremen, Germany). Two ml of blood samples collected in Vacuette K3EDTA tubes were digested using an oxidizing mixture of concentrated nitric acid and hydrogen peroxide with a microwave system (Mars 5; CEM Corporation, Matthews, NC, US). The digested samples were then clarified by filtration, and the concentrations of the metals were determined after dilution with ultrapure water by a factor of 10. An HNO_3 blank was run during the analysis to ensure that the memory effect, due to the more refractory elements, was negligible.

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Statistical Analysis

An expected difference in FMD between the two groups was estimated to be at least 3%. The power analysis showed that with an α error of 0.05, a pooled standard deviation of 2.5, and a power of 0.80, 12 participants were needed per group. All data are presented as mean ± standard deviation (SD) or as prevalence (%). Comparisons between groups were tested for statistical significance with the unpaired Student's t-test or Fisher's χ^2, when appropriate. Linear regression analysis assessed the relationships between variables. A two-tailed $P<0.05$ was considered significant. All analyses were performed with Systat (Windows version 11.0; San Jose, CA, USA).

RESULTS

A total of 54 participants were evaluated: 29 HFC and 25 LFC (Figure 1). Serum concentrations of metals and metalloids could be measured in 24 HFC and in 19 LFC participants. The characteristics of the two groups are presented in Table 1. In terms of dietary patterns, the LFC group had a lower prevalence of participants who followed the Mediterranean diet, and a higher prevalence of Unhealthy dietary pattern compared with the HFC group. However, differences were not significantly different between the two groups (P=0.06). FMD was significantly higher in the HFC group than in the LFC group (9.7±1.8 vs. 7.3±1.9%; P<0.001). The HFC group had also higher blood concentrations of both mercury (5.87±2.69 vs. 1.65±1.10; P<0.001) and arsenic (6.04±3.25 vs. 2.30±1.58; P<0.001) than the LFC group. No significant difference between the two groups was observed concerning anthropometric and other clinical and laboratory data (Tables 2 and 3). The FPG concentrations were significantly correlated with the blood concentration of both mercury (r=0.39; P=0.01) and arsenic (r=0.55; P<0.001) (Figure 2). No other significant correlation between metal concentration and the variables considered in the study were observed.

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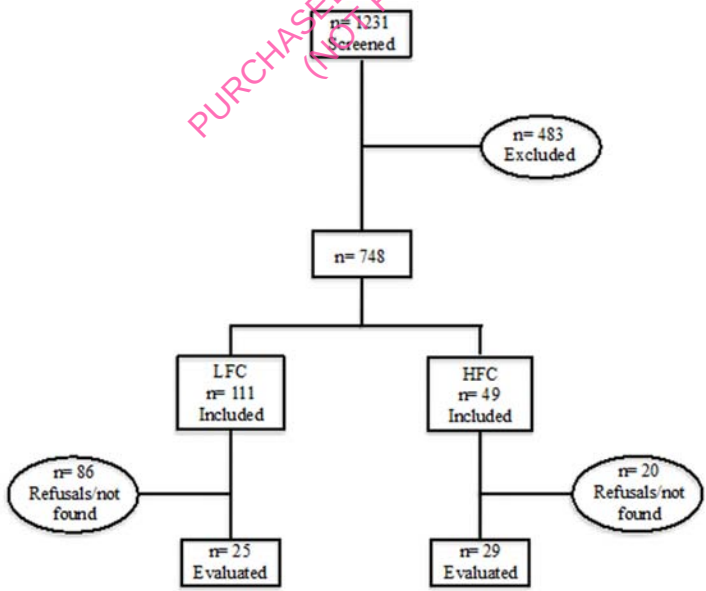


Figure 1: Participant Selection Flow (LFC=Low Habitual Fish Consumers; <1 Portion a Week. HFC=High Fish Consumers; ≥3 Portions a Week).

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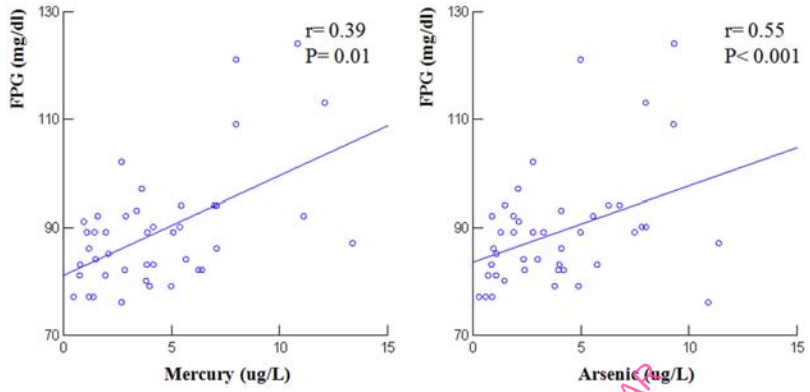


Figure 2: Correlations between Fasting Plasma Glucose (FPG) and Both Mercury and Arsenic Serum Concentrations.

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Table 1: Demographic and Clinical Characteristics of the Cohort Categorized according to the Level of Habitual Fish Consumption

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	Fish consumers		
	High (n = 29)	Low (n = 25)	P[1]
Age (y)	54±12	50±12	0.22
Gender (male/female)	13/16	7/18	0.20
Smokers, n (%)	9 (16.7)	11(20.4)	0.15
Number of Offspring	1.5±1.3	1.6±1.0	0.85
Prevalence of hypertension, n (%)	6 (11.1)	9 (16.7)	0.21
Use of anti-hypertensives, n (%)			
diuretics	1 (1.9)	2 (3.7)	0.47
beta-blockers	3 (5.6)	1 (1.9)	0.38
ACEI or ARBs	1 (1.9)	4 (7.4)	0.11
Dietary pattern, n (%)			0.06
Mediterranean	12 (22.2)	7 (12.9)	
Intermediate	15 (27.8)	10 (18.5)	
Unhealthy	2 (3.7)	8 (14.8)	
Fish consumption (portions/week)	4.4±1.5	0.04±0.2	<0.001

All values are presented as means ± SD or in absolute values (percentage in parenthesis).
[1]Student's unpaired t-test or Fisher's χ^2, when appropriate.
ACEI: angiotensin converting enzyme inhibitors. ARBs: angiotensin receptor blockers.
doi:10.1371/journal.pone.0112478.t001

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Table 2: Anthropometric, Echographic and Laboratory Data of the Cohort Categorized according to the Level of Habitual Fish Consumption

https://doi.org/10.1371/journal.pone.0112478.t002

	Fish consumers		
	High (n = 29)	Low (n = 25)	P[1]
Body weight (kg)	70.4±10.7	72.9±15.1	0.50
BMI (kg/m^2)	26.6±4.4	26.7±3.9	0.91
Waist circumference (cm)	92±10	92±12	0.78
Waist-to-hip ratio	0.89±0.07	0.88±0.08	0.93
Blood pressure (mmHg)			
systolic	131±16	127±11	0.26
diastolic	76±9	79±7	0.20
Heart rate (beats/min)	75±13	73±9	0.48
Carotid-IMTmax (mm)	0.61±0.12	0.62±0.12	0.80
FMD (%)	9.7±1.8	7.3±1.9	<0.001
Blood concentration of:			
glucose (mg/dL)	92±16	91±13	0.57
glycated hemoglobin (%)	5.4±0.6	5.4±0.5	0.87
insulin (μU/ml)	7.8±4.8	9.3±4.9	0.26
total cholesterol (mg/dl)	226±34	211±32	0.11
HDL-cholesterol (mg/dl)	67±20	63±17	0.39
triglycerides (mg/dl)	88±40	99±47	0.37
LDL-cholesterol (mg/dl)	144±31	129±28	0.06
uric acid (mg/dl)	5.0±1.4	4.4±1.0	0.10
creatinine (mg/dl)	0.81±0.18	0.78±0.13	0.53
GFR - MDRD (mL/min/1.73 m^2)	106.3±12.9	103.7±11.6	0.44
HOMA-IR	1.95±1.78	2.64±2.38	0.24

All values are presented as means ± SD.
[1]Student's unpaired t-test.
FMD: flow-mediated dilation of the brachial artery; GFR: glomerular filtration rate; HDL: high-density lipoproteins; MDRD: Modification of Diet in Renal Disease study; LDL: low-density lipoproteins.
doi:10.1371/journal.pone.0112478.t002

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Table 3: Serum Concentrations of Metals, Including Some Transition Metals and Metalloids, in the Cohort Categorized according to the Level of Habitual Fish Consumption

https://doi.org/10.1371/journal.pone.0112478.t003

	Fish consumers		
	High (n = 24)	Low (n = 19)	P[1]
Mercury (202) ug/L	5.87±2.69	1.65±1.09	<0.001
Cobalt (59) ug/L	0.74±0.18	0.65±0.19	0.12
Lead (208) ug/L	25.4±12.4	26.6±14.2	0.77
Copper (63) ug/L	511±202	694±184	0.17
Zinc (66) ug/L	4618±261	4784±314	0.07
Arsenic (75) ug/L	6.04±3.25	2.30±1.58	<0.001
Selenium (78) ug/L	158.3±54.5	141.8±44.2	0.29
Strontium (88) ug/L	24.1±9.8	22.5±6.3	0.54
Antimonium (121) ug/L	4.07±0.88	3.53±1.01	0.07

All values are presented as means ± SD. Atomic weight in brackets.
[1]Student's unpaired t-test.
doi:10.1371/journal.pone.0112478.t003

DISCUSSION

We investigated two groups of apparently healthy people with similar anthropometric, nutritional and clinical characteristics, but quite different habitual fish consumption. In particular, we had no clinical evidence of a different atherosclerotic involvement between the two groups. Levels of c-IMTmax, a marker of subclinical atherosclerosis, were similar. Therefore, even in people without established atherosclerosis, we found that consuming fish at least 3 times a week was associated with better endothelial function, measured in terms of FMD, than was consuming fish less than once a week. These results are in agreement with studies that found an inverse association between habitual fish intake and markers of inflammation or endothelial dysfunction [20], [41]. Few studies have investigated endothelial function in frequent fish eaters and in people who habitually do not eat fish. In contrast with our results, Petersen et al. did not find any significant difference in FMD comparing a group of people who habitually consumed at least 300 g of fish per week (corresponding to the Danish recommendations) with a group that habitually did not consume fish [42]. A possible explanation is that the effect of fish intake on FMD is significant for habitual intake of high amounts of fish. In fact, the intake of fish of our HFC participants was on average 4.4 portions/week, and assuming that a portion of fish is about 150–200 g, their weekly intake of fish was at least of 600–800 g/week, which is well above the Danish recommendations. Furthermore, the cardiovascular health benefits of fish are due, at least in part, to the n-3 PUFAs present in many species. Even in this case, when the effect of low doses of n-3 PUFAs on FMD was tested in healthy adults no significant improvement was observed [43]. On the contrary, higher amounts of n-3 PUFA consumption sharply improved FMD following a saturated-fatty-acid-rich drink [44]. Similarly, the effect of fish oil supplementation on FMD has not been firmly established [21], and a study by Plat et al. failed to demonstrate beneficial effects of fish oil on biomarkers of endothelial function and inflammation [45]. However, n-3 PUFAs are only one of the components of fish that may contribute to its favorable cardiovascular effects, including those on FMD. Vitamins A, B and D, calcium, phosphorus, iron, copper and selenium should in fact be considered, though we did not investigate them, with the exception of selenium, which had blood concentrations slightly but not significantly higher in the HFC group. Indeed, consumption of fish as a source of n-3 PUFAs has been found to be superior to the use of fish oil supplements, making fish intake the most effective source of n-3 PUFAs [46]. Our study reinforces the notion that fish is an important component of a healthy diet,

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with protective cardiovascular effects that are mediated, at least in part, by beneficial effects on the endothelium. Beyond its beneficial effects on coronary artery disease [47], [48], fish consumption has also been associated with reduced risk of ischemic stroke [49]–[52] and, interestingly, it has been suggested that racial and geographic differences in fish consumption may help explain racial and geographic differences observed in stroke incidence and mortality [53].

The favorable cardiovascular effects of consuming significant amounts of fish should, however, be balanced with the possible risks of exposure to contaminants, mainly metals. The presence of select toxic metals has been reported even in fish from Sicily, the largest island in the Mediterranean Sea, which was the site of our study [54], [55]. This is likely why we found significantly higher blood concentrations of mercury and arsenic in the HFC group. Studies that measured the toxic metal content of fish have generally concluded that despite the fact that they are significantly present, their concentrations are well below those indicated as having toxic effects on human health [54], [56]–[58]. It is perhaps appropriate to relate risk of exposure to toxic metals, including the transition metals and some metalloids, to the amount and frequency of habitual fish intake. We found that the serum concentrations of mercury and arsenic were significantly higher in HFC participants than in non-consumers, but we do not know if a different health risk corresponds to such differences in blood concentrations. Based on data from Moon et al., in a South Korean population, the average blood concentrations of mercury that we observed in the HFC group correspond to the fourth quartile of distribution in the Moon study, and should therefore be considered elevated [59]. Mercury may increase the risk of myocardial infarction [23], and may induce oxidative stress [60]. Our short term study indicates that despite higher serum mercury and arsenic concentrations, improved endothelial function was observed in the HFC group. Our study therefore seems to agree with other reports [61] that the cardiovascular benefits of fish intake exceed the potential risks. However, longer follow-up would be required to determine whether increased levels of metal contaminants have negative effects on health. There are, for example, reports suggesting that mercury and arsenic exposure increase the risk of diabetes [27], [28]. Our finding of a significant correlation between fasting plasma glucose and both mercury and arsenic concentrations supports this idea.

Our study has important limitations. We did not obtain metal measurements in all study participants, and we measured concentrations of

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metals in serum and not in hair or erythrocytes, as usually required when exposure to these substances needs to be investigated. However, the choice of measuring metals in serum was based on the availability of samples, and it has been demonstrated that serum Hg concentration is also a reasonably good index of exposure to mercury [62]. In addition, serum concentrations may be related to exposure to metals in the short term, compared with measurements in hair and nails, which reflect longer exposures. This may explain why we found significant correlations between FPG and mercury and arsenic serum concentrations, while we found no significant correlations with other measures of glucose control, such as glycated hemoglobin, which reflect glucose levels for the preceding 3 months.

Habitual fish intake may, generally speaking, reflect healthier dietary habits, which are responsible for the favorable effects on the endothelial function that we found associated with fish consumption. However, in this study we reported the prevalence of dietary patterns as obtained in the complete cohort we presented elsewhere [31]. The HFC group exhibited healthier dietary habits, but differences with LFC group did not reach statistical significance. Given the cross-sectional design of the study, we cannot exclude the possibility of residual confounding factors. Also, we cannot determine whether differences in the favorable endothelial effects of fish consumption exist according to differences in fish cooking-procedures (fried or not) or in its provenance (farm-raised fish or their wild counterparts), storage (frozen or fresh) or typology (fatty or lean fish).

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The strengths of this study are the modality of participant recruitment, which allowed for the characterization of study participants, and the use of a strict ultrasound procedure for FMD measurement by one operator, which may have contributed to reducing possible biases.

In conclusion, despite higher levels of toxic metals such as mercury and arsenic, we observed a favorable association between fish consumption and endothelial function in apparently healthy people. Though these results may encourage the consumption of fish, intervention studies in the medium to long term are needed to test the effects of this food on metabolic and cardiovascular end-points.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: S. Buscemi S. Vasto FD GG S. Berganti FG S. Verga. Performed the experiments: S. Buscemi S. Vasto FD

FMM EA GR. Analyzed the data: S. Buscemi GG S. Berganti FG FMM GR S. Verga. Contributed reagents/materials/analysis tools: S. Buscemi S. Vasto FD FMM EA. Contributed to the writing of the manuscript: S. Buscemi S. Vasto GG S. Berganti FG EA GR S. Verga.

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SECTION VI: VETERINARY TOXICITY PESTICIDES, INSECTICIDES, RODENTICIDES AND HERBICIDES

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Chapter 11

Honey Bee Exposure to Pesticides: A Four-Year Nationwide Study

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ABSTRACT

Pollinators, including honey bees, are responsible for the successful reproduction of more than 87% of flowering plant species: they are thus vital to ecosystem health and agricultural services world-wide. To investigate honey bee exposure to pesticides, 168 pollen samples and 142 wax comb samples were collected from colonies within six stationary apiaries in six U.S. states. These samples were analyzed for evidence of pesticides. Samples were taken bi-weekly when each colony was active. Each apiary included thirty colonies, of which five randomly chosen colonies in each apiary were sampled for pollen. The pollen samples were separately pooled by apiary. There were a total of 714 detections in the collected pollen and 1008 detections in collected wax. A total of 91 different compounds were detected: of these, 79 different pesticides and metabolites were observed in the pollen and 56 were observed in the wax. In all years, insecticides were detected more frequently than were fungicides or herbicides: one third of the detected pesticides were found only in pollen. The mean (standard deviation (SD)) number of detections per pooled pollen sample varied by location from 1.1 (1.1) to 8.7 (2.1). Ten different modes of action were found across all four years and nine additional modes of action occurred in only one year. If synergy in toxicological response is a function of simultaneous occurrence of multiple distinct modes of action, then a high frequency of potential synergies was found in pollen and wax-comb samples. Because only pooled pollen samples were obtained from each apiary, and these from only five colonies per apiary per year, more data are needed to adequately evaluate the differences in pesticide exposure risk to honey bees among colonies in the same apiary and by year and location.

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Keywords: honey bees, pesticides, exposure, insecticides, herbicides, fungicides, pollen, wax

INTRODUCTION

Pollinators, including honey bees, are responsible for the successful reproduction of more than 87% of flowering plant species: therefore, they are vital to ecosystem health and agricultural services world-wide [1]. Agriculture depends strongly on the services of the honey bee, which is responsible for providing more than 90% of commercial pollination services [2]. Thus, elevated loss rates seen in managed honey bee colonies [3] threatens

the pollination services they provide. A variety of different stressors—including pathogens, parasites, floral resource loss, environmental stresses, and socioeconomic factors—have been suggested as contributing to the elevated levels of bee population decline [4,5,6,7,8,9,10,11,12,13]. One stressor, exposure to pesticides, has been, and remains, an important focus for research.

Until the early 1980s, the primary, if not exclusive, exposure to pesticides experienced by honey bees, and the source of hive product contamination, was from pesticides applied to agricultural or horticultural landscapes. With the introduction, nearly three decades ago, of the parasitic *Varroa* mite (*Varroa destructor*) into North America, concerns about pesticide residues in wax, honey, bees, pollen, bee bread, and other honey bee-related matrices expanded to include residues resulting from direct application of pesticides within colonies [14,15,16,17,18,19,20,21,22,23,24]. The most frequently reported compounds in honey bee products are acaricides used to control *V. destructor*, including coumaphos, fluvalinate, and amitraz; however, many other compounds have been detected, including some at levels that may result in sublethal and lethal outcomes [20].

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To date, studies of pesticide residue in honey bee colonies have been cross-sectional. Residues have been evaluated in a variety of matrices, including but not exclusive to honey, beebread/pollen, wax, propolis, and bees [15,16,17,18,19,20,22,23,24,25,26,27,28,29,30]. These cross-sectional studies have described contaminants from samples obtained from multiple colonies/hives at a single time during the foraging season, or after the application of an acaricide to colonies. Most have investigated a limited range of pesticide residues, those primarily resulting from the management of honey bee pests [15,16,17,23,24,29,30]. Some studies used analytical methods capable of detecting of a wide range of pesticide residues found in the foraging environment as well as those used in colony management [18,19,20,22,25,26,28,31].

Mullin et al. [22], in their cross-sectional study of North American apiaries, detected 121 different herbicides, fungicides and insecticides, along with metabolites, in multiple matrices, e.g., wax, pollen, bee bread, and bees. In 259 wax samples, 87 compounds were detected with a mean of eight detections per sample. One sample contained residues of 39 compounds. The four most frequently detected pesticides in wax were fluvalinate (98.1%), coumaphos (98.1%), coumaphos oxon (89.9%, a metabolite of coumaphos) and chlorpyrifos (63.2%). Of these, fluvalinate,

coumaphos, and coumaphos oxon are all compounds used by beekeepers against *Varroa*. In 350 pollen samples, a combination of bee bread (320 samples), trapped pollen (28 samples) and anther pollen (2 samples), 98 compounds were detected with a mean of 7.1 detections per sample. One sample contained residues of 31 compounds. The most frequently detected compounds were fluvalinate (88.3%), coumaphos (75.1%), and chlorpyrifos (43.7%), again with fluvalinate and coumaphos likely being introduced as compounds targeting *Varroa*.

Cross-sectional studies like these provide valuable information on bee colonies at a single point in time, or immediately following a single exposure. They are not capable of assessing the impact on bee health from multiple exposures, the cumulative and possibly synergistic effects of multiple sequential or simultaneous exposures, or the effect of multiple residues within a single matrix or spread throughout multiple matrices (e.g., wax, pollen, bee bread, nectar, honey, propolis, and adult bees, within a hive) [8,32]. The order of exposure is also important: Johnson et al. [8] demonstrated that the toxicity of amitraz was not changed when honey bees were exposed to sublethal doses of tau-fluvalinate or coumaphos prior to amitraz exposure, but tau-fluvalinate and coumaphos toxicity increased when bees were treated with amitraz prior to tau-fluvalinate or coumaphos.

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This study is the first to sample the same colonies, from the time of their establishment until their death, using a prospective study design. Its objective is to extend the study of honey bee health impacts by assessing the level of pesticide contaminants, over a period of four years, carried to honey bee colonies by foragers in pollen loads. Colonies established in six states were included in the study.

MATERIALS AND METHODS

Apiary Establishment and Management

Three trials (2009, 2010, and 2011) of stationary hives in six states were established for this study. In 2009, six apiaries with 30–32 colonies each were established. Three-pound packages were obtained from sources in the southeastern and western U.S. and introduced into new 10-frame wooden hive boxes (all deep boxes, except in PA, which used medium boxes) using Pierco® wax-coated plastic foundation (Riverside, CA, USA). All Pierco® wax-coated plastic foundation was purchased at the same time and

the wax used for coating the foundation was from the same lot number (one wax source). Queens accompanying the packages were replaced with Italian queens from Koehnen & Sons, Inc. (Glenn, CA, USA).

In 2010 an additional 15-colony apiary was established in Orono, ME with packages originating from Florida. As in 2009, Italian queens from Koehnen & Sons, Inc. replaced the package queens.

In 2011, six apiaries with 30 colonies each were again established using 3-pound packages and new equipment as described above. Carniolan queens replacing those accompanying the packages were obtained from Koehnen & Sons, Inc. Because insufficient supplies of the Pierco® wax-coated plastic foundation from 2009 were available, additional Pierco® wax-coated plastic foundation was purchased in 2011 and the wax used for coating the foundation was from the same lot number (one wax source).

The habitat surrounding the apiaries varied from 68% to 4% agriculture (vegetable, fruit and field crops) and from 35% to 0% residential suburban. The general climate varied from the hot, dry summers and cold winters of eastern Washington, to the hot, humid summers and mild to cool winters of southern Texas, to the short warm, humid summers and cold winters of Minnesota, and the long hot humid summers and short cool winters of Florida.

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Colonies were placed at least 2 m apart, where possible, in each apiary and managed in a manner typical for each region of the country (supering when necessary, wrapping in cold climates, etc.) except that *no pest management practices were employed*: i.e., no miticides, no medications for pathogens, no colony splitting resulting in intentional broodless periods, no screened bottom boards, no sugaring for mite control, and no drone frames were used during the lifespan of the colonies during the study.

Table 1 summarizes the location and location characteristics, year of establishment, number and survivorship after one year, of the study colonies in each apiary.

Table 1: Number of colonies alive (dead) by location and year including the number of wax and pollen samples. A pooled pollen sample is a composite bi-weekly sample of pollen collected from five colonies per apiary. The number of analyzed pollen samples reported in column 6 corresponds to pooled samples from five colonies in each apiary, except that analysis of unpooled samples, taken in 2009 from the WA apiary, is also reported in parentheses. The wax samples are comb wax; each sample is from a separate colony. See Section 2.1 (Apiary Establishment and Management) and Section 2.2 (Pollen and Wax Sample Collection) for further details

		Initial # of Colonies	End of Year # of Colonies Alive (Dead)	# of Wax Samples	# of Analyzed Pollen Samples	Location	Surrounding Habitat
Florida (FL)	2009	30	22 (8)		8	Citra	25% agriculture * 19% forest, 38% pasture/ grazing, 8% wetlands
	2010		6 (16)		12		
	2011	30	24 (6)	1	10		
	2012		0 (30)				
Maine (ME)	2009	30	26 (4)		6	Stockton Springs	30% agriculture, 35% forest, 20% shrub/old field, 8% wetland
	2010		10 (16)	2	4		
	2010	15	14 (1)		4	Orono	18% agriculture, 25% forest, 35% residential/ suburban
	2011	30	28 (2)	6	7	Glenburn	30% agriculture, 35% forest, 20% shrub/old field, 8% wetland
	2012		25 (3)		8		
Minnesota (MN)	2009	32	31 (1)		5	Minnestrista	4% agriculture, 27% forest, 6% residential/ suburban, 42% pasture/ grazing, 20% other
	2010		20 (11)	4	5		
	2011	30	27 (3)	6	9		
	2012		6 (21)	22	10		
Pennsylvania (PA)	2009	30	27 (3)		3	Rock Springs	50% agriculture, 46% forest 4% shrub/old field
	2010		0 (27)	15			
	2011	30	27 (3)	1	4		
	2012		3 (24)		7		
Texas (TX)	2009	30	29 (9)	6	8	Weslaco	68% agriculture, 14% shrub/old field, 5% pasture/grazing, 13% other
	2010		3 (26)	2	11		
	2011	30	29 (1)	4	11		
	2012		7 (22)	16	10		
Washington (WA)	2009	30	29 (1)	20	4 (20 **)	Pullman	65% agriculture, 25% residential/suburban, 10% pasture/grazing
	2010		15 (14)	4	5		
	2011	30	27 (3)	6	10		
	2012		27 (0)	27	7		

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* Agriculture land was vegetable, fruit and field crops. ** Twenty pollen samples were taken from five hives. These pollen samples were not pooled.

Pollen and Wax Sample Collection

Pollen Samples among Apiaries and over Time

In all 4 years of the study (2009–2012), pollen samples were collected at each of the six apiary sites from the same five, randomly selected, living colonies every two weeks during the foraging season. If a colony died, another colony was randomly selected to replace it. Samples were collected every two weeks using a pollen trap operating for a brief period. Due to inclement weather (resulting in poor or non-existent foraging) or fungal growth resulting in pollen degradation, some locations did not have pollen samples for every two-week sampling period. The length of the foraging season varied by location. Florida (FL) and Texas (TX) had the longest season, with collection occurring from January/February through November/December. Pennsylvania (PA) and Maine (ME) had the shortest foraging seasons, with collection taking place from May/June through August/September. After each collection, pollen was sent to The Connecticut Agricultural Experiment Station (CAES) for pesticide residue analysis. Samples from each apiary were pooled by CAES for each bi-weekly collection. A total of 168 pooled samples were analyzed.

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To evaluate if compounds detected in a colony are predictive of compounds likely to be detected in another colony, from 15 June–21 July 2009, 20 additional pollen samples were taken from five hives located in Washington (WA). A total of 20 unpooled samples were analyzed.

Table 1 includes a summary of the sampling at each apiary by year. The 20 unpooled samples from WA are noted separately.

Wax Comb and Foundation Samples among States and over Time

Foundation

In 2009, prior to the installation of foundation in hives, two samples (minimum of 25 g/sample) were taken from randomly selected frames Pierco® wax coated plastic foundation. These samples were sent to the CAES for pesticide analysis. In 2011, before packages were installed in the hives, three composite wax samples were obtained as described above from

the unused Pierco® wax coated plastic foundation purchased in 2009, and from Pierco® wax coated plastic foundation newly purchased in 2011.

Wax Comb

In contrast to the pollen sampling, wax comb sampling was less frequent and not at consistent intervals. A total of 142 wax comb samples were taken. Most of these were obtained soon after colonies died, but not all hives in all apiaries were sampled after colony loss. A total of 107 wax comb samples were collected from dead colonies between 2010 and 2012 (FL (1), ME (8), MN (27), PA (16), TX (25), WA (30)). In addition, 35 samples of comb from living colonies were obtained in 2009, 2010, and 2012 (MN (5), WA (27), TX (3)). All wax comb samples were of 25 g or greater and taken from brood-rearing areas of hive. After each wax comb collection, samples were sent to CAES for pesticide residue analysis.

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Pesticide Residue Quantification

Sample Preparation

CAES performed the pesticide analysis for all wax and pollen samples. Except for the 20 pollen samples taken to assess the variability in compounds detected among individual colonies (see Section 2.2.1), all pollen analyses took place on pooled samples from each location. These pooled samples, one for each apiary and sampling period, were obtained by thoroughly mixing the samples from the five pollen-trapped colonies in each of the respective apiary. Pollen and wax samples were analyzed using a modified Quechers extraction protocol [25]. In brief, pollen samples (5 g if sufficient pollen was available or entire sample if less) were combined with water to a final volume of 15 mL. To this sample was added 100 ng of isotopically labeled (d-4) imidacloprid (Cambridge Isotope Laboratories Andover, MA, USA) as an internal standard. The samples were combined with 15 mL of acetonitrile, 6 g magnesium sulfate and 1.5 g sodium acetate, and 150 uL of acetic acid. After shaking and centrifuging, 10 mL of the supernatant was combined with 1.5 g magnesium sulfate, 0.5 g PSA, 0.5 g C-18 silica and 2 mL toluene. The samples were further shaken and centrifuged. Finally, 6 mL of the supernatant was concentrated to 1 mL for instrumental analysis. Wax samples were analyzed in a similar manner except that after addition of the water they were placed in a waterbath at 80 °C for 45 min to allow the wax to melt before shaking and centrifuging with the acetonitrile.

Analysis

Extracts were analyzed with liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). In 2009, the LC system was an Agilent 1100 LC; 6 µL of the extract was injected onto a Zorbax SB-C18, 2.1 × 150 mm, 5 µm column. The column was gradient eluted at 0.25 mL per minute from 12.5% methanol in water to 100% methanol. Both solvents had 0.1% formic acid added. In 2010 the LC system was an Agilent 1200 Rapid Resolution system with a Zorbax SB-C18 (Agilent, Santa Clara, CA, USA) Rapid Resolution HT 2.1 × 50 mm, 1.8 µm column using a 3 µL injection with the gradient going from 5% methanol in water to 100% methanol at 0.45 mL/min. In both cases the LC was coupled to a Thermo-LTQ (Thermo-Fisher, Waltham, MA, USA, a linear ion trap mass spectrometer. The system was operated in the positive ion electrospray mode, with a unique scan function for each compound allowing for MS/MS monitoring. Detection limits varied with the exact sample matrix and the amount of sample available but were in the single digit part per billion (ppb) level or lower for pesticides reported herein.

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Though our procedures were similar to those reported by Mullin et al. [22], we do not report the on the same set of pesticides as does that study. For enhanced specificity in pesticide detection at very low concentrations, we chose to use only our available LC/MS-MS. While providing greater confidence in our data on pesticides detected during a multi-residue screen, this choice precluded detection of some classes of pesticides (such as pyrethroid insecticides, also we could not detect chlorothalonil only its hydroxyl metabolite), which can require additional sample cleanup steps and GC/MS in electron impact or negative chemical ionization modes.

Estimation of Oral and Contact LD_{50}s

For the 82 pesticides (parent compounds), eight metabolites and one synergist detected in pollen and wax, we searched the literature for honey bee oral and contact LD_{50} [33,34,35,36,37]. Where LD_{50} values differed among literature sources, we used the value provided by the US EPA Ecotoxicity Database; if more than one LD_{50} value was reported in the EPA Ecotoxicity Database, we used the lowest value. Unless information specific to a metabolite was available, we used the LD_{50} value for the parent compound, acknowledging that some metabolites have lower toxicity than the parent compound and some have higher toxicity than the parent compound [34,38]. For 35 compounds only a contact LD_{50} was found; for three compounds only an

oral LD_{50} was found. No contact or oral LD_{50} was found for two compounds (diphenylamine and octhilinone). Oral and contact LD_{50} values reported in terms of µg/bee were converted to ppb relative to body weight (ng pesticide per g bee) by multiplying each value by a factor of 10,000; this is equivalent to 1000 ng per µg, divided by a mean bee weight of 0.1 g [39].

Statistical Analysis

Pollen, Comb Wax, and Foundation Wax

Goodness of Fit and Tests of Independence were conducted to determine similarities and differences between pollen and wax pesticide residues or oral versus contact LD_{50}. Wax sample (comb and foundation) residues were logarithm +1 (base 10), transformed to achieve homogeneity and normality of variances. Pearson and Spearman's correlation coefficients were used to assess evidence of association among types and classes of pesticide residues in detected pollen. An analysis of variance (RCBD) was used to determine if significant differences existed among residue concentrations in a paired study involving wax comb (near the brood rearing area) taken from hives with living colonies and hives where the colonies had died.

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Can Residues Detected in Pollen Samples a Colony Predict the Residues in Pollen in an Unsampled Colony?

To evaluate if compounds detected in a colony are predictive of compounds likely to be detected in another colony, from 15 June–21 July 2009, 20 pollen samples were taken from five hives located in WA. These samples were individually analyzed by CAES: i.e., they were not pooled before analysis. Each sample was either positive or negative for the presence of any detectable pesticide. To quantify the degree of variability we introduce the Predictive Power of individual pollen samples:

Predictive Power = (# of correct predictions)/(# of incorrect predictions).

To evaluate the (# of correct predictions) and (# of incorrect predictions), first pool the pesticide detections found, in any sample, at each sampling time. A correct prediction at a sampling time corresponds to a detection of a pesticide from that pooled list in an individual sample. For example, if—in a given sampling time—there were ten pesticides detected in five samples, the number of correct predictions at that sampling time would be at least ten, but no greater than fifty. The number of correct predictions is the total of correct

predictions across all sample times less the number of pesticides detected.

Likewise, an incorrect prediction at a sampling time corresponds to a pesticide in the pooled list that was not found in one of the individual sample assays from which the list was assembled. Using the same example numbers, the number of incorrect predictions at a sampling time could be as low as zero, or as high as forty. The number of incorrect predictions is the total of incorrect predictions across all sample times.

It is important to note that, by our definition, a prediction occurs only when a pesticide is detected: i.e., the absence of a pesticide detection in a sample does not count as a prediction that the same pesticide will not be found in other samples taken at the same time. This choice was made to eliminate false negatives: i.e., situations in which the pesticide was not detected because the pesticide was not in the environment on the day the pollen was sampled. Subtracting the number of pesticides detected at each sampling time is necessary to eliminate the bias introduced by counting only pesticide detections as predictions.

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As defined, a Predictive Power of unity means there is 50% chance of that a pesticide detection in a single hive correctly signals the presence of that pesticide in any other hive. A predictive power greater than unity corresponds to a greater than 50% chance that other hives will show presence of the same pesticide, while a predictive power less than unity means that detection of a pesticide in one hive is anti-correlated with the presence of the pesticide in other apiary hives.

RESULTS

Table 2, Table 3, Table 4 and Table 5, provide different perspectives on the number of detections in the different matrices (pollen, wax comb, and foundation wax), the number of compounds detected in each matrix, and the years in which each compound was detected. Salient features of the data are described in the subsections that follow.

Table 2: Honey bee contact and oral LD_{50} for 91 compounds detected in pollen (P) wax comb (WC) and wax from coated foundation (WF). Mean, range, and LD_{50} values reported as ppb. The LD_{50} for the parent compound was used for a detected metabolite. Abbreviations: F = fungicide; H = herbicide, I = insecticide; U = unknown mode of action (MOA); nhbd = no honey bee data available. See Section 3.1 for details

Pesticide	Detected in Pollen, Wax Comb or Wax Foundation	Pesticide Class MOA	Number of Detections: Pollen, Wax Comb, or Wax Foundation	Years Found: Detection Years	Detection		Oral LD_{50}	Contact LD_{50}	Source
					Mean	Range			
FUNGICIDES									
4-Hydroxy-chlorothalonil (chlorothalonil)	P		5	2009–2011	19.5	5.5–42		1,812,900	[35] *
	WC	Multi-site	7	2010	7.3	1.4–17			
Azoxystrobin	P		41	2009–2012	12.2	0.5–214	250,000	2,000,000	[35,37]
	WC	F-C3	56	2010–2012	2.8	0.5–33			
	WF		4	2009 & 2011					
Benalaxyl	P	F-A1	2	2010, 2011	1.4	1–1.9	1,000,000		[35]
Boscalid	P		10	2010, 2011	2.75	1.2–5.2	1,660,000	2,000,000	[35,37]
	WC	F-C2	14	2010–2012	19	0.6–43			
	WF		5	2009 & 2011	5.4	2.2–10			
Carbendazim	P		48	2009–2012	22.7	0.8–548		500,000	[35]
	WC	F-B1	40	2010–2012	61.5	0.7–137			
	WF		1	2009		1			
Cymoxanil	P	U	1	2010		7.5	852,900	250,000	[35,37]
Cyprodinil	P		1	2011		21		7,840,000	[35]
	WF	F-D1	3	2009 & 2011	49.4	8.7–90			
Difenoconazole	P	F-G1	2	2010, 2011	4.2	3.9–4.5	1,770,000	1,010,000	[35]

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Dimethomorph	P		3	2009, 2011, 2012	0.55	0.5–0.6	324,000	100,000	[35,37]	
	WF	F-H5	1	2011		3.9				
Diphenylamine	WC	U	3	2011	100.7	3.5–250	nhbd	nhbd		
Epoxiconazole	WC	F-G1	1	2010		0.5	830,000	1,000,000	[35]	
Fenbuconazole	WF	F-G1	1	2009		2.5		2,920,000	[35]	
Fluazinam	P	F-C5	1	2009		4.7	1,000,000	40,000	[35,37]	
Fluoxastrobin	WC	F-C3	1	2012		13	8,433,000	2,000,000	[35]	
Iprodione	WF	F-E	3	2009, 2011	133.3	110–165	250,000	2,000,000	[35]	
Imazalil (Enilconazole)	P	F-G1	1	2009		1.6	351,000	390,000	[35]	
Mandiproamid	P		1	2009		1.2	2,000,000	2,000,000	[35]	
	WC	F-H5	1	2010		2.9				
Metalaxyl	P		4	2010–2012	1.7	0.8–3.5	250,000	1,000,000	[35,37]	
	WF	F-A1	1	2011		1.5				
Metconazole	P	F-G1	1	2011		1.3	850,000	1,000,000	[35]	
Myclobutanil	P		2	2011, 2012	3.6	1.1–6.1		3,620,000	[25]	
	WC	F-G1	2	2010, 2012	2.9	1.7–4.1				
Octhilinone	P		1	2011		24	nhbd	nhbd		
	WC	F-A3	7	2010, 2011	8	1.2–14				
	WF		1	2011		3.1				
Propiconazole-1	P		32	2009–2012	13.1	1.1–64	1,000,000	250,000	[35,37]	
	WC	F-G1	36	2010–2012	6.3	0.8–66				
	WF		1	2009		2.6				
Pyraclostrobin	P		12	2010–2012	5.8	0.8–12	731,000	1,000,000	[35,37]	
	WC	F-C3	53	2011, 2012	4.8	1.2–24.5				
Pyrimethanil	P	F-D1	1	2010		29	1,000,000	1,000,000	[35]	

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Spiroxamine	P	F-G2	2	2011	1.5	1.0–2.0	1,000,000	42,200	[35,37]
Tebuconazole	P		8	2011, 2012	65.6	2.0–104	850,500	2,000,000	[37]
	WC	F-G1	32	(2010–2012)	7.6	1.3–24			
Thiabendazole	P		2	2009, 2012	1.2	0.8–1.5	340,000	40,000	[37]
	WC	F-B1	9	2010–2012	46.1	0.4–91			
Thiophanate-methyl	P	F-B1	4	2010, 2011	13	4.6–40		1,000,000	[35]
Trifloxystrobin	P		7	2009–2012	6.8	0.2–23	2,000,000	2,000,000	[35,37]
	WF	F-C3	2	2009	1.8	1.3–2.4			
HERBICIDES									
Acetochlor	P	H-K3	4	2011, 2012	14.6	1.8–22		2,000,000	[35]
Alachlor	P	H-K3	1	2009		1.3		362,000	[35]
Atrazine	P		84	2009–2012	5.4	0.1–58		970,000	[33]
	WC	H-C1	49	2010–2012	0.9	0.01–6.5			
Bentazon	P		1	2010		3.1	2,000,000	2,000,000	[37]
	WC	H-C3	19	–2012	5.9	1.3–17			
Dithiopyr	P		3	2009, 2010	1.85	0.6–3		810,000	[35]
	WC	H-K1	4	2010–2012	2.4	1.7–5.1			
Diuron	P		27	2009–2012	19.5	1.1–275		1,450,300	[35]
	WC	H-C2	41	2010–2012	10.7	0.4–30			
Fenuron	P		2	2010	1.4	0.7–2.1		250,000	[35]
	WC	H-C2	2	2011	29.5	24–35			
Halosulfuron-methyl	WC	H-B	3	2010	307	213–386		1,000,000	[35]
Linuron	P		3	2010, 2011	5.2	2.2–14.4	1,600,000	1,208,600	[35]
	WC	H-C2	5	2010, 2011	2.4	1–5.5			
MCPA	WC	H-O	1	2012		4	100,000	250,000	[35]

PURCHASED BY DR. N B SHRIDHAR (NOT FOR RESALE)

Metolachlor	P		32	2009–2012	15.3	0.2–130	1,100,000	1,100,000	[27]
	WC	H-K3	12	2011, 2012	1.4	1–4.5			
Metribuzin	P	H-C1	3	2011	1.4	0.6–2.6	1660,000	604,000	[35,37]
Metsulfuron-methyl	P	H-B	1	2011		7	443,000	250,000	[35,37]
Napropamide	WC	H-K3	1	2011		2.8	1,000,000	1,000,000	[37]
Pendimethalin	P		41	2009–2012	6.6	1.2–55		498,000	[35]
	WC	H-K1	28	2010–2012	5.4	0.4–20			
	WF		4	2009, 2011	15.5	11–17			
Prodiamine	P		1	2009		2.3		1,000,000	[35]
	WF	H-K1	2	2009	15.2	13.4–17			
Prometon	P		3	2011	2.4	1–3.3		360,000	[35]
	WC	H-C1	1	2012		3.5			
	WF		1	2011		0.5			
Prometryn	P		1	2011		2.2		966,900	[35]
	WF	H-C1	1	2009		0.5			
Propyzamide	P	H-K1	3	2012	2.2	1.5–6.2		1,810,000	[35]
Sethoxydim	P	H-A	3	2010, 2011	13.4	1.75–23		100,000	[35]
Siduron	P	H-C2	3	2012	9.1	5.6–13		1,200,000	[35]
Simazine	P	H-C1	3	2009, 2011, 2012	1.5	1.1–2		967,000	[35]
Sulfentrazone	P	H-E	1	2011		2.5	250,000	250,000	[35]
INSECTICIDES									
Acephate	P		2	2012	2.4	1.6–3.2		12,000	[35]
	WC	I-1B	1	2010		5.8			
Acetamiprid	P	I-4A	3	2010, 2011	150.3	10–436	145,300	81,000	[35,37]
Aminocarb	P	I-1A	1	2011		1.7		600	[33]

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DMPF (amitraz)	P		2	2010, 2011	2.5	2.5		1636	[35] *
	WC	I-19	2	2010, 2011	4.2	3.7–4.6			
	WF		5	2009, 2011	440.9	16.5–670			
Azinphos-methyl	P		3	2009–2011	1.7	0.4–3.5	1500	4200	[35]
	WC	I-1B	8	2010	0.3	0.2–0.3			
Bendiocarb	P		3	2010	1.7	1.2–1.9		4280	[35]
	WC	I-1A	1	2010		4.5			
Carbaryl	P		43	2009–2012	7.1	1–95	2310	11,000	[35]
	WC	I-1A	1	2010		0.9			
Carbofuran	P		6	2009–2011	7.9	1-24		1600	[35]
	WC	I-1A	10	2010	5.7	0.8–10.8			
OH-Carbofuran (carbofuran)	P	I-1A	6	2009, 2011	8.8	1.2–29		1600	[35] *
	WC		15	2010	28.1	5.4–112			
Chlorantraniliprole	P	I-28	2	2010, 2011	7.6	5.5–9.6	1,041,000	40,000	[37]
Chlorpyrifos	P		36	2009–2012	11	0.9–61	2500	100	[35]
	WC	I-1B	26	2010–2012	6.5	0.8–44			
	WF		4	2009, 2011	14.7	1-28			
Clothianidin	P	I-4A	6	2009, 2010, 2012	2	1.2–4.8	36.8	439	[25]
Coumaphos	P		31	2009–2012	10.2	0.2–196		240,000	[25]
	WC	I-1B	122	2010–2012	30	0.2–2060			
	WF		5	2009, 2011	1546.25	109–2500			
Coumaphos oxon (coumaphos)	P		1	2012		2.5		240,000	[25] *
	WC	I-1B	38	2010–2012	4.2	0.9–33			
	WF	=	5	2009, 2011	38.6	6.3–63			

PURCHASED BY DR N B SHRIKHAR (NOT FOR RESALE)

Diazinon	P		4	2011, 2012	6.8	1–8.6	2000	2200	[35]
	WC	I-1B	11	2011	0.8	0.4–2			
	WF		1	2009		0.3			
Dichlorvos	P		2	2010, 2012	18	1.5–35		5000	[35]
	WC	I-1B	6	2010, 2011	3.6	2-13			
	WF		1	2011		10			
Diflubenzuron	P		1	2011		19			[35]
	WF	I-15	1	2011		3.8	351,000	390,000	
Dimethoate	P		27	2009–2012	23	0.3–194	560	1600	[35]
	WC	I-1B	47	2010–2012	23.9	0.6–945			
Fenpyroximate	P		2	2009	0.4	0.2–0.6	500,000	1,185,000	[35,37]
	WC	I-21A	10	2010–2012	23.2	1–207			
	WF		4	2009, 2011	98.4	4.1–366			
Fluvalinate	P		12	2009–2011	9.6	0.9–27		2000	[35]
	WC	I-3	111	2009–2012	157.5	1–862			
	WF		5	2009, 2011	8064.8	290–15,400			
Imidacloprid	P	I-4A	9	2009, 2011, 2012	7.4	1.5–51	37	439	[25]
Imidacloprid, Olefin	P	I-4A	1	2011		9.5	3600		[33]
Imidacloprid, urea	P	I-4A	1	2011		19	995,000		[33]
Malathion	P		33	2009–2012	65.8	1.4–148	3800	2000	[35,37]
	WC	I-1B	29	2010–2012	35.5	1.2–91			
Methiocarb	P	I-1A	7	2010–2012	3.7	1-20	3750	2300	[37]
Methomyl	P	I-1A	6	2011, 2012	10.4	2.5–26	2900	1600	[35]
Methoxyfenozide	P	I-18	4	2012	45.3	2.2–162	1,000,000	1,000,000	[35]

PURCHASED BY DRN B SHRIDHAR (NOT FOR RESALE)

Omethoate (methoate)	P	I-1B	9	2011, 2012	4	1-12	560	1600	[35] *
Phorate	P	I-1B	1	2009		22	4400	3200	[35]
Phorate Sulfoxide (phorate)	P	I-1B	1	2011	3.3	1.7–9	4400	3200	[35] *
Phosmet	P		17	2009–2012	46.4	1.1–483	3700	10,600	[35]
	WC	I-1B	30	2010, 2012	15.8	1.1–220			
Propargite	WF	I-12C	4	2009, 2011	36.7	19–60		181,300	[35]
Pyridaben	WC	I-21A	1	2012		31		236	[35]
Resmethrin	WC	I-3	5	2010, 2011	8.6	2.4–17	690	150	[35]
Rotenone	P		8	2011, 2012	21.2	1.6–82	300,000	2400	[35]
	WC	I-21B	3	2010	25.5	23–28			
Spinetoram	P	I-5	5	2010, 2011	183.9	4.4–645		7340	[22]
Spiridoclofen	P		4	2012	18.5	5.6–55	1,960,000	2,000,000	[35,37]
	WC	I-23	1	2012		12			
Thiamethoxam	P		5	2009, 2011 2012	2.8	1.1–3.8	50	240	[35]
	WC	I-4A	4	2010	1.6	1.1–2.8			
SYNERGIST									
Piperonyl butoxide	WC		16	2012	15.2	5.3–42		110,000	[35]
	WF	U	3	2011	128.9	6.6–370			

* The parent compound oral and/or contact LD_{50} was used. The parent compound is in parentheses.

PURCHASED BY DR N B SHRIDHAR (NOT FOR RESALE)

Table 3: Pesticide detections in pollen by year and pesticide: number of detections (#), and the range and mean (standard deviation (SD)) of the amount detected (in ppb). See Section 3.1, Section 3.3–Section 3.5for a summary discussion

2009			2010			2011			2012			
	#	Range	Mean (SD)	#	Range	Mean (SD)	#	Range	Mean (SD)	#	Range	Mean (SD)
FUNGICIDES												
4-Hydroxychlorotha-lonil	2	5.5–42	23.8 (25.8)	2	5.5–22	13.8 (11.7)	1	21				
Azoxystrobin	3	0.5–56	19.4 (31.7)	12	0.5–214	19.6 (61.2)	15	0.7–14	3.8 (4.0)	11	0.5–37	6.0 (11.0)
Benalaxyl				1	1.9		1	1				
Boscalid				6	1.2–2.8	1.8 (0.6)	4	1.8–5.2	3.7 (1.6)			
Carbendazim	9	0.8–13	4.2 (4.3)	16	1-14	4.0 (3.8)	13	2.1–548	64.7 (146.2)	10	1–56	17.8 (21.5)
Cymoxanil				1	7.5							
Cyprodinil							1	21				
Difenoconazole				1	4.5		1	3.9				
Dimethomorph	1	0.6					1	0.5		1	1	
Fluazinam	1	4.7										
Imazalil (Enilcon-azole)	1	1.6										

PURCHASED BY DR. N.B. SRIDHAR (NOT FOR RESALE)

Mandiproamide	1	1.2										
Metalaxyl				1	1.5					3	0.8–3.5	1.9 (1.4)
Metconazole							1	1.3				
Myclobutanil							1	1.1		1	6.1	
Octhilinone							1	24				
Propiconazole-1	2	1.2–3.9	2.6 (1.9)	7	1.1–57	16.5 (19.9)	15	1.1–12	6.7 (6.8)	8	11–64	26.1 (18.5)
Pyraclostrobin				1	12		3	1–3.9	2.1 (1.6)	8	0.8–8.8	3.3 (3.1)
Pyrimethanil				1	29							
Spiroxamine							2	1-2	1.5 (0.7)			
Tebuconazole							7	2–90	27.1 (31.4)	1	104	
Thiabendazole	1	0.8								1	1.5	
Thiophanate-methyl				1	4.6		3	10–40	21.3 (16.3)			
Trifloxystrobin	2	0.2–1.2	0.7 (0.7)	1	23		3	1.4–2	1.7 (0.3)	1	1.6	
HERBICIDES												
Acetochlor							1	22		3	1.8–17	7.2 (8.5)
Alachlor	1	1.3										

PURCHASED BY DR N B SHRIDHAR (NOT FOR RESALE)

Atrazine	21	0.1–10	1.9 (2.7)	19	0.2–41	7.4 (10.5)	21	0.2–29	5.4 (8.4)	23	0.4–58	6.8 (14.3)
Bentazon				1	3.1							
Dithiopyr	2	0.6–0.8	0.7 (0.1)	1	3							
Diuron	3	2.4–8.9	5.1 (3.4)	5	4.3–33	13.2 (11.6)	12	3.1–275	48.6 (73.9)	7	1.1–27	11.2 (10.1)
Fenuron				2	0.7–2.1	1.4 (1.0)						
Linuron				2	2.2–14.4	8.3 (8.6)	1	2				
Metolachlor	8	0.2–8.3	2.1 (2.6)	5	5.5–130	48.9 (49.2)	10	0.6–31	6.01 (9.6)	9	0.7–20	4.3 (6.7)
Metribuzin							3	0.6–2.6	1.4 (1.1)			
Metsulfuron-methyl							1	7				
Pendimethalin	13	1.2–14	5.2 (4.8)	11	1-26	9.2 (8.7)	4	2.1–4.1	2.8 (0.9)	13	1.6–55	9.0 (14.1)
Prodiamine	1	2.3										
Prometon							3	1–3.3	2.4 (1.2)			
Prometryn							1	2.2				
Propyzamide										3	1.5–6.2	3.2 (2.6)

PURCHASED BY DR. N B SHRIDHAR (NOT FOR RESALE)

Sethoxydim				1	23		2	1.7–2.5	2.1 (0.6)			
Siduron										3	5.6–13	9.1 (3.7)
Simazine	1	1.1					1	2		1	1.4	
Sulfentrazone							1	2.5				
INSECTICIDES												
Acephate										2	1.6–3.2	2.4 (1.1)
Acetamiprid				2	10–436	223.0 (301.2)	1	4.8				
Aminocarb							1	1.7				
Amitraz-Metab. DMPMF				1	2.5		1	2.5				
Azinphos-methyl	1	0.4		1	1.1		1	3.5				
Bendiocarb				3	1.2–1.9	1.5 (0.4)						
Carbaryl	3	1.5–8.3	5.1 (3.4)	13	1–6.1	2.9 (1.5)	11	1–95	14.8 (27.3)	16	2.1–21	5.5 (4.4)
Carbofuran	2	1–6.7	3.8 (4.0)	3	12-24	18.0 (6.0)	1	2				
Chlorantraniliprole				1	9.6		1	5.5				

PURCHASED BY DR N B SHRIDHAR (NOT FOR RESALE)

Chlorpyrifos	10	0.9–27	10.4 (11.1)	5	2–10.6	5.4 (3.2)	11	1.6–61	15.5 (18.7)	10	1.4–34	12.8 (11.1)
Clothianidin	1	2		1	1.2					4	1.3–4.8	2.8 (1.7)
Coumaphos	17	0.2–196	20.3 (52.6)	5	1.3–33	8.8 (13.6)	3	1.3–20	8.4 (10.1)	6	1.6–6.1	3.1 (1.7)
Coumaphos oxon										1	2.5	
Diazinon							3	1–8.5	5.1 (3.8)	1	8.6	
Dichlorvos				1	35					1	1.5	
Diflubenzuron							1	19				
Dimethoate	8	0.3–181	27.3 (62.6)	5	0.7–79	18.6 (33.8)	7	2.5–12	7.2 (3.5)	7	0.7–194	33.6 (71.1)
Fenpyroximate	2	0.2–0.6	0.4 (0.3)									
Fluvalinate	7	0.9–4.7	2.5 (1.3)	2	21–27	24.0 (4.2)	3	1.6–2.8	2.4 (0.7)			
Imidacloprid	2	1.5–2	1.8 (0.4)				4	4.4–51	17.8 (22.2)	3	1.8–4.2	2.7 (1.3)
Imidacloprid, Olefin							1	9.5				
Imidacloprid, urea							1	19				
Malathion	1	7.9		9	1.4–138	32.8 (42.0)	11	3.8–397	168.1 (160.1)	12	5.5–148	54.5 (45.6)

PURCHASED BY DR. M.B SIRSIDHAR (NOT FOR RESALE)

Methiocarb				1	2		1	1		5	1.9–20	8.2 (7.8)
Methomyl							3	2.5–26	13.2 (11.9)	3	1.4–19	7.5 (10.0)
Methoxyfenozide										4	2.2–162	45.3 (77.9)
OH-Carbofuran	5	1.2–29	15.8 (11.2)				1	1.9				
Omethoate							6	1–2.4	1.9 (0.6)	3	1-12	6.1 (5.5)
Phorate	1	22										
Phorate Sulfoxide							1	1.7–9	3.3 (3.2)			
Phosmet	4	2.4–57	20.7 (25.1)	4	2.1–483	125.1 (238.6)	5	1.6–82	32.2 (39.4)	4	1.1–26	7.8 (12.1)
Rotenone							5	1.6–82	32.2 (39.4)	3	2.3–16	10.1 (7.0)
Spinetoram				1	4.4		4	8.8–645	363.4 (311.7)			
Spiridoclofen										4	5.6–55	18.5 (23.3)
Thiamethoxam	1	2.8					1	3.1		3	1.1–3.8	2.4 (1.4)

PURCHASED BY DR N B SHRIDHAR (NOT FOR RESALE)

Table 4: Pesticides in wax from active colonies: number of detections (#) and amount detected—highest, lowest and mean (SD) from hives (1) Established Spring 2009 and Sampled Fall 2010 [one hive in Maine established in 2010, in brackets and marked by *], (2) Established in Spring 2009 and Sampled Fall 2011 (3) Established Spring 2011/ Sampled Fall 2011 and (4) Established in Spring 2011 and Sampled Fall 2012

(1) Established Spring 2009/ Sampled Fall 2010 * (n = 52)			(2) Established Spring 2009/Sampled Spring 2011 (n = 18)			(3) Established Spring 2011/ Sampled Fall 2011 (n = 7)			(4) Established Spring 2011/Sampled Fall 2012 (n = 65)			
	#	Range	Mean (SD)	#	Range	Mean (SD)	#	Range	Mean (SD)	#	Range	Mean (SD)
FUNGICIDES												
4-hydroxychlorothalonil	7	1.4–17	7.3 (5.3)									
Azoxystrobin	4	1–1.3	1.1 (0.2)	4	2.5–3.5	2.9 (0.4)				48	0.5–33	4.9 (6.8)
Boscalid	12	0.6–22	3.9 (5.9)	1	10					1	43	
Carbendazim	6	0.7–56	14.6 (21.2)	9	64–137	89.9 (48.6)	7	1.2–152	101 (49.9)	18	1.5–109	40.6 (28.7)
Diphenylamine				3	25–227	100.7 (110.1)						
Epiconazole	1	0.5										
Fluoxastrobin										1	13	
Mandiproamide	1	2.9										

PURCHASED BY DR. N B SHRIDHAR (NOT FOR RESALE)

Myclobutanil	1	4.1								1	1.7	
Octhilinone	3	12-14	13 (1)	4	1.2–4.2	2.9 (1.3)						
Propiconazole-1	13	0.8–66	2.5 (1.5) [1.6] *	4	4.8–8	6.7 (1.4)				19	6.2–27	14.4 (6.9)
Pyraclostrobin				2	1.2–2.1	1.6 (0.6)				51	1.2–47	7.8 (9.4)
Tebuconazole	2	1.3–2.8	2.1 (1.1)	7	1.9–21	10.8 (7.6)	7	1–23.1	10.8 (7.6)	16	2.7–24	6.9 (5.6)
Thiabendazole	7	0.4–92	44.6 (37.0)	1	91					1	2.8	
HERBICIDES												
Atrazine	17	0.01–6.5	1.2 (1.5)	6	0.4–2.1	1.0 (0.6)	3	0.5–1.6	1.0 (0.3)	23	0.2–5.4	1.3 (1.2)
Bentazon										19	1.3–17	5.9 (4.8)
Dithiopyr	1	1.8								3	1.7–5.1	3.0 (1.9)
Diuron	9	0.4–3.3	1.4 (0.8)	9	6.4–30	18.1 (8.4)	7	1.7–29.4	16.1 (8.4)	16	2.2–20	7.1 (5.7)
Fenuron				2	24–35	29.5 (7.8)						
Halosulfuron-methyl	3	213–386	307 (87.5)									

PURCHASED BY DR N B SHRIDHAR (NOT FOR RESALE)

Linuron	3	1–2.4	1.5 (0.8)	2	1–5.5	3.3 (3.2)						
MCPA										1	4	
Metolachlor				1	0.7					11	1–4.5	2.2 (1.1)
Napropamide				1	2.8							
Pendimethalin	18	0.4–5.9	2.1 (1.5)							10	2.8–20	6.5 (5.6)
Prometon										1	3.5	
INSECTICIDES												
Acephate	1	5.8										
Amitraz Metab DMPF	1	4.6		1	3.7							
Azinphos-methyl	8	0.2–0.3	0.3 (0.1)									
Bendiocarb	1	4.5										
Carbaryl	1	0.9										
Carbofuran	10	0.8–10.8	5.7 (4.0)									
OH-Carbofuran	15	5.4–112	28.1 (34.6)									
Chlorpyrifos	2	0.8–5.5	3.2 (3.3)	9	3.1–15	6.8 (3.6)	7	1.6–14.3	6.6 (4.2)	8	1.4–44	9.5 (14.0)

PURCHASED BY DR N B SHRIDHAR (NOT FOR RESALE)

Coumaphos	32	0.2–75	24.6 (17.8) [17] *	18	8.5–87	25.8 (19.2)	7	8.5–40.1	19.8 (11.8)	65	1.8–2060	62.8 (288.9)
Coumaphos—oxon	21	0.9–5.8	3.1 (1.6) [1.8] *	6	1-12	3.3 (4.3)				11	1.1–43	8.6 (15.1)
Diazinon				6	0.4–2	0.8 (0.6)	5	0.1–1.6	0.8 (0.7)			
Dichlorvos	2	2–2.3	2.2 (0.2)	3	2.3–13	6.2 (5.9)	1	2.3				
Dimethoate	20	1.1–945	63.9 (208.0)	2	0.6–1.9	1.3 (0.92)				25	0.8–52	6.4 (10.2)
Fenpyroximate	1	1.9		4	1–207	72.1 (97.2)	2	1–1.3	1.2 (0.2)	3	5.5–26	17.8 (10.9)
Fluvalinate	24	1–862	348.1 (223.4) [15] *	18	7.6–512	169.3 (151.3)	7	63–407	202.6 (136.1)	62	1.7–537	52.5 (94.9)
Malathion	2	35/49	42 (9.9)	6	1.2–85	26.8 (30.2)	4	11.9–31.2	18.6 (8.8)	17	4.7–91	50.5 (25.7)
Phosmet	18	1.1–220	19.0 (51.0)	1	6.5					11	1.5–32	12.7 (10.5)
Pyridaben										1	31	
Resmethrin	3	6.3–17	13.4 (6.2)	2	2.4–5.5	3.9 (2.2)						

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Rotenone	2	23–28	25.5 (3.5)									
Spiridoclofen										1	12	
Thiamethoxam	4	1.1–2.8	1.6 (0.8)									
SYNERGIST												
Piperonyl butoxide	21	5.3–53	20.52 (13.8)	4	3.5–37	18.1 (13.9)	7	7.1–22.9	11.4 (7.4)	14	1.7–35	15.7 (11.8)

Table 5: Pesticides in 2009 and 2011 Foundation Wax: number of detections, highest, lowest amount detected (ppb)

	2009 (n = 2)			2011 (n = 3)		
	Number of Detections	Amount Detected		Number of Detections	Amount Detected	
		Lowest	Highest		Lowest	Highest
FUNGICIDES						
Azoxystrobin	2	2.3	3.4	2	1.8	3.9
Boscalid	2	2.2	6.5	3	3	10
Carbendazim	1		1			
Cyprodinil	1		90	2	8.7	19
Dimethomorph						3.9
Fenbuconazole	1		2.5			
Iprodione	2	110	125	1		165
Metalaxzyl				1		1.5
Octhilinone				2	3.1	6.8
Propiconazole-1	1		2.6			

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Trifloxystrobin	2	1.3	2.4			
HERBICIDES						
Pendimethalin	2	11	17	2	17	17
Prodiamine	2	13.4	17			
Prometon				1		0.5
Prometryn	1		0.5			
INSECTICIDES						
Amitraz Metab DMPF	2	510	670	3	16.5	567
Chlorpyrifos	1		17	3	15	28
Coumaphos	2	1584	2500	3	109	1992
Coumaphos oxon	2	48	47	3	6.3	63
Diazinon	1		0.3			
Dichlorvos				1		10
Diflubenzuron				1		3.8
Fenpyroximate	2	4.1	5.5	2	18	366
Fluvalinate	2	7851	15,400	3	290	8718
Propargite	2	31	60	2	19	20
SYNERGIST						
Piperonyl butoxide				3	6.6	370

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Pollen and Wax Pesticide Residues—General Findings

Viewed as a whole, 716 compound (pesticides, metabolites and/or synergists) detections occurred in the 168 pooled pollen samples and 993 detections occurred in the 142 wax comb samples. These 1709 detections were of 91 different compounds. Of the 91 compounds detected, 42% (38) were insecticides, 32% (29) were fungicides, 25% (23) were herbicides, and one was a synergist.

Of these 91 different compounds detected, 79 pesticides and metabolites were detected in the pollen samples, while 58 pesticides, metabolites and/or synergists were detected in wax.

Also, of the 91 compounds detected, 32 were found only in pollen, 12 were found only in wax, and 47 were found in both wax and pollen. If a pesticide or metabolite was found only in one matrix (pollen or wax), the pesticide or metabolite was significantly more likely to be found only in pollen rather than in wax ($\chi^2_{(1)} = 11.5$; $p < 0.001$).

Table 2, Table 3, Table 4 and Table 5 show all detected compounds, the matrix (pollen, wax comb, or foundation wax) in which the detections were made, and how many detections were made of each compound in each matrix. Table 2 organizes this information by pesticide and includes each pesticide's oral and contact LD_{50}, matrix and years of detection, mean concentration and range and mode of action (MOA) [40,41,42]. Table 3organizes the residues in pollen by year. Table 4 organizes the wax comb residues by year and Table 5 describes the foundation wax residues.

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Predictive Power of Pollen Samples from a Single Colony to an Apiary

Twenty pesticides were detected in the 20 unpooled pollen samples obtained from the five WA colonies. The mean (SD) number of residues detected per unpooled pollen sample was 6 (1.7); no sample had fewer than three detections. Dimethoate was detected in all samples. Acephate, carbaryl, chlorpyrifos, dithiopyr, epiconazole, and fluvalinate were each detected in a single sample.

The predictive power, defined in Section 2.4.2, of a pollen sample from one colony to indicate the presence of a pesticide in other colonies in the same apiary was 2.75: i.e., the presence (absence) of a pesticide in a pooled sample was a good predictor of the presence (absence) of the compound throughout the apiary.

Fungicides in Pollen, Wax Comb and Foundation Wax

Of the 1709 compound detections in pollen and wax, the number of fungicide and their metabolites detections in pollen, wax comb, and wax foundation were 192, 262, 24, respectively. These fungicide detections were of 29 different fungicides. Of these 29 fungicides, 24 (83% of all fungicides found) were found in pollen and 20 (69%) were found in wax. Also of these 29 fungicides, 9 (31%) were found only in pollen samples, while five (17%) were found only in the wax samples.

Table 3 summarizes the assay results for pesticides in pollen. Focusing on the 24 fungicides detected in the pollen samples, nine (38%) were detected in only a single year, while four (17%) were detected in all years. The fungicides carbendazim, azoxystrobin, and propiconazole-1 were the most frequently detected compounds in pollen: 48 (28% of the 168 samples), 41 (24%), and 32 (19%), respectively. The greatest number of fungicides detected in any one year was in 2011, when 17 different fungicides were detected. Also in 2011, the highest concentration of any single fungicide, carbendazim (548 ppb), was observed.

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Table 4 summarizes the assay results for pesticides in wax comb samples. Focusing on the 14 fungicides detected in the wax comb samples, five (36%) were detected in only a single year while two (14%) were detected in all four years. The fungicides azoxystrobin and pyraclostrobin were the most frequently detected compounds in wax: 56 (39% of the 142 wax comb samples) and 53 (37%), respectively. The greatest number of fungicides detect in any one year was in fall 2010, when 11 different fungicides were detected. The highest concentration of any single fungicide in wax was 137 ppb of carbendazim in the (established Spring 2009)/(sampled Fall 2011) samples of drawn comb.

Table 5 summarized the results for pesticides in the wax used to coat the plastic foundation. Focusing on the 11 fungicides detected in the foundation wax, seven (64%) were detected only once and one fungicide, boscalid, was detected all five samples. Iprodione was found in the highest concentration—165 ppb.

Herbicides in Pollen, Wax Comb and Foundation Wax

In the 168 pollen samples, 142 wax comb samples, and 5 wax foundation samples, there were a total of 220, 166, and 8 detections of 23 different herbicides, respectively. Of the 23 detected herbicides, 20 herbicides were detected in pollen samples and 14 were detected in wax (comb and

foundation) samples. Also of the 23 detected herbicides, 11 were found in both pollen and wax, nine (39%) were found only in pollen, and three (13%) were found only in wax.

Focusing on the herbicides detected in the pollen samples, 12 herbicides (60% of 20 herbicides detected in pollen) were detected only in a single year while four (20%) were detected in all 4 years. Atrazine was detected most frequently: it was found in 84 (50%) of the 168 pooled pollen samples. In pollen, the highest concentration of a single herbicide was of diuron (275 ppb) in 2011 (Table 3).

In comb wax, six herbicides (50% of 12 herbicides detect in wax comb) were detected only in a single year, while two, atrazine and diuron, were detected in all years and these two herbicides were the most frequently detected in the wax samples: atrazine was found in 49 (34%) of the 142 samples, and diuron in 41 (29%). In wax, the highest concentration of a single herbicide was of halosulfuron-mythyl (386 ppb) in 2010 (Table 4).

In foundation wax, only four herbicides were detected (Table 5). The herbicides at highest concentrations were pendimethalin and prodiamine (17 ppb).

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Insecticides in Pollen, Wax Comb, and Foundation Wax

In the 168 pollen samples, 142 wax comb samples, and 5 foundation wax samples there were a total of 304, 481, and 38 detections of 38 different insecticides, respectively. Of the 38 detected insecticides, 35 (92%) were detected in pollen samples and 24 (63%) in wax (comb and foundation) samples. Also, of the 38 detected insecticides, 21 (55%) were detected in both pollen and wax, 14 (37%) were found only in pollen, two (5%) only in wax comb and one (3%) only in wax foundation (Table 2, Table 4 and Table 5). The synergist piperonyl butoxide was detected only in wax samples: 56 times in wax comb and 3 times in foundation wax.

Pollen sample assays detected 35 different insecticides. Of these, 12 (34% of insecticides detected in pollen) were detected only in a single year while six (17%) were detected in all 4 years. Carbaryl was most frequently detected insecticide: it was found in 26% of the 168 pooled pollen samples. Imidacloprid or its metabolites were detected only 11 times (6% of the 168 samples) and it was not detected at all in 2010. In pollen, the highest concentration of a single insecticide was of spinetoram (645 ppb) in 2011 (Table 3). In comb wax, 22 different insecticides were detected (Table 4). Of these, 10 insecticides (45% of insecticides detected in wax) were

detected only in a single year while four (18%) were detected in all years. Even though no miticides were used in the colonies in this study, coumaphos was the most frequently detected insecticide/miticide: it was found in 122 (86%) of the 142 wax comb samples and 100% of the five foundation wax samples. Fluvalinate, another insecticide/miticide approved for use in honey bee colonies but never used in this study, was the second most detected insecticide: it was found in 111 (78%) of the 142 wax comb samples (Table 4) and 100% of the five foundation wax samples (Table 5). A metabolite of amitraz, another miticide used to control Varroa mites, also was found in 100% of the wax foundation samples.

Annual Pattern of Residues

In all years, more types of insecticides were observed in pollen and wax than were types of fungicides or herbicides: 38 (42%), 29 (32%), and 23 (25%), respectively (see Table 2). In pollen, the greatest number of detections (223) and the greatest number of compounds (56) occurred in 2011. Also, in pollen, the smallest number of detections (138) and compounds (33) were found in 2009 (Table 3). In wax comb, the greatest number of detections (458 detections in 65 samples (7 detections/sample)) occurred in fall 2012 and the greatest number of compounds (37) were found in fall 2010. However, the greatest number of detections per wax comb sample occurred in fall 2012: 64 detections in 7 samples (9.1 detections/sample) (Table 4).

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Annual Pattern of Residues within Pollen Samples

The largest number of residues detected in a single pooled pollen sample was 10, 9, 13, and 12 compounds in 2009, 2010, 2011, and 2012, respectively. No detectable residues were found in eight of the 168 pooled samples: one from Maine in 2009, two each from ME and FL in 2011, and three from PA in 2012. The maximum number of compounds found in a single pollen sample was 13 (WA, 2011). The overall mean (SD) number of detections per sample was 4.1 (2.7). Table 6 gives the mean (SD) number of detections per pollen sample broken down by year. The mean (SD) number of detections per pooled pollen sample varied by location from a low of 1.1 (1.1) in ME in 2011 to a high of 8.7 (2.1) in TX in 2012.

Table 6: Mean number of distinct residue detections per pollen sample by year, aggregated across all locations

Year	Mean # Detections/(Pooled Sample)	Standard Deviation
2009	3.6	2.20
2010	3.6	2.06
2011	4.4	3.06
2012	4.7	3.20

Table 7 presents the three most common pesticides found in pollen samples for each year of the study (summarized from Table 3). Over the four years of the study, the herbicide atrazine was the most commonly detected pesticide; it was also the most frequently detected pesticide in each individual study year. Carbaryl, an insecticide, was among the three most frequently detected pesticides detected in years 2010 and 2012. It was also the third most commonly detected pesticide over the four study years. The fungicide carbendazim made the "top three" most frequently detected pesticides in only one study year (2011); however, it was the second most commonly detected pesticide over the four study years.

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Table 7: Most common pesticides detected in pollen samples broken down by year. See Section 3.6.1 for discussion. Pesticides are labeled (F) for fungicides, (H) for herbicides, and (I) for insecticides

Year	# Pollen Samples	Most Common Pesticides	
		Pesticide	# Sample Detections
2009	34	Atrazine (H)	21 (62%)
		Coumaphos (I)	17 (50%)
		Chlorpyrifos (I)	10 (29%)
2010	41	Atrazine (H)	19 (46%)
		Carbendazim (F)	16 (39%)
		Carbaryl (I)	13 (32%)
2011	51	Atrazine (H)	21 (41%)
		Azoxystrobin (F)	15 (29%)
		Propiconazole (F)	15 (29%)
2012	42	Atrazine (H)	23 (55%)
		Carbaryl (I)	16 (38%)
		Pendimethalin (H)	13 (31%)

Most pollen residues tended to be episodic—occurring in some but not all locations and in some but not all months or years. Thus, predicting the presence of a residue based upon prior detection was not possible. For example, imidacloprid was detected in PA in July and August of 2009, MN in August and September of 2011, TX in July and MN in August of 2012 but in no other locations or months/years. Tebuconazole was not detected in any of the six locations in 2009 and 2010. In 2011, it was detected once in TX in August, twice in WA in June and August and in 2012 it was found only in TX in March. Propiconazole in 2009 was observed in WA in two non-sequential months—June and August—but then was found in 2010 through 2012 from May–September. In ME, FL and MN propiconazole was only episodic. It was detected in ME in three months—May 2010 and June and July 2011 while in FL and MN the only detection was in November 2010 and August 2011, respectively. In MN and FL dimethoate was detected only in June 2009 and it was never detected in PA or ME.

While most pollen residues were episodic, in some locations a few residues occurred more continuously. Dimethoate was found in WA from May/June through September in all four years. Azoxystrobin was detected in WA in June and July 2009 and from May/June through September 2010, 2011, and 2012, and in MN from July through September 2011 and 2012. Atrazine was found in all locations but somewhat continuous only in MN, PA, and WA. It was detected in MN from May/June through September 2010, 2011 and 2012, in PA from June through August 2012, and in TX from May through December 2009, February through July 2010 and in March through July 2012.

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Annual Pattern of Wax Comb and Wax Foundation Residues

Table 4 summarizes detections in wax comb taken from active colonies, while Table 5 summarizes detections in foundation wax. A total of 58 different compounds were detected in these samples. More compounds were detected in drawn wax comb (49) than in wax-coated foundation (27). Not all compound detections in wax comb and wax foundation were the same. Thirty-three compounds were found only in wax comb and eight were found only in the foundation wax. Fifteen compounds (five fungicides, two herbicides, and eight insecticides) were found in both comb and foundation wax.

In drawn wax comb, there was a mean (SD) of 7.1 (1.7) compounds per sample, and a total of 49 compounds detected. Of the 49 detected compounds,

14 were fungicides, 22 were insecticides, 12 were herbicides, and one was a synergist. The greatest number of fungicides (11 of 14) and insecticides (19 of 22) detected in any single year were detected in the fall 2010 wax comb samples, while the greatest number of herbicides (8 of 12) detected in any one year was in the fall 2012 wax comb samples. The maximum number of residues per wax comb sample varied by location from three in FL to ten in TX.

When colonies were established in 2011, the wax used to coat the plastic foundation was from two different batches (lot numbers) of wax (see Section 2.1). Therefore, foundation wax samples from PA 2009 (purchased and sampled in 2009), PA 2011 (purchased in 2009 but sampled in 2011) and ME 2011 (purchased and sampled in 2011) were compared. There was a weak but positive association in contaminants between the samples from the same manufacturer but different batches; the PA 2009 and PA 2011 (same batch) and ME (different batch, same manufacturer); $r = +0.433$, $p = 0.0273$ and $r = +0.326$, $p = 0.105$; PA 2009 vs. ME 2011 and PA 2011 vs. ME 2011, respectively. There was a high correlation between the PA 2009 and 2011 foundation wax samples ($r = +0.948$, $p < 0.001$); they were from the same manufacturer and batch.

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The foundation used to initiate the colonies in 2009, 2010, and 2011, was found to have 26 pesticides or metabolites with a mean (SD) of 14.2 (4.2) detections per sample. Of the 26 compounds detected in the foundation wax, 11 were fungicides, four were herbicides, 10 were insecticides, and one was a synergist. The number of detections per sample ranged from nine to 19. Two metabolites (amitraz DMPF and coumaphos oxon), two insecticides (fluvalinate and coumaphos), and one fungicide (boscalid) were found in all of the wax foundation samples. One herbicide (pendimethalin), one fungicide (azoxystrobin), and three insecticides (chlorpyrifos, fenproximate, and propargite) were found in 80% of wax foundation samples.

There were 16 contaminants found in common to both the wax comb and foundation wax (five fungicides, two herbicides, and eight insecticides/miticides, and one synergist).

In contrast to the pollen sampling, wax sampling was less frequent and not at consistent intervals (see Section 2.2.2). Whether the data were log transformed or not, there was no evidence of a correlation between the frequency of detection of insecticides in wax comb and foundation wax for compounds that were found in both in either 2009 or 2011 ($p > 0.10$). When data were pooled over years there was a weak correlation when the

data were transformed (log ppb) between concentrations of pesticides in foundation wax and wax comb ($r = +0.448$, $p = 0.054$).

The contaminants in the wax foundation used to initiate the hives in 2009 was not correlated with the mean monthly, maximum, or total seasonal contaminants found in pollen brought into hives in 2009 for any of the six apiaries in the 2009 trial. Only three of the apiaries in 2009 shared some of the top five most abundant contaminants in pollen with those most abundant compounds in the foundation wax (fluvalinate in two apiaries and coumaphos in three apiaries).

Comparison of Wax Comb Residues from Living vs. Dead Colonies

Comparison of wax comb sampled from both living and dead colonies in the same apiary on the same date revealed that pesticide detection number did not differ ($p > 0.10$); however, the total pesticide concentration (sqrt ppb) showed a possible trend of lower total concentration in wax combs from living colonies than from wax comb from dead colonies ($F_{(1,6)} = 5.3566$, $p = 0.06$).

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Lethal Dose Levels of Residues in Pollen and Wax Comb

Of the parent pesticides and metabolites for which both honey bee oral and contact LD_{50} values were available (51 compounds), 12 (24% of the 91 detected compounds) had the same LD_{50} values for the contact and oral mode of entry, 18 (35%) had lower oral than contact LD_{50} values (oral exposure was more toxic than contact exposure) and 21 (41%) had lower contact than oral LD_{50} values (contact exposure was more toxic than oral exposure). There is no evidence, based upon the oral versus contact LD_{50} values for the detected compounds, to suggest that, independent of quantity of pesticide used, oral exposure to a pesticide of a honey bee is more lethal than contact exposure ($\chi^2_{(2)} = 2.0799$; $p = 0.35$).

While some pesticides were frequently detected, most never were detected at a level that was lethal to honey bees. For example, the herbicide atrazine, was never detected at a level higher than 10^{-6} of its contact LD_{50}. Likewise, the most frequently detected fungicide in pooled pollen was carbendazim, which was never detected higher than 0.1% of its contact LD_{50}. Among the compounds detected in pollen, the greatest acute risks were posed by insecticides: while imidacloprid and its metabolites were infrequently detected in the pooled pollen samples (11 of 168 samples), in

one sample imidacloprid was measured at 137% of its oral LD_{50}. Another insecticide, dimethoate, was detected at near 35% of its oral LD_{50}. The largest measured residue of two other insecticides, phosmet, and pyridaben, were 13% of their respective LD_{50}s.

The herbicide, fungicide and insecticide residues in comb wax posed a lower acute risk than pollen. In wax comb, the highest concentration of a single fungicide was diphenylamine at 227 ppb, for which no oral or contact LD_{50} has been determined. No fungicide was detected in wax comb at a level greater than 0.2% of its LD_{50}; no herbicide was found in wax comb at a level greater than 0.01% of its LD_{50}. In wax comb the greatest risk was, again, for insecticide exposure: in 2010, 2011, and 2012, the maximum fluvalinate concentrations were 862 ppb, 512 ppb, and 537 ppb, respectively, which correspond to 43%, 25%, and 26% of fluvalinate's 2000 ppb contact LD_{50}.

In foundation wax the most acute risk was posed by the insecticide fluvalinate, which was measured at 7.7 times its LD_{50}. Amitraz, because of its ability to interact with fluvalinate and coumaphos [8], likely also posed a significant risk (a metabolite of amitraz was detected at 582.3 ppb); however, its LD_{50}, acting alone or in combination with fluvalinate and coumaphos, is unknown. Of the remaining pesticides, the insecticide phosmet, posed a low—though not negligible—risk with its largest measured residue level being 5% of its LD_{50}. The variation among replicate samples of foundation, estimated by the coefficient of variation when including all 26 contaminants, was 105.6%. This level indicates the vast majority of the 21 contaminants have low mean concentrations.

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Modes of Action Distributions in Pollen, Wax Comb, and Wax Foundation

Different pesticides may act on target organisms in similar or in different ways. Pesticides that act in the same way are said to be of the same class; pesticides that act in different ways are of different classes. The different classes of pesticides detected in pollen, wax comb, or foundation wax, are summarized in Table 8. Whether in pollen, wax comb, or foundation wax, a greater number of classes of insecticides (14) were observed than of fungicides (11) or herbicides (8). The fungicides that were detected ranged from benalaxyl, an inhibitor of nucleic acid synthesis to chlorothalonil, a multi-site activity fungicide. Herbicides detected varied from atrazine, an inhibitor of photosynthesis, to sethoxydim, an inhibitor of acetyl CoA carboxylase. Insecticides detected varied from spiriclofen, an inhibitor of

acetyl CoA carboxylase (as is the herbicide sethoxydim) to imidacloprid, a nicotinic acetylcholine receptor agonist. Among the fungicides, the largest number of compounds (9) was found in class G (MOA: sterol biosynthesis in membranes). The herbicide group with the greatest number of compounds (10) was class C (MOA: inhibition of photosynthesis at photosystem II). Insecticide class 1B contained the greatest number (11) of compounds (MOA: acetylcholinesterase inhibition).

Table 8: Pesticide MOA for compounds detected in pollen and wax

CLASS	MODE OF ACTION	PESTICIDES DETECTED
Fungicides		
A1	Nucleic acid synthesis: RNA polymerase I	benalaxyl, metalaxyl
A3	Nucleic acid synthesis: DNA/RNA synthesis (proposed)	octhilinone
B1	Mitosis and cell division: β-tubuline assembly in mitosis	carbendazim, thiabendazole, thiophanate-methy
C2	Respiration: Complex II: succinate-dehydrogenase	boscalid
C3	Respiration: Complex III: cytochrome bc1	azoxystrobin, fluoxastrobin, pyraclostrobin, trifloxystrobin,
C5	Respiration: Oxidative phosphorylation uncoupler	fluazinam
D1	Amino acid and protein synthesis: Methionine biosynthesis (proposed)	cyprodinil, pyrimethanil
E	MAP/Histidine-kinase in osmotic signal transduction	iprodione
G1	Sterol biosynthesis in membranes: C14-demthylase in sterol biosynthesis	difenoconazole, epoxiconazole, fenbuconazole, imazali, metconazole, myclobutanil, propiconazole-1, tebuconazole
G2	Sterol biosynthesis in membranes: Δ^{14}-reductase and Δ^{8}-Δ^{7}-isomerase in sterol biosynthesis	spiroxamine
H5	Cell wall biosynthesis: Cellulose synthase	dimethomorph, mandipropamid

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Multi-site	Multi-site contact activity	chlorothalonil
Unknown	Mode of action is unknown	cymoxanil, diphenylamine
Herbicides		
A	Inhibition of acetyl CoA carboxylase	sethoxydim
B	Inhibition of acetolactate synthase ALS	halosulfuron-methyl, metsulfuron-methyl
C1-C3	Inhibition of photosynthesis at photosystem II	C1: atrazine, metribuzin, prometon, prometryn, simazine C2: diruon, fenuron, linuron, siduron C3: bentazon
E	Inhibition of protoporphyrinogen oxidase	sulfentrazone
K1	Mitosis inhibition: Microtubule assembly inhibition	dithiopyr, pendimethalin, prodiamine, propyzamide
K3	Mitosis inhibition: Inhibition of very long chain fatty acid synthesis (inhibition of cell division)	acetochlor, alachlor, metolachlor, napropamide
O	Synthetic auxins	MCPA
Insecticides		
1A	Nerve action: Reversible acetylcholinesterase inhibition	aminocarb, bendiocarb, carbaryl, carbofuran, methiocarb, methomyl
1B	Nerve action: Irreversible acetylcholinesterase inhibition	acephate, azinphos-methyl, chlorpyrifos, coumaphos, diazinon, dichlorvos, dimethoate, malathion, omethoate, phorate, phosmet
3	Nerve action: Sodium channel modulator	fluvalinate, resmethrin
4A	Nerve action: Nicotinic acetylcholine receptor agonist	acetamiprid, clothianidin, imidacloprid, thiamethoxam
5	Nerve action: Nicotinic acetylcholine receptor allosteric activator	spinetoram
12C	Inhibitor of mitochondrial ATP synthase	propargite
15	Inhibition of chitin biosynthesis, type 0	diflubenzuron

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18	Growth regulation: Ecdysone receptor agonist	methoxyfenozide
19	Nerve action: Octopamine receptor agonist	amitraz
21	Energy metabolism: Mitochondrial complex I electron transport inhibition	A: fenpyroximate, pyridaben B: rotenone
23	Lipid synthesis and growth regulation: Acetyl CoA carboxylase inhibition	spiriclofen
28	Nerve and muscle action: Ryanodine (calcium) receptor modulator	chlorantraniliprole
Unknown	Piperonyl butoxide—synergist, Cytochrome P450-dependent monoxygenase inhibitor	piperonyl butoxide

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In pollen, the most frequently detected pesticide was the herbicide atrazine, a photosynthesis inhibitor. Of the insecticides detected in pollen, acetylcholinesterase inhibitors were detected in more samples than were any other class of insecticides. Among the fungicides, three compounds with different modes of action (B1, C2 and G1: see Table 5) occurred most frequently. If aggregated, the acetylcholinesterase inhibitors (239 detections) were detected more frequently in pollen than were the photosynthesis inhibitors such as atrazine (127 detections). If we suppose that each acetylcholinesterase inhibitor insecticide acts independently, then we might reasonably estimate the cumulative risk to this class of insecticides, in the "LD_{50}" sense, as the sum of the ratios of each compound's measured residue to its own LD_{50}. If all acetylcholinesterase inhibitor insecticides exposure occurred simultaneously at the maximum detected level, then the total exposure for this class of insecticide would be 130% of the "net" acetylcholinesterase inhibitor LD_{50}.

In wax comb and foundation wax, a greater number of classes of insecticides (10) were observed than classes of fungicides (9) and herbicides (7). Some modes of action were more commonly observed than others: the most frequently detected modes of action were the irreversible and reversible acetylcholinesterase inhibitors (358 of 559 insecticide detections, or 64%), followed by photosynthesis inhibitors such as atrazine (119 of 173 herbicide detections, or 69%), followed by respiration inhibitors such as azoxystrobin

(131 of 275, or 48%, of fungicide detections) [40,41,42].

Potential Residue Interactions—Multiple Compounds

Linear correlations (co-occurrence) of pesticides in pollen were evaluated for pesticides of the same class, and between different pesticide classes. All correlations with individual $p < 0.05$ are reported in Table 9. Likely co-occurrences in pollen were observed between class B1 fungicides and C2 herbicides ($r = 0.9660$; $p < 0.0001$), between class C3 fungicides and class A herbicides ($r = 0.9561$; $p < 0.0001$), between class D1 fungicides and class C1 herbicides ($r = 0.9955$; $p < 0.0001$), between class C2 fungicides and class 28 insecticides ($r = 0.9910$; $p < 0.0001$), and between class A herbicides and class 28 insecticides ($r = 0.9962$; $p < 0.0001$).

Table 9: Bivariate association (co-occurrence) among different pesticide MOA occurring in pollen

PESTICIDE INTERACTION	ASSOCIATION		R [1]	*p*
	MOA CLASS	MOA CLASS		
Fungicide & Fungicide	Fungicide	Fungicide		
	A1	H5	0.4802	0.0188
	B1	G2	0.8986	<0.0001
	B1	Multiple targets	0.4514 *	0.0266
	C3	G1	0.4362	0.0332
Herbicide & Herbicide	Herbicide	Herbicide		
	C1	K1	0.7488	<0.0001
Insecticide & Insecticide	Insecticide	Insecticide		
	A1	3	0.6645	0.0004
	A1	19	0.4041	0.0507
	3	19	0.6575	0.0005
	4A	21B	0.5302	0.0077
	21B	28	0.7170	<0.0001
Fungicide & Herbicide	Fungicide	Herbicide		

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	A1	C1	0.7754	<0.0001
	A1	K1	0.4523 *	0.0265
	B1	C2	0.9660	<0.0001
	C2	A	0.6728 *	0.0003
	C3	A	0.9561	<0.0001
	G1	A	0.4387 *	0.0320
	D1	C1	0.9955	<0.0001
	G1	C2	−0.4366 *	0.0338
Fungicide & Insecticide	Fungicide	Insecticide		
	A1	1A	0.4817	0.0171
	A1	4A	0.4054 *	0.0494
	C2	21B	0.7289	<0.0001
	C2	28	0.9910	<0.0001
	C3	28	0.4311 *	0.0354
	G1	28	0.4328	0.0346
	C3	1B	0.4255 *	0.0382
	D1	1B	0.4153 *	0.0436
	G2	1B	0.7726	<0.0001
	G2	4A	0.5213	0.0090
	H5	1A	0.4794 *	0.0178
Herbicide & Insecticide	Herbicide	Insecticide		
	A	28	0.9962 *	<0.0001
	C1	4A	0.6761 *	0.0003
	C2	1B	0.8857	<0.0001
	K1	1A	0.6629	0.0004
	K1	1B	0.5345 *	0.0071
	K1	3	0.7512	<0.0001
	K1	19	0.4628	0.0228

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DISCUSSION

This is the only honey bee study to date to use a prospective study design sampling the same colony from the time of its establishment until its death. The colonies, once established, were not moved; thus the samples can be used to describe the temporal changes in pesticide residues in bee forage.

Differences in the number of compounds detected among Mullin et al. [22] and Stoner and Eitzer [25] and this study may be due to differences in the sampling protocols (same colonies sample throughout the foraging season and over multiple years versus single sample per colony), matrices sampled (pollen only versus pollen/bee bread and wax versus pollen and wax), time of year when sampling occurred, migratory versus stationary bee management, in-hive pest management methods, land use differences, length of time wax is in the hive, or the use of wax-coated plastic foundation or pre-drawn wax comb with pesticide residues. To fully characterize the exposure of honey bees to pesticides it is important to sample multiple colonies at multiple locations, with dissimilar management strategies, various colony longevities and different study methodologies.

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A total of 91 pesticides, metabolites, and synergists from 335 pooled and individual pollen and comb and foundation wax samples, collected over four years, were detected in this study. Mullin et al. [22] detected 121 compounds in wax and pollen/bee bread while Stoner and Eitzer [25] detected 70 parent compounds and metabolites in pollen. A higher proportion (72% of 121) of pesticides in wax was reported by Mullin et al. [22] than in this study (48% of 91). It is not surprising that fewer compounds (44) were detected in 1 to 2-year-old wax from this study while a greater number of compounds (85) were reported by Mullin et al. [22] where some wax samples where older than 2 years. In this study atrazine, carbendazim, and carbaryl were the most frequently detected in pollen (48.6%, 27.7% and 25%, respectively). Mullin et al. [22] reported fluvalinate, coumaphos and chlorpyrifos (88.3%, 75.1%, and 43.7%, respectively) were the most frequently detected residues in pollen/bee bread and Stoner and Eitzer [27] reported coumaphos, carbaryl, and phosmet were most frequently detected residues in pollen (46.6%, 40.6%, and 32.9%, respectively).

The frequency of residue detection in pollen was similar between Stoner and Eitzer [25] and our study; <50% of the pollen samples were positive for at least one pesticide residue. This differed substantially from Mullin et al. [22], who reported 88.3% of the pollen samples positive for at least one pesticide residue. Differences in the types of compounds found

in pollen and number of samples positive for residues may be due to an artifact of data reporting. Mullin et al. [22] reported pollen and beebread residues as a single category while our data and Stoner and Eitzer [25] are pollen samples collected from foragers prior to their entry into the hive. In-hive contamination of the beebread resulting from the use of amitraz, fluvalinate or coumaphos in the management of *Varroa* may explain the difference in the frequency of detection. The source of coumaphos found in the pollen analyzed in our study or by Stoner and Eitzer [25] is unknown. The pollen was never inside the colony, thus eliminating contamination by in-hive use of coumaphos, fluvalinate or amitraz. At this time there are no approved crop uses of coumaphos (the only approved use other than in honey bee management of *V. destructor* is as a cattle dip) unlike all the other compounds detected. As none of the pollen collected in this study or by Stoner and Eitzer [25] was ever in the colony, the source of coumaphos is of great interest. Possibly the source of coumaphos (and possibly fluvalinate and the amitraz metabolite) in the pollen in both our study and Stoner and Eitzer [25] could be due to residue transfer from the surface of the foragers' body to the pollen. The source of surface contamination of the forager's body could be bees from a package source where coumaphos was used, contamination picked up from wax residues in the colony or contamination of bee forage from unknown sources. Another potential source is the wax-coated foundation but a number of pesticides residues present in the wax-coated foundations samples were never detected in the drawn comb wax. A third possibility is that some coumaphos- or fluvalinate-contaminated pollen could have been obtained by bees stealing from honey bee colonies not a part of this project but located within flying distance to our apiaries. The remaining pesticides detected in pollen likely originated from pollen contamination from crop application.

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Generally, insecticides were present more frequently and at higher concentrations than fungicides or herbicides. It would be interesting to explore the possible reasons for this result. One possibility would be to investigate potential correlations between prevalence of different types of pesticides to high versus low humidity conditions, differences in nighttime temperatures or the length of the growing season. Other possible explanations for the differences insecticide versus herbicide and fungicide detection that could be explored include similarities and differences in the chemical characteristics among insecticides, herbicides and fungicides, the types crops grown, regional differences in recommendations by Extension or pesticide company representatives and differences in the research emphasis

by land grant universities. The pattern of pollen and wax residues differed substantially by month and year. The number of detections was highest in 2011 and lowest in 2009. A compound may have been detected only once across all locations and years, at one or all locations but only once in a foraging season, multiple times (months) within one foraging season but at only one location or, at multiple locations or multiple years at multiple locations and multiple times (months) per foraging season. The differences by month, year and location are likely due to differences in target organism and the types of pesticides applied by growers, homeowners, and others to honey bee forage. Different pesticides may be used by growers because different crops have been planted, to address the occurrence of different pests or to reduce the development of pesticide resistance by pests. Differences among the apiaries may be a result of the surrounding land use or differences in climate. Two of the three apiaries surrounded by the highest concentration of agricultural land, TX and WA, tended to have the greatest number of residues and the most continuous exposure to the same compounds. Surprisingly, FL with its high humidity and long growing season had consistently fewer and more episodic detections than TX and WA.

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Substantial variation in the quantity of residue detected in wax was found. Without information on the history of a wax sample, it is difficult to determine the reasons for the large variation in the quantity and types of pesticides detected. All the comb wax samples in this study were from stationary hives less than two years old, managed without the use of miticides or other pesticides and with a variety of environments surrounding the apiaries. Mullin et al. [22] obtained their samples from a variety of sources including colonies managed by small beekeepers, migratory beekeepers and commercial sources. Martel et al. [27] used commercially available wax. It is likely that the wax from these two studies included some old (>2 years) wax samples, samples from hives where beekeeper management likely included both registered and unregistered miticides, and wide-ranging surrounding environments. More studies with detailed information concerning the conditions experienced by the wax samples are needed to arrive at a clearer understanding of the presence and absence of compounds when studies are compared.

Interestingly, the type and the quantity of pesticides found in the same batch of wax used for coating foundation wax were not consistent. These data show that a better understanding of the dispersion of pesticide contaminants in wax is needed to explain the variation in types and quantities of compounds observed from the same batch of wax. Two likely reasons

for the variation are the uneven distribution of residues in wax and the degradation of residues over time. Not knowing the causes of the variation nor the degree to which each cause influences the variation limit our ability to predict honey bee exposure.

Fewer studies of honey bee oral exposure to pesticides have been conducted than contact exposure. Of the 91 compounds detected in this study, two (2.2%) had neither an oral nor a contact LD_{50}, two (2.2%) lacked a contact LD_{50} and 35 (38.5%) lacked an oral LD_{50} (Table 2). If the oral and contact LD_{50} for the remaining 52 compounds are compared, excluding the eight compounds with identical oral and contact LD_{50}, the same number of compounds posed a greater oral exposure risk (lower oral LD_{50}) as compounds posing a greater contact risk (lower contact LD_{50}) (Table 2). Thus, the lack of oral LD_{50} studies may be less of a problem than expected.

Increased attention is needed on the MOA of various pesticides. Currently, the data on pesticide modes of action focus on the pests for which the pesticide has been developed and on resistance management for the target organism. Thus, important the modes of action for honey bees and other beneficial or non-target insects may be missed. An example is the herbicide atrazine with plants as the target organism; its published MOA is photosynthesis inhibition [41]. Atrazine has been studied in several non-target organisms including honey bees where the carotenoid-retinoid system is altered at exposure levels observed in agricultural systems. In rats, fish and quail the endocrine system is altered [43,44,45]. Thus, additional research is needed on pesticide modes of action for honey bee, other pollinators and non-target organisms. An additional area requiring research is, at minimum, to determine methods for summing exposures within the same MOA and how multiple modes of action may interact with one another.

Though the number of potential pesticide interactions and the varied types of target and non-target organisms is daunting, studies have been conducted on the combined impact from simultaneous and sequential exposure to pesticides [8,11,46,47,48,49,50,51,52,53,54,55,56,57]. Our study provides likely real-world examples of multi-residue exposure for future research. Because multiple residue exposure can result from the presence of more than one pesticide within a single pollen sample and from the all the residues in all pollen within a colony, it is important to gather data on pesticide residues in pollen and other hive products over multiple years and from many locations. One group of compounds found in our study to frequently co-occur was the reversible and irreversible acetylcholinesterase inhibitors and three of

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the miticides (amitraz, coumaphos, and fluvalinate) used for *Varroa*control. Other multiple exposures of interest, based upon pesticides detected in this study, include the possibility interaction of fluvalinate with resmethrin, the only other insecticide detected with the same MOA as fluvalinate, and the interaction of coumaphos with 10 other insecticides with the same MOA—acephate, azinphos-methyl, chlorpyrifos, diazinon, dichlorvos, dimethoate, malathion, omethoate, phorate, and phosmet.

Most efforts to understand pesticide interactions have focused on developing predictive models, not studies of specific compounds [49,50,51]. Due to the sheer number of potential combinations of pesticides, predictive models are a logical method to evaluate potential risk. Unfortunately, at least for our understanding of the risks to honey bees, the models have been built using common laboratory animal and plant model organisms, i.e., *Daphnia magna*, *Vibrio fischeri*, and *Stellaria media* [50]. Thus, the models are of little use to those attempting to predict real-world impacts of multiple pesticide exposure on honey bees.

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Most of the pesticides detected in our study are for plant protection rather than honey bee colony management. Fungicides and herbicides may be applied to crops or horticultural plantings but would not be applied within a honey bee colony. Even among the insecticides detected, most are never used within the hive. Potential interactions between pesticides used and never used for colony management need to be explored along with interactions between pesticides only used for crop or horticultural plants.

Some studies focused on honey bees exposed to multiple pesticides have been conducted usually including at least one pesticide that may be used in honey be management. Colin and Belzunces [52], Pilling and Jepson [47], and Pilling et al. [48] explored the synergistic impact from paired pyrethroid and fungicide exposures. Sub-lethal exposures, either simultaneous or sequential, resulted in an increase in honey bee mortality; a reduction in the rate of pyrethroid biotransformation in the presence of the fungicide was proposed as the mechanism of action. More recently, Thompson and Wilkins [53] obtained similar results when they paired eight different fungicides and two pyrethroid insecticides but the evidence provided suggested a different mechanism of action. The presence of a fungicide decreased the repellency of the pyrethroid resulting in a higher exposure to the pyrethroid. Johnson et al. [8] reported that acaricides with different modes of action interact, usually resulting in decreased the LD_{50} compared to the single compound control in adult bees. Additionally, they showed that some fungicides interact with

acaricides to lower the LD_{50} of the acaricide and that piperonyl butoxide lowers the LD_{50} of tau-fluvalinate. Data from our study show that fluvalinate and coumaphos could be found in the same sample of pollen, drawn comb, and wax-coated foundation and the piperonyl butoxide may co-exists with fluvalinate in wax.

Using honey bee larvae, Zhu et al. [54] tested fluvalinate, coumaphos, chlorothalonil, and chlorpyrifos alone and in combination. At moderate levels of exposure, the toxicity of fluvalinate-chlorothalonil and coumaphos-chlorothalonil increased the toxicity of the insecticides but at very low doses (10-fold reduction) the fluvaninate-chlorothalonil exposure became antagonistic. Complicating the picture further, when coumaphos was added to the fluvalinate-chlorothalonil mixture, the toxicity was reduced. The risk potential to adult and immature bees from pollen consumption, and to nurse bees, who recycle hive wax, and to new adult bees, who chew through wax to emerge from their brood cells, should be explored.

If pesticide exposure is evaluated using only the LD_{50} from each individual pesticide, based upon the results from this study, the risk posed to honey bees is low. In our pollen samples, one sample out of 168 exceeded the pesticide residue's oral LD_{50}. While much attention has been focused on the newer types of pesticides, ie. Neonicotinoids, and their impact on bee health, from this study it is clear that some of the older pesticides, specifically the carbamates and organophosphates, may continue to pose a risk. When the ratio of exposure (ppb) to its LD_{59} for the acetylcholinesterase inhibitors was summed, the ratio exceeded 1. While few samples had residues approaching the pesticide's LD_{50}, the pesticides residues detected are likely to pose a high sublethal risk to honey bee survival [7,46,47,48,52,53,54,55]. Currently it is difficult to evaluate the risks from sublethal exposures as there are no agreed upon outcome measures similar to the LD_{50} for the various sublethal outcomes. Additional work is needed, first, to define the most important sublethal outcomes and, second, to quantify the impact of sublethal exposures from various pesticides so a risk evaluation can be conducted.

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CONCLUSIONS

Exposure, as estimated by pollen and wax comb contamination, is very complex and difficult to parse. Our study, and several others [22,25], has shown that many compounds at varying orders of magnitude of concentration are involved. In general, we found that insecticides/miticides were present

more frequently and at higher concentrations than fungicides or herbicides. This was true in all four years. Our study also found that residues vary by month and by year making it difficult to generalize, without additional research, about which pesticides are most likely to be encountered by honey bees and which residues pose the greatest risk. Pollen was more contaminated than wax comb and foundation wax; one-third of the pesticides detected were only observed in pollen. We also found, on average, the potential oral toxicity of these residues was no greater than the potential contact toxicity. The modes of actions of the all pesticide residues in pollen and wax comb were diverse with 10 separate modes of action being found in all four years and nine additional modes of action occurring in only one year. If synergy in toxicological response is a function of simultaneous occurrence of multiple distinct modes of action, then we found high frequency of potential synergies in our pollen and wax comb samples. This prospective, longitudinal study expands our knowledge by providing pollen, wax comb, and wax foundation pesticide residue data throughout the lifespan of honey bee colonies in six locations across the U.S. over four years. (Approximately one-third of the 377 colonies in the study were sampled directly while the residues in other colonies were estimated indirectly using the predictive power—Section 2.4.2 and Section 3.2.) Previous residue analysis studies were cross-sectional, providing a single snapshot of honey bee exposure to pesticides. More data are needed to adequately evaluate the differences in pesticide exposure risk to honey bees, and other pollinators, by week, month, year and location; the pooled pollen sampling method used in this study did not provide the level of detail necessary to adequately describe the risk to honey bees and other pollinators.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.O., F.A.D., K.A., B.E., J.D.E., M.S. and W.S.S.; Data curation, N.O. and F.A.D.; Formal analysis, N.O. and F.A.D.; Funding acquisition, N.O., F.A.D., K.A., B.E., M.S. and W.S.S.; Investigation, N.O., F.A.D., K.A., B.E., J.D.E., M.S. and W.S.S.; Methodology, N.O., F.A.D., K.A., B.E., J.D.E., M.S. and W.S.S.; Project administration, N.O. and F.A.D.; Resources, N.O., F.A.D., K.A., B.E., J.D.E., M.S. and W.S.S.; Supervision, N.O., F.A.D., K.A., J.D.E., M.S. and W.S.S.; Visualization, N.O. and F.A.D.; Writing—original draft, N.O.; Writing—review & editing, N.O., F.A.D., K.A., B.E. and J.D.E.

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Chapter 12

Insecticide Mixtures Could Enhance the Toxicity of Insecticides in a Resistant Dairy Population of Musca domestica L

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ABSTRACT

House flies, *Musca domestica* L., are important pests of dairy operations worldwide, with the ability to adapt wide range of environmental conditions. There are a number of insecticides used for their management, but development of resistance is a serious problem. Insecticide mixtures could enhance the toxicity of insecticides in resistant insect pests, thus resulting as a potential resistance management tool. The toxicity of bifenthrin,

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cypermethrin, deltamethrin, chlorpyrifos, profenofos, emamectin benzoate and fipronil were assessed separately, and in mixtures against house flies. A field-collected population was significantly resistant to all the insecticides under investigation when compared with a laboratory susceptible strain. Most of the insecticide mixtures like one pyrethroid with other compounds evaluated under two conditions (1:1-"A" and LC_{50}: LC_{50}-"B") significantly increased the toxicity of pyrethroids in the field population. Under both conditions, the combination indices of pyrethroids with other compounds, in most of the cases, were significantly below 1, suggesting synergism. The enzyme inhibitors, PBO and DEF, when used in combination with insecticides against the resistant population, toxicities of bifenthrin, cypermethrin, deltamethrin and emamectin were significantly increased, suggesting esterase and monooxygenase based resistance mechanism. The toxicities of bifenthrin, cypermethrin and deltamethrin in the resistant population of house flies could be enhanced by the combination with chlorpyrifos, profenofos, emamectin and fipronil. The findings of the present study might have practical significance for resistance management in house flies.

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INTRODUCTION

The house fly, *Musca domestica* L., has been considered as one of the major pests of dairies and public health with a potential role in disease transmission, nuisance to animals and humans, and have the ability to disperse off-farm areas [1]. Various insecticides from organochlorine, organophosphate, carbamate, pyrethroid and new chemistry classes have been used for their management worldwide. However, they have the ability to develop resistance to most of the insecticides used for their management all over the world.

Insecticide mixtures, rotation and/or fine scale mosaics have been proposed as important tools for resistance management in different insect pests [2]. Mixtures consisted of organophosphate, pyrethroid or carbamate insecticides have been found very effective in enhancing the toxicity of insecticides in different resistant insect pests worldwide like *Bemisia tabaci* [3], *Culex quinquefasciatus* [4], *Helicoverpa armigera* [5], [6], *Musca domestica* [7] and *Spodoptera litura* [8]. This type of potentiation or synergism is explained by the inhibition of esterases [9], [10] or monooxygenases activity [6]. However, cases of insecticide resistance to different classes of insecticides are increasing day by day. This situation is alarming, as there are very few classes, and the development of new insecticides is limited owing

to the rising standards for environmental safety [11]. In this, alternative strategies like mosaics or rotational use of insecticides with different modes of action should also be included in resistance management programs [12].

Theoretically, mixing insecticides (with different modes of action) usually prove very effective in resistance management programs compared to mosaics or rotational use of insecticides [13]because, if a resistance mechanism to each insecticide in the mixture is independent and initially rare, the chances for the occurrence of resistance to both insecticides at the same time would be minimum [14]. There are a number of studies on mixture toxicities (particularly pyrethroids with other compounds) in different dipteran insect pests worldwide, however, to the best of authors' knowledge such studies are rare in the house fly research particularly in Pakistan. Since pyrethroids and organophosphates have different modes of action, their mixtures have commonly been in practice against a variety of pests worldwide for the last many years [8]. Increased metabolic detoxification is one of the major mechanisms in house flies for the development of insecticide resistance [15]. Previously it has been assumed that organophosphates, when used in combination with pyrethroids, inhibit the enzymes responsible for metabolic detoxification in different insect pests [6], [9], [10]. Given the development of insecticide resistance in field populations of house flies from the dairies in Punjab, Pakistan [16], [17], an investigation was made using different combination of pyrethroids (bifenthrin, cypermethrin, deltamethrin) with organophosphates (chlorpyrifos, profenofos) and new insecticides (emamectin benzoate, an avermectin; and fipronil, a phenypyrazole), to study the synergistic or antagonistic interaction between insecticides used in mixtures against house flies. The paper also addresses the question of synergistic interaction of two synergists, piperonyl butoxide (PBO) and S,S,S-tributylphosphorotrithioate (DEF), with different insecticides under investigation.

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METHODS

Insects

The adult flies were collected from a dairy farm of the province Punjab, Pakistan. Recently, resistance to different insecticides have been observed in house flies from the said area [16], [17]. No specific permit was required to collect house fly samples from the dairy farm as it was privately owned

and collection was made merely by speaking with the private owner. Since the house fly is not an endangered species, no such permission was required from any concerned authority in Punjab, Pakistan. The collected flies were fed on powdered milk, sugar and water while larvae were reared on a medium of milk powder, sugar, yeast, grass meal and wheat bran with a ratio of 0.3:0.3:1:2:4 by weight, respectively [18]. A population of house flies was collected from zero or very low chemical use zone and maintained in the laboratory without any chemical exposure. This population was designated as the laboratory susceptible strain. The fly cultures were maintained in the laboratory at 25±2°C, 60±5% RH and 12L: 12D (h) photoperiod.

Chemicals

Commercial formulations of the following insecticides were used for bioassays: bifenthrin (Talstar ® 100 EC, FMC) cypermethrin (Arrivo® 10 EC, FMC), deltamethrin (Decis Super®, 10.5 EC, Bayer Crop Science), profenofos (Curacron® 50 EC, Syngenta), chlorpyrifos, emamectin benzoate (Proclaim® 019EC, Syngenta) and fipronil (Regent® 36EC, Bayer Crop Sciences). Two synergists, *S*,*S*,*S*-tributylphosphorotrithioate (DEF; Sigma Ltd, UK), the esterase specific inhibitor, and piperonyl butoxide (PBO; Sigma Ltd, UK), an inhibitor of cytochrome P450 monooxygenases and of esterases were also used to check their effect on insecticide toxicity.

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Bioassays

Feeding bioassay method was used to assess the toxicities of insecticides under investigation [19]. Briefly, twenty 3–5 days old female flies were introduced in plastic containers (250 ml) and provided two pieces of cotton dental wick (2 cm length) moistened with 20% sugar water solution containing an insecticide or an insecticide mixture concentration. Five to eight concentrations were prepared for each insecticide and each concentration was replicated three times. In control plastic jars, cotton wicks soaked in 20% sugar solution without toxicant were given to flies. To avoid drying, cotton wicks were hydrated at 24 hours [19]. Bioassays were conducted at 25±2°C, 60±5% RH and 12:12 (L/D) photoperiod. Synergists PBO and DEF at the maximum sublethal dose of 10 µg per fly (in 0.5 µl acetone) were applied 1 h before the insecticide treatment as reported elsewhere [20]. Final mortality was assessed after 48 h of exposure to insecticides and the flies were assumed dead if they were ataxic. LC_{50s} were calculated by Probit Analysis using the software SPSS 10 for windows.

Synergism analysis

The combination index (CI) method proposed by Chou and Talalay [21], was used to evaluate possible additive, antagonistic or synergistic interaction between the insecticides in the mixture. To determine which of the above said interaction exist between the insecticides in the mixture, two insecticides in the mixtures were tested under two conditions viz., 1:1-"A" and LC_{50}: LC_{50}-"B", with serial concentrations. **To assess synergism, antagonism and/or additive effect**, the combination index was determined by using the following equation:

$$CI_x = \frac{LC_x^{1m}}{LC_x^1} + \frac{LC_x^{2m}}{LC_x^2} + \left(\frac{LC_x^{1m}}{LC_x^1} \times \frac{LC_x^{2m}}{LC_x^2}\right)$$

Where the values in the numerator of the equation are the lethal concentrations of insecticide 1 and 2 respectively, giving the mortality x, while the values in the denominator are the lethal concentrations of insecticide 1 and 2 respectively, producing the same mortality x when used alone. The resultant values of CIs were scaled to categorize the mixture effect: additive effect when combination index =1, antagonistic effect when combination index >1, and synergistic effect when combination index <1 [21]. These values were calculated at 50% mortality level.

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RESULTS

Toxicity of Insecticides Alone or in Mixture to the Laboratory Susceptible Strain

The relative toxicities of organophosphates and new chemical insecticides were significantly higher (non overlapping of 95% CL; $P<0.01$) than that of the pyrethroids (Table 1).

Table 1: Toxicity of insecticides alone and in combination against laboratory susceptible strain of *Musca domestica*

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Insecticide	Ratio	n*	LC_{50} (95% CL)**	Slope ± SE	χ^2	df	P
Bifenthrin	1:0	360	10.89 (9.18–13.11)	2.23±0.23	2.52	3	0.47
Cypermethrin	1:0	360	4.90 (4.24–5.59)	2.86±0.27	2.56	3	0.47
Deltamethrin	1:0	420	13.18 (11.18–15.59)	2.25±0.19	1.66	4	0.79
Chlorpyrifos	1:0	420	1.85 (1.55–2.18)	2.28±0.20	0.49	4	0.98
Profenofos	1:0	420	1.81 (1.53–2.14)	2.23±0.19	1.55	4	0.82
Emamectin benzoate	1:0	360	2.89 (2.46–3.46)	2.34±0.24	1.44	3	0.69
Fipronil	1:0	360	1.94 (1.62–2.40)	2.19±0.24	1.83	3	0.61
Bifenthrin + Chlorpyrifos	1:1	420	6.69 (5.29–8.90)	1.53±0.17	0.86	4	0.93
Bifenthrin + Profenofos	1:1	420	5.32 (4.20–7.06)	1.47±0.16	0.64	4	0.96
Bifenthrin + Emamectin	1:1	420	4.52 (3.75–5.51)	1.90±0.18	6.64	4	0.17
Bifenthrin + Fipronil	1:1	420	2.96 (2.39–3.69)	1.57±0.16	1.79	4	0.78
Cypermethrin + Chlorpyrifos	1:1	420	3.33 (2.71–3.55)	1.81±0.18	6.71	4	0.15
Cypermethrin + Profenofos	1:1	360	7.10 (5.51–10.12)	1.71±0.23	0.61	3	0.90
Cypermethrin + Emamectin	1:1	420	2.51 (2.02–3.20)	1.59±0.16	0.96	4	0.92
Cypermethrin + Fipronil	1:1	420	4.21 (3.98–5.44)	1.71±0.18	3.29	4	0.51
Deltamethrin + Chlorpyrifos	1:1	420	8.77 (7.25–10.97)	2.00±0.18	3.08	4	0.55
Deltamethrin + Profenofos	1:1	420	8.82 (6.03–15.11)	1.93±0.19	7.81	4	0.10
Deltamethrin + Emamectin	1:1	480	4.90 (4.12–5.89)	2.04±0.16	6.79	5	0.24
Deltamethrin + Fipronil	1:1	360	9.88 (7.83–13.18)	1.63±0.17	0.83	3	0.94
Chlorpyrifos + Bifenthrin	1:5.89	360	3.45 (2.76–4.32)	1.63±0.19	0.86	3	0.83
Profenofos + Bifenthrin	1:6.02	360	3.44 (2.86–4.15)	2.04±0.22	2.41	3	0.49
Emamectin + Bifenthrin	1:3.77	420	2.65 (2.18–3.23)	1.79±0.16	3.09	4	0.54
Fipronil + Bifenthrin	1:5.61	420	1.38 (1.33–1.95)	1.91±0.18	1.47	4	0.83

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Chlorpyrifos + Cypermethrin	1:2.65	360	2.28 (1.91–2.79)	2.03±0.22	4.97	3	0.17
Profenofos + Cypermethrin	1:2.71	360	3.22 (2.23–5.40)	2.34±0.35	5.34	3	0.15
Emamectin + Cypermethrin	1:1.70	360	1.24 (0.83–1.90)	2.36±0.23	6.28	3	0.10
Fipronil + Cypermethrin	1:2.53	360	1.55 (1.30–1.84)	2.22±0.23	3.77	3	0.29
Chlorpyrifos + Deltamethrin	1:7.12	360	5.22 (4.35–6.36)	2.08±0.22	3.17	3	0.37
Profenofos + Deltamethrin	1:7.28	360	5.80 (4.85–7.11)	2.11±0.22	1.83	3	0.61
Emamectin + Deltamethrin	1:4.56	360	3.46 (2.92–4.07)	2.35±0.24	3.82	3	0.28
Fipronil + Deltamethrin	1:6.79	420	2.71 (2.30–3.20)	2.25±0.19	2.37	4	0.67

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The toxicities of pyrethroids, based on their LC_{50s}, increased when mixed with other insecticides. For bifenthrin, this effect was observed in all combinations: bifenthrin/other non-pyrethroids. The toxicity of bifenthrin increased significantly when mixed with fipronil over that of mixtures with the other non-pyrethroids at the both ratios tested. For cypermethrin, the increased toxicity was observed in the following combinations: cypermethrin/chlorpyrifos or emamectin at the 1:1 ratio, and cypermethrin/other insecticides except profenofos, at the LC_{50}: LC_{50} ratio. At the 1:1 ratio, the mixtures of cypermethrin/chlorpyrifos or emamectin were significantly more toxic compared to the mixture with profenofos or fipronil while at LC_{50}: LC_{50}ratio, the mixture of cypermethrin with either emamectin or fipronil was significantly more toxic than the rest of the two mixtures. For deltamethrin, the mixture of deltamethrin with either chlorpyrifos or emamectin at the 1:1 ratio, and with all the other insecticides at the LC_{50}: LC_{50}ratio, showed significantly increased toxicities compared to the toxicity of deltamethrin used alone. At the 1:1 ratio, the toxicity of deltamethrin/emamectin was higher than the rest of the three mixtures while at the LC_{50}: LC_{50} ratio, the mixtures of deltamethrin with either emamectin or fipronil were significantly more toxic than the rest of the two mixtures. Moreover, the toxicities of pyrethroids/non-pyrethroids were significantly higher at the LC_{50}: LC_{50} ratio (non overlapping 95% CL; $P<0.01$) than the combinations mixed at the 1:1 ratio, except the toxicities of following combinations: cypermethrin/chlorpyrifos and deltamethrin/profenofos (Table 1).

The mixtures of bifenthrin, except with fipronil revealed antagonistic effect at the 1:1 ratio with combination index values >1, however, at

the LC_{50}: LC_{50} ratio all combinations produced synergistic effect with combination index values <1 (Table 2). For cypermethrin at the 1:1 ratio, only cypermethrin/emamectin mixture produced a synergistic effect while at the LC_{50}: LC_{50}ratio, except cypermethrin/profenofos, all the mixtures produced a synergistic effect. Similarly, the mixtures of deltamethrin/other insecticides at the 1:1 ratio revealed antagonistic effect, while snyrgistic effect was observed at the other ratio tested (Table 2).

Table 2: Combination index (CI) values of insecticide mixtures against the laboratory susceptible strain of *Musca domestica*

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(Insecticide mixture (A+B	Active ingredient ratio			At LC_{50} level
	Ratio	*A	*B	**CI
Bifenthrin + Chlorpyrifos	1:1	3.35	3.35	1.20
Bifenthrin + Profenofos	1:1	2.66	2.66	2.07
Bifenthrin + Emamectin	1:1	2.26	2.26	1.15
Bifenthrin + Fipronil	1:1	1.48	1.48	1.00
Cypermethrin + Chlorpyrifos	1:1	1.66	1.66	1.54
Cypermethrin + Profenofos	1:1	3.55	3.55	4.10
Cypermethrin + Emamectin	1:1	1.26	1.26	0.80
Cypermethrin + Fipronil	1:1	2.11	2.11	1.99
Deltamethrin + Chlorpyrifos	1:1	4.38	4.38	2.88
Deltamethrin + Profenofos	1:1	4.41	4.41	2.95
Deltamethrin + Emamectin	1:1	2.45	2.45	1.191.19
Deltamethrin + Fipronil	1:1	4.94	4.94	3.88
Chlorpyrifos + Bifenthrin	1:5.89	0.50	2.95	0.62
Profenofos + Bifenthrin	1:6.02	0.49	2.95	0.62
Emamectin + Bifenthrin	1:3.77	0.55	2.09	0.42
Fipronil + Bifenthrin	1:5.61	0.21	1.71	0.23
Chlorpyrifos + Cypermethrin	1:2.65	0.62	1.66	0.80
Profenofos + Cypermethrin	1:2.71	0.87	2.35	1.19
Emamectin + Cypermethrin	1:1.70	0.46	0.78	0.35
Fipronil + Cypermethrin	1:2.53	0.44	1.11	0.50
Chlorpyrifos + Deltamethrin	1:7.12	0.64	4.58	0.81
Profenofos + Deltamethrin	1:7.28	0.70	5.10	0.91

Emamectin + Deltamethrin	1:4.56	0.62	2.84	0.49
Fipronil + Deltamethrin	1:6.79	0.35	2.36	0.39

*calculated value of insecticide A and B (µg/ml) respectively.

**combination index.

Toxicity of Insecticides Alone or in Mixture to the Field Collected Strain

The field collected population showed resistance to all the tested insecticides when compared with the laboratory reared susceptible strain (Table 3). The resistance ratios were 11.72, 64.79, 15.87, 8.82, 22.02, 43.11 and 22.29 for bifenthrin, cypermethrin, deltamethrin, chlorpyrifos, profenofos, emamectin and fipronil, respectively, when these insecticides used alone.

Table 3: Toxicity of insecticides alone and in combination against field strain of *Musca domestica*

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Insecticide	Ratio	n*	LC_{50} (95% CL)**	Slope ± SE	χ^2	df	P	RR***
Bifenthrin	1:0	420	127.62 (104.75–157.69)	1.74±0.17	1.74	4	0.78	11.72
Cypermethrin	1:0	480	317.45 (250.39–418.56)	1.39±0.14	1.73	5	0.88	64.79
Deltamethrin	1:0	540	209.12 (170.00–262.11)	1.55±0.12	7.93	6	0.24	15.87
Chlorpyrifos	1:0	360	16.31 (13.17–21.29)	1.81±0.22	0.02	3	0.99	8.82
Profenofos	1:0	480	39.85 (32.89–49.41)	1.84±0.16	7.74	5	0.17	22.02
Emamectin benzoate	1:0	420	124.59 (97.82–168.87)	1.54±0.17	0.38	4	0.98	43.11
Fipronil	1:0	420	43.24 (30.34–67.40)	2.01±0.19	7.56	4	0.11	22.29
Bifenthrin + Chlorpyrifos	1:1	420	44.64 (37.03–55.00)	1.94±0.18	4.20	4	0.38	6.67
Bifenthrin + Profenofos	1:1	420	58.39 (46.23–77.22)	1.60±0.16	1.23	4	0.87	10.98

Bifenthrin + Emamectin	1:1	360	141.00 (113.02–187.47)	1.81±0.22	1.48	3	0.69	31.19
Bifenthrin + Fipronil	1:1	360	100.36 (80.04–134.71)	1.81±0.22	3.74	3	0.29	33.91
Cypermethrin + Chlorpyrifos	1:1	360	74.09 (60.73–90.64)	1.86±0.21	0.15	3	0.98	22.32
Cypermethrin + Profenofos	1:1	420	51.87 (41.94–64.17)	1.60±0.16	1.68	4	0.80	7.31
Cypermethrin + Emamectin	1:1	480	77.45 (64.50–94.45)	1.91±0.16	7.27	5	0.20	30.86
Cypermethrin + Fipronil	1:1	420	64.74 (50.43–85.00)	1.29±0.15	1.50	4	0.82	15.38
Deltamethrin + Chlorpyrifos	1:1	480	93.01 (76.81–115.31)	1.88±0.17	5.75	5	0.33	10.64
Deltamethrin + Profenofos	1:1	420	152.72 (116.77–216.41)	1.39±0.12	1.16	4	0.89	17.32
Deltamethrin + Emamectin	1:1	480	90.67 (73.35–115.36)	1.60±0.15	3.30	5	0.65	18.50
Deltamethrin + Fipronil	1:1	480	69.54 (54.96–90.73)	1.35±0.13	0.51	5	0.99	7.04
Chlorpyrifos + Bifenthrin	1:7.82	420	29.33 (24.16–35.85)	1.78±0.17	4.25	4	0.37	8.50
Profenofos + Bifenthrin	1:3.20	420	26.57 (17.10–40.91)	1.67±0.16	7.89	4	0.10	7.72
Emamectin + Bifenthrin	1:1.02	480	60.51 (48.78–76.74)	1.50±0.14	1.02	5	0.96	22.92
Fipronil + Bifenthrin	1:2.95	420	96.82 (73.54–139.51)	1.40±0.17	1.10	4	0.89	70.16
Chlorpyrifos + Cypermethrin	1:19.46	420	54.79 (45.09–66.50)	1.80±0.17	2.25	4	0.69	24.03
Profenofos + Cypermethrin	1:7.97	420	45.85 (35.96–60.26)	1.35±0.15	0.34	4	0.99	14.23
Emamectin + Cypermethrin	1:2.55	420	93.46 (64.89–138.78)	1.97±0.18	7.76	4	0.10	75.37
Fipronil + Cypermethrin	1:7.34	480	90.39 (75.49–109.67)	1.94±0.16	1.73	5	0.89	58.32
Chlorpyrifos + Deltamethrin	1:12.82	420	45.59 (37.15–56.36)	1.66±0.16	2.14	4	0.71	8.73
Profenofos + Deltamethrin	1:5.25	360	53.05 (32.93–82.71)	2.01±0.23	5.56	3	0.14	9.51
Emamectin + Deltamethrin	1:1.68	480	102.84 (79.24–140.15)	1.24±0.12	0.78	5	0.98	29.72

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Fipronil + Deltamethrin	1:4.84	420	44.08 (36.03–53.91)	1.72±0.16	3.08	4	0.55	16.24

*number of females exposed.

**lethal conc. (µg/ml) to kill the fifty percent population.

***resistance factor, calculated as [LC_{50} of field population ÷ LC_{50} of laboratory susceptible strain].

In most of the cases, the toxicities of pyrethroids, based on their LC_{50s}, increased significantly when mixed with other insecticides. For bifinthrin, the toxicity was increased significantly with the following mixtures: bifenthrin/organophosphates at the 1:1 ratio, and bifenthrin/other insecticides except fipronil at the LC_{50}: LC_{50} ratio. However, bifenthrin with either emamectin or fipronil at 1:1, and bifenthrin/fipronil at LC_{50}: LC_{50} ratio did not increase toxicity since their LC_{50}values were similar with bifenthrin alone based on overlapping of 95% CLs. In case of cypermethrin, either it was used at the ratio of 1:1 or LC_{50}: LC_{50} with the other insecticides, the toxicities increased significantly compared to the toxicity of cypermethrin alone. Similarly, the toxicity of deltamethrin increased significantly with all the combinations at both tested ratios except the combination with profenofos at the 1:1 ratio where the LC_{50} value was the same as for deltamethrin alone (Table 3).

For each mixture, the combination index was calculated for the purpose to assess possible additive, antagonistic or synergistic interaction between insecticides. The mixtures of bifenthrin/other insecticides produced an antagonistic effect (combination index >1) at the 1:1 ratio, however, synergistic effect was observed (combination index <1) with all the combinations when they were tested in the ratio of LC_{50}: LC_{50}, except bifenthrin/fipronil mixture (combination index >1) (Table 4). For cypermethrin, all the tested combinations produced a synergistic effect in both ratios tested, except the combination cypermethrin/chlorpyrifos which was antagonistic when used at the 1:1 ratio. In case of deltamethrin at the 1:1 ratio, only the combination deltamethrin/emamectin produced synergistic effect. In contrast, all the combinations of deltamethrin with other insecticides gave synergistic effect when used in the ratio of LC_{50}: LC_{50}(Table 4).

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Table 4: Combination index (CI) values of insecticide mixtures against field strain of *Musca domestica*

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Insecticide mixture (A+B)	Active ingredient ratio			At LC_{50} level
	Ratio	A*	B*	CI**
Bifenthrin + Chlorpyrifos	1:1	22.32	22.32	1.79
Bifenthrin + Profenofos	1:1	29.20	29.20	1.13
Bifenthrin + Emamectin	1:1	70.50	70.50	1.42
Bifenthrin + Fipronil	1:1	50.18	50.18	2.01
Cypermethrin + Chlorpyrifos	1:1	37.05	37.05	2.66
Cypermethrin + Profenofos	1:1	25.94	25.94	0.78
Cypermethrin + Emamectin	1:1	38.73	38.73	0.47
Cypermethrin + Fipronil	1:1	32.37	32.37	0.93
Deltamethrin + Chlorpyrifos	1:1	46.51	46.51	3.71
Deltamethrin + Profenofos	1:1	76.36	76.36	3.00
Deltamethrin + Emamectin	1:1	45.34	45.34	0.66
Deltamethrin + Fipronil	1:1	34.77	34.77	1.11
Chlorpyrifos + Bifenthrin	1:7.82	3.33	26.00	0.45
Profenofos + Bifenthrin	1:3.20	6.33	20.24	0.34
Emamectin + Bifenthrin	1:1.02	29.96	30.55	0.54
Fipronil + Bifenthrin	1:2.95	24.51	72.31	1.46
Chlorpyrifos + Cypermethrin	1:19.46	2.68	52.11	0.36
Profenofos + Cypermethrin	1:7.97	5.11	40.74	0.27
Emamectin + Cypermethrin	1:2.55	26.33	67.13	0.47
Fipronil + Cypermethrin	1:7.34	10.84	79.55	0.57
Chlorpyrifos + Deltamethrin	1:12.82	3.30	42.29	0.44
Profenofos + Deltamethrin	1:5.25	8.49	44.56	0.40
Emamectin + Deltamethrin	1:1.68	30.37	72.47	0.72
Fipronil + Deltamethrin	1:4.84	7.55	36.53	0.38

*calculated value of insecticide A and B (µg/ml), respectively.

**combination index.

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Effect of Enzyme Inhibitors on the Toxicities of Insecticides

The use of two enzyme inhibitors viz., PBO and DEF, against the field population of *M. domestica* largely overcame resistance to bifenthrin, cypermethrin, deltamethrin, chlorpyrifos, profenofos and emamectin.

PBO significantly reduced LC_{50} values (non overlapping of 95% CLs) for bifenthrin from 127.62 to 21.94 (6 fold), cypermethrin from 317.45 to 43.86 (7 fold), deltamethrin from 209.12 to 38.76 (5 fold), chlorpyrifos from 16.31 to 6.19 (3 fold), profenofos from 39.85 to 14.77 (3 fold), and emamectin from 124.59 to 60.61 (2 fold). However, PBO did not produce a synergistic effect with fipronil. DEF also reduced LC_{50} values for bifenthrin from 127.62 to 64.35 (2 fold), cypermethrin from 317.45 to 162.50 (2 fold), deltamethrin from 209.12 to 100.09 (2 fold), chlorpyrifos from 16.31 to 3.66 (4 fold), and profenofos from 39.85 to 5.48 (7 fold). However, DEF did not show synergism with emamectin and fipronil (Table 5). Moreover, PBO and DEF did not synergize any insecticide against the laboratory susceptible strain.

Table 5: Effect of enzyme inhibitors on insecticide toxicity against *Musca domestica*

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Strain	Insecticide	LC_{50} (95% CL)	Slope±SE	χ^2	df	P	RR	SR*
Lab	Bifenthrin	10.89 (9.18–13.11)	2.23±0.23	2.52	3	0.47		
	Cypermethrin	4.90 (4.24–5.59)	2.86±0.27	2.56	3	0.47		
	Deltamethrin	13.18 (11.18–15.59)	2.25±0.19	1.66	4	0.79		
	Chlorpyrifos	1.85 (1.55–2.18)	2.28±0.20	0.49	4	0.98		
	Profenofos	1.81 (1.53–2.14)	2.23±0.19	1.55	4	0.82		
	Emamectin	2.89 (2.46–3.46)	2.34±0.24	1.44	3	0.69		
	Fipronil	1.94 (1.62–2.40)	2.19±0.24	1.83	3	0.61		
	Bifenthrin+PBO	8.02 (6.53–9.86)	1.80±0.21	0.86	3	0.84		1
	Bifenthrin+DEF	11.83 (8.06–19.69)	1.92±0.19	8.19	4	0.09		1
	Cypermethrin+PBO	3.84 (2.49–5.95)	2.32±0.23	6.36	3	0.10		1
	Cypermethrin+DEF	5.60 (4.74–6.70)	2.34±0.24	4.33	3	0.22		1
	Deltamethrin+PBO	9.59 (7.78–11.96)	1.73±0.20	3.72	3	0.29		1
	Deltamethrin+DEF	16.14 (13.84–18.91)	2.54±0.22	2.18	4	0.70		1
	Chlorpyrifos+PBO	2.54 (2.10–3.03)	2.23±0.23	0.30	3	0.96		1
	Chlorpyrifos+DEF	1.54 (1.29–1.83)	2.26±0.22	1.42	3	0.70		1
	Profenofos+PBO	1.97 (1.66–2.37)	2.08±0.18	2.62	4	0.62		1
	Profenofos+DEF	1.27 (1.02–1.62)	1.60±0.20	1.14	3	0.77		1
	Emamectin+PBO	3.26 (2.73–3.99)	2.17±0.23	1.18	3	0.78		1
	Emamectin+DEF	3.15 (2.60–3.94)	1.92±0.21	2.44	3	0.49		1
	Fipronil+PBO	2.11 (1.71–2.73)	1.91±0.23	1.91	3	0.59		1
	Fipronil+DEF	1.85 (1.48–2.45)	1.66±0.21	1.71	3	0.64		1
Field	Bifenthrin	127.62 (104.75–157.69)	1.74±0.17	1.74	4	0.78	11.72	
	Cypermethrin	317.45 (250.39–418.56)	1.39±0.14	1.73	5	0.88	64.79	
	Deltamethrin	209.12 (170.00–262.11)	1.55±0.12	7.93	6	0.24	15.87	

	Chlorpyrifos	16.31 (13.17–21.29)	1.81±0.22	0.02	3	0.99	8.82	
	Profenofos	39.85 (32.89–49.41)	1.84±0.16	7.74	5	0.17	22.02	
	Emamectin benzoate	124.59 (97.82–168.87)	1.54±0.17	0.38	4	0.98	43.11	
	Fipronil	43.24 (30.34–67.40)	2.01±0.19	7.56	4	0.11	22.29	
	Bifenthrin+PBO	21.94 (17.81–27.04)	1.65±0.16	0.56	4	0.97	3.33	6
	Bifenthrin+DEF	64.35 (41.91–100.77)	2.44±0.24	6.73	3	0.08	5.44	2
	Cypermethrin+PBO	43.86 (35.62–54.65)	1.53±0.13	8.02	5	0.16	11.42	7
	Cypermethrin+DEF	162.50 (124.05–233.37)	1.51±0.18	2.88	4	0.58	29.93	2
	Deltamethrin+PBO	38.76 (32.24–46.83)	1.82±0.15	5.79	5	0.34	4.90	5
	Deltamethrin+DEF	100.09 (80.84–128.62)	1.68±0.17	0.97	4	0.91	6.20	2
	Chlorpyrifos+PBO	6.19 (5.70–7.78)	1.85±0.21	1.60	3	0.66	2.44	3
	Chlorpyrifos+DEF	3.66 (3.03–4.41)	2.03±0.22	3.38	3	0.34	2.38	4
	Profenofos+PBO	14.77 (11.75–19.55)	1.63±0.17	4.15	4	0.39	7.50	3
	Profenofos+DEF	5.48 (3.72–8.13)	1.78±0.16	7.21	4	0.13	5.43	7
	Emamectin+PBO	60.61 (47.72–78.54)	1.36±0.15	1.57	4	0.81	18.59	2
	Emamectin+DEF	94.04 (73.89–125.83)	1.42±0.16	0.50	4	0.97	29.85	1
	Fipronil+PBO	34.28 (27.88–43.00)	1.65±0.16	4.49	4	0.35	16.24	1
	Fipronil+DEF	38.45 (30.88–49.27)	1.55±0.15	4.48	4	0.44	20.78	1

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DISCUSSION

The present study was conducted for the purpose to evaluate the toxicities of pyrethroids alone and in mixture with non-pyrethroids against the insecticide resistant dairy population of house flies. The antagonistic or synergistic interactions among the tested insecticides depended on the type of insecticides used, ratios and strains. In the laboratory susceptible strain, the combination between one pyrethroid and the other compounds produced an antagonistic interaction (in the ratio 1:1), except the following combinations: bifenthrin/fipronil and cypermethrin/emamectin where the interactions were additive and synergistic, respectively. In contrast, all the mixtures of pyrethroid/non-pyrethroid produced a synergistic effect when used in the ratio of LC_{50}: LC_{50}, except the combination cypermethrin/profenofos. In the field strain, all the mixtures of pyrethroid/non-pyrethroid when used in the ratio of 1:1, except the following combinations where responses were synergistic: cypermethrin/profenofos or emamectin or fipronil, and deltamethrin/emamectin. However, at the LC_{50}: LC_{50} ratio, all the mixtures of pyrethroid/non-pyrethroid produced synergistic effect, except the combination bifenthrin/fipronil. The synergistic interaction between pyrethroids and organophosphate insecticides has previously been

reported in different pests like *Bemisia tabaci* [22], *Culex quinquefasciatus* [4], *Helicoverpa armigera* [23], [24], *Musca domestica* [7], *Pectinophora gossipiella* [25], *Plutella xylostella* [26], *Spodoptera litura* [12], *S. littoralis* [27] and *Tetranychus urticae* [28]. However, the antagonistic interactions between pyrethroid and organophosphate have also previously been reported in different insect species like *B. tabaci* from Pakistan [29], *H. armigera* from Africa [6] and *S. littoralis* from Egypt [30]. The results of the present study also revealed that the toxicity of pyrethroids could be enhanced by the addition of new insecticides like emamectin benzoate and fipronil. Previously, Attique et al. [26] has documented the enhanced toxicity of bifenthrin and chlorpyrifos by new chemical insecticides in *P. xylostella*.

According to the results of present study the toxicity of bifenthrin, cypermethrin and deltamethrin against house flies could be enhanced by the addition of chlorpyrifos, profenofos, and in some cases by emamectin or fipronil, which reveal that these insecticides might be countering the resistance mechanisms in the present population. Corbett [31] proposed a general theory to explain the synergistic interactions among insecticides. According to this, one product or toxicant in the mixture interferes with the metabolic detoxification of the other toxicant, thereby synergizing the toxicity of the latter toxicant. In addition, insecticides from pyrethroid and organophosphate classes may be potential or competitive substrates for the same oxidase, as demonstrated by Kulkarni and Hodgson [32], thus potentiating the toxicity of the insecticide mixture. Moreover, it was also proposed that when pyrethroid/organophosphate mixtures applied against resistant insects, OP insecticide might bind to the monooxygenase that the first result in the activation of molecules, and then prevent the binding and subsequent degradation of pyrethroid insecticide by monooxygenase enzymes. These enzymes when bind to the OP insecticide could also lead to non-toxic metabolites by the process of hydroxylation of either oxon or thioate forms, which ultimately results in the degradation via oxidative ester cleavage. In this way, the binding of monooxygenase enzymes with OP insecticide would prevent or delay the degradation, and enhanced the toxicity of pyrethroid insecticide by competitive substrate inhibition mechanism [26], [32]. Previously it has been assumed that organophosphates, when used in combination with pyrethroids, inhibit the enzymes (monooxygenases and/or esterases) responsible for metabolic detoxification in different insect pests [6], [9], [10]. In the present study, the organophosphates largely overcame the resistance of pyrethroids, may be due to the inhibition of metabolic detoxification enzymes. We have no direct evidence of enzyme inhibition

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by organophosphates, but this hypothesis could be checked in the future studies.

The use of two enzyme inhibitors in the present study viz., PBO and DEF, against the field population of house flies largely overcame the resistance to the pyrethroids and organophosphates, which indicate that the resistance was mixed function oxidase or esterase based. However, both inhibitors did not synergise any insecticide against the laboratory strain. Previously, the effect of enzyme inhibitors in enhancing insecticide toxicities has been studied in different insect pests. In house flies, for example, the mixture of PBO with different pyrethroids increased the toxicity of cypermethrin, deltamethrin and permethrin [33]. Although PBO is a very good synergist, it should be used cautiously in field due to possible ill effects on non-target organisms. Moreover, the environmental protection agency (EPA) has categorized it as class C human carcinogen [34]. The occurrence of mixed function oxidase or esterase based resistance has been reported in different insect pests. PBO and DEF have also showed good synergism with cypermethrin in *Agrotis ipsilon* and *H. zea* [35], *S. litura* [12], [36] and *S. exigua* [37]. Compared with other insecticides, emamectin and fipronil seem to resist the enzymatic attack, as there was no synergism by PBO and DEF for fipronil, and DEF for emamectin and fipronil. Such type of results indicates that other mechanisms of insecticide resistance like decreased cuticular penetration and target-site insensitivity may be of major importance in the present population for emamectin and fipronil; however, further studies are required to confirm the exact mechanisms of resistance to these insecticides in Pakistani populations of house flies.

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The findings of the present study showed the presence of resistance to all the insecticides under investigation that suggest that multiple resistance mechanisms may be present in field population of house flies. In conclusion, the occurrence of resistance to different insecticides in disease vectors of public health importance has stressed the need to search out ways and develop strategies to manage resistance in field conditions [2]. The results reveal that the toxicity of bifenthrin, cypermethrin and deltamethrin to house flies could be enhanced by the addition of chlorpyrifos, profenofos, and in some cases by emamectin or fipronil. Insecticide mixtures could be very helpful in the management of house flies, especially in cases where synergistic interactions may occur between insecticides. Since, owing lack of systematic management plans for house flies in Pakistan [16], [17], such resistance management tools are not in practice here. Recently, it has been reported that house flies got indirect exposure to chemicals used for the management of

different dairy pests [16]. Insecticide mixtures, as a resistance management tool, should be given importance whenever a systematic management plan for dairy pests including flies will be designed by the policy makers. However, the fact of the occurrence of multiple resistance when insecticide mixtures are used for long, and effects on beneficial organisms should not be ignored [26]. For this, alternative tactics for the management of insecticide resistance like fine scale mosaics and/or rotation of insecticides should also be given importance. Moreover, studies on basic and operational aspects of interaction between insecticides in mixtures should be strengthened to set up adequate resistance management plans under field conditions.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: HAAK, WA SAS JL. Performed the experiments: HAAK. Analyzed the data: HAAK. Contributed reagents/materials/analysis tools: HAAK WA JL. Wrote the paper: HAAK.

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SECTION VII: ENVIRONMENTAL TOXICOLOGY: ECOTOXICOLOGY

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Chapter 13

Graphing Ecotoxicology: The MAGIC Graph for Linking Environmental Data on Chemicals

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ABSTRACT

Assessing the impact of chemicals on the environment and addressing subsequent issues are two central challenges to their safe use. Environmental data are continuously expanding, requiring flexible, scalable, and extendable data management solutions that can harmonize multiple data

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sources with potentially differing nomenclatures or levels of specificity. Here, we present the methodological steps taken to construct a rule-based labeled property graph database, the "Meta-analysis of the Global Impact of Chemicals" (MAGIC) graph, for potential environmental impact chemicals (PEIC) and its subsequent application harmonizing multiple large-scale databases. The resulting data encompass 16,739 unique PEICs attributed to their corresponding chemical class, stereo-chemical information, valid synonyms, use types, unique identifiers (e.g., Chemical Abstract Service registry number CAS RN), and others. These data provide researchers with additional chemical information for a large amount of PEICs and can also be publicly accessed using a web interface. Our analysis has shown that data harmonization can increase up to 98% when using the MAGIC graph approach compared to relational data systems for datasets with different nomenclatures. The graph database system and its data appear more suitable for large-scale analysis where traditional (i.e., relational) data systems are reaching conceptional limitations.

Keywords: Ecotoxicology, graph database, environmental data, data harmonization, chemical use types, organic contaminants, synonyms, nomenclature, specificity

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SUMMARY

The primary concern of ecotoxicology is the impact of chemicals on the environment [1]. To assess this impact at a large-scale, i.e., continental or global context, data of environmental concentrations, effects, use types or application rates have to be incorporated into a consistent structure. Today, science can rely on numerous databases providing these data (Table 1) for potential environmental impact chemicals (PEICs, e.g., pesticides, industrial chemicals, flame retardants, and solvents). However, the process of linking them takes significant harmonization efforts, even after a common semantic framework has been established, i.e., even after their integration into a coherent base. Among the most fundamental reasons hindering instant data linkage and affecting dimensions of the ecotoxicological data are differing nomenclatures and differing levels of specificity (see Table 2 for examples). For instance, when linking data spatially, some problems typically arise around issues of specificity, i.e., data present at different spatial scales or resolutions, while linking data within chemical dimension is often impeded by the usage of different nomenclatures. For ecotoxicology, however, the

specific interest lies in the chemical dimension, as it applies to all core data (Table 1).

Table 1: Core data, their dimensions and exemplary datasets, providing these data and being used in this study

Type of Data	Dimensions	Example Datasets
Environmental Concentrations	Space, time, medium, chemical	WQP [1]
Biological effects	Species, medium, chemical	ECOTOX [2], FOODTOX [3]
Use types	Chemical	PAN [4], (WQP)
Application rates	Space, time, chemical	USE [5]

[1] National Water Quality Monitoring Council—Water Quality Portal (WQP) [2]. [2] U.S. EPA ECOTOX database (ECOTOX) [3]. [3] European Food Safety Authority—OpenFoodTox (FOODTOX) [4]. [4] Pesticide Action Network—Pesticide Database (PAN) [5]. [5] U.S. Geological Survey—Estimated Annual Agricultural Pesticide Use (USE) [6].

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Table 2: Examples of problems occurring when linking data from different sources

Field of Problem	Affected Dimension	Problem	Example
Nomenclature	Chemical	Different spellings	Lambda-cyhalothrin–cyhalothrin, lambda
		Different nomenclatures	Thiametoxam (trivial name)–153719-23-4 (CAS RN)
	Space	Different languages	United States of America–Estados Unidos de América
Specificity	Chemical	Different stereo-chemical information	Concentration of beta-cyfluthrin–threshold of cyfluthrin
	Space	Different spatial resolution	Concentration at GPS coordinate–use rate at county-level
	Time	Different temporal resolution	Concentration with date–use data in yearly resolution

The usage of different nomenclatures is a well-known issue in chemistry [7,8]. Although a chemical compound is defined by its molecular structure, there is no exclusive convention for naming or identifying it. Instead, there are many concurring schemes based on two different approaches: naming

chemicals based on their molecular structure (International Chemical Identifier (InChI) and Simplified Molecular Input Line Entry Specification (SMILES) [7,9]) or assigning arbitrary, yet unique, identifiers to them (e.g., Chemical Abstract Services registry number (CAS RN) and Distributed Structure-Searchable Toxicity substance identifier (DTXSID) [10,11]). In addition, many chemicals, such as pesticides, also have various other names (e.g., trivial, brand, and formulation names) that may also differ among languages (Table 2). The co-existence of these naming schemes results in a high number of synonyms making nomenclature an important issue, particularly when linking larger datasets. This issue further aggravates in analyses that operate in a trans-national or global context, consider many PEICs and require harmonizing many different data sources.

Differing data specificity is also an issue when linking chemical data. Measured environmental concentrations or effect endpoints may be provided in different databases specifically for any kind of isomers, including data specific to enantiomers or diastereomers, or at the level of unique compound structures, ignoring stereo-compositions and, thus, including isomeric and racemic mixtures. Differing specificity, if not addressed, substantially hinders the integration of data from different sources for some of the ecotoxicologically most important groups of compounds, e.g., insecticides [12], that may act substantially differently based on their stereo-chemical composition [13].

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For analyses that cover only relatively small sets of PEICs, problems of nomenclature and specificity can be handled manually by expert judgment. Knowledge of PEICs thereby allows constructing data analysis workflows that cover all deviations in chemical names and that reasonably span different levels of specificity. Larger analyses that cover several dozens, or more, of PEICs are often based on relational data representations [12]. In the case only two different naming schemes are involved, e.g., if only two data sources are linked, differing identifiers of the same chemical can still be resolved by establishing a synonym table. However, relational database joins are costly, and linking more than two different data sources by joining their chemical identifiers (e.g., chemical name) with synonym tables increases the processing complexity significantly, quickly reaching points where complex data analyses become cumbersome [14]. Moreover, resolving different levels of specificity within and among relational datasets requires sophisticated techniques that entail even more effort to develop and that can hardly be established without significant lack of performance [15]. At least when combining more than two data sources—a requirement of

many ecotoxicological meta-analyses—relational data representations are suboptimal due to their constraints in performance and usability [15,16,17].

Labeled property graph databases represent an effective tool to address the aforementioned issues of extendibility, scalability, and flexibility [14,18,19]. Briefly, a labeled property graph consists of nodes (vertices) that are connected through relationships (directed edges) [19]. Both nodes and relationships can be labeled to distinguish functional roles and can be enriched with properties (see Appendix A for further details). In contrast to relational database systems, the number and type of relationships between entities is, thus, not strictly defined and allows linking information very flexibly [18,20]. This flexibility and the graph's emphasis on relationships appear well suited for the establishment of a synonym database that can also resolve hierarchical relationships [14,15]. Consequently, over the last years, graph databases have evolved as a technical alternative to the established relational database systems, featuring large-scale business (e.g., logistics, social media, and health management) and scientific applications (e.g., web science and sociology) where relational solutions become unfeasible [21,22]. However, even after an extensive literature review, we could not find any published approach that uses a graph for managing and analyzing data in ecotoxicology.

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The aim of this study was to assess the usability of graph databases for large-scale ecotoxicological meta-analyses that integrate and link a wide range of relevant data (Table 1) and was conducted by the research group "Meta-Analysis of the Global Impact of Chemicals" (MAGIC). In addition, multiple ecotoxicologically-relevant databases were used to perform a data harmonization, using U.S. EPA Chemical Dashboard (CDDB) [11] as a synonym provider, demonstrating the method's applicability in a large-scale ecotoxicological scope and quantifying the method's advantages compared to relational joins. The data were subsequently reprojected into tabular form, granting easy accessibility to researchers and professionals. The Microsoft® Excel worksheet published with this data description summarizes the information that is currently contained in the MAGIC graph in a tabular format, while an up-to-date version of the MAGIC graph can be explored using our website (https://magic.eco; see User Notes). Harmonized data for 16,739 PEICs in the MAGIC graph contain information about unique identifiers (CAS RN and DTXSID), valid synonyms, respective chemical classes, use type classification and their inclusion in various databases. We are positive that the MAGIC graph can serve as a reliable proof that, with graph databases, one already has a suited data integration tool at hand.

With it already being actively integrated in large-scale risk analysis at the national level [23], the MAGIC graph will find further applications and become a central tool in trans-national or global risk analyses in the future (DFG SCHU 2271/6-2). The MAGIC graph is publicly available and will provide a continuously expanding feature set, allowing researchers to take advantage of graph database solutions.

DATA DESCRIPTION

Database

The MAGIC graph contains 16,731 PEICs (see Microsoft® Excel worksheet for complete list). For these chemicals, chemical identifiers (n = 66,636) used by relevant datasets (Table 1) are stored and linked to the chemicals they identify. Each chemical has a preferred name for consistent creation of output. Use types and chemical classes, as provided by external datasets, are included in the MAGIC graph and linked with the chemical identifier used by the external dataset. The resulting schema (Figure 1) allows collecting chemical information over multiple databases regardless of the individually used identifiers by navigating the graph (Figure 2). Up-to-date contents of the graph can be retrieved using the website https://magic.eco (also see user notes).

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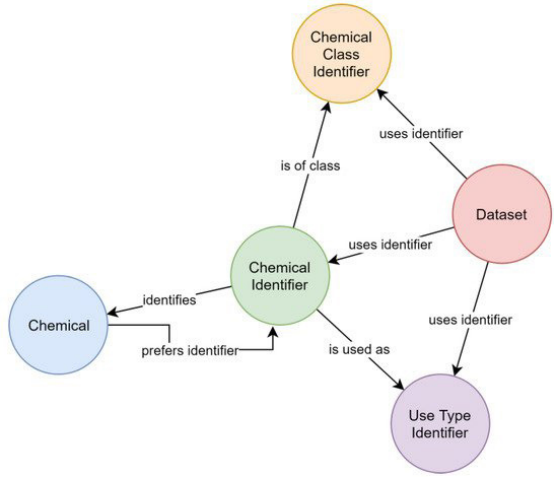
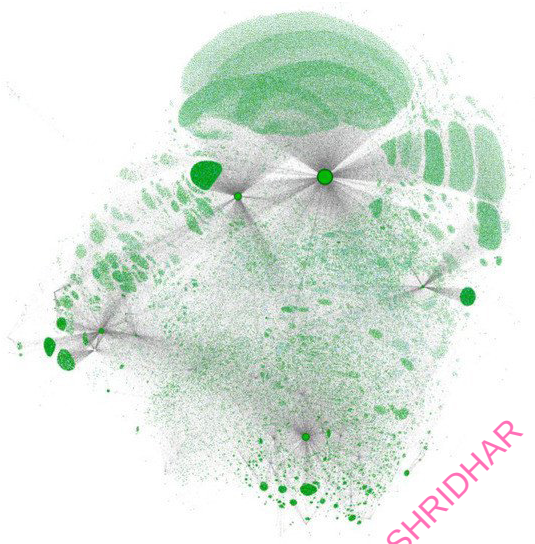


Figure 1: Schema of the "Meta-analysis of the Global Impact of Chemicals" (MAGIC) graph depicting typed relationships (arrows) between labeled nodes (circles).



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Figure 2: Network map of the MAGIC graph. A subset of chemical identifiers is shown as green dots. Larger circles outlined in black represent the datasets included in the graph. Grey lines connect the datasets with chemical identifiers, and individual identifiers with chemicals (cyan circles, rarely visible).

Summary Microsoft® *Excel Worksheet*

Published with this data descriptor is a Microsoft® Excel worksheet that summarizes the content of the MAGIC graph. The columns of this worksheet are described in Table 3.

Table 3: Description of the submitted Microsoft® Excel worksheet

Column	Description
Chemical	The preferred name of the chemical as derived from the CDDB. In most instances, the name given here equals the preferred name of the CDDB.
CAS RN	The currently valid Chemical Abstract Service registry number as given by the CDDB. Alternative CAS RNs, such as deleted numbers, are given under synonyms if they are used by at least one of the databases included in the MAGIC graph.
DTXSID	The substance identifier of the distributed structure-searchable toxicity database as provided by the CDDB

Synonyms	Additional identifiers of the chemical. Synonyms are only listed if they are used by at least one of the databases included in the MAGIC graph.
Chemical Class	The chemical class according to the PAN and WQP dataset. Only chemicals occurring in one of these datasets are classified and classifications are given here as is. Chemical classifications will be extended by considering further databases and harmonized among databases in the future.
Stereochemical	An "x" indicates stereo-chemical information is associated with the chemical.
Insecticide	An "x" indicates that the chemical is used as an insecticide according to the PAN database.
Herbicide	An "x" indicates that the chemical is used as an herbicide according to the PAN database.
Fungicide	An "x" indicates that the chemical is used as a fungicide according to the PAN database.
Microbiocide	An "x" indicates that the chemical is used as a microbiocide according to the PAN database.
Other Uses	A list of other uses of this chemical (excluding insecticide, herbicide, fungicide and microbiocide) according to the PAN and WQP databases. As with chemical classes, use type classification will be improved continuously over the next versions of the MAGIC graph.
WQP	Entries marked "x" indicate that the WQP database contains records of this chemical, using any of its identifiers.
ECOTOX	Entries marked "x" indicate that the ECOTOX database contains records of this chemical, using any of its identifiers.
FOODTOX	Entries marked "x" indicate that the FOODTOX database contains records of this chemical, using any of its identifiers.
USE	Entries marked "x" indicate that the USE database contains records of this chemical, using any of its identifiers.
PAN	Entries marked "x" indicate that the PAN database contains records of this chemical, using any of its identifiers.

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Database Linkage and Pesticide Use Types

The databases listed in Table 1 were integrated into the MAGIC graph and subsequently analyzed individually regarding the chemical identifiers they contain (Table 4). These databases were selected because they are the most comprehensive resources for large-scale ecotoxicological core data from governmental and non-governmental sources. The MAGIC graph made

it possible to evaluate how many of the identifiers used by each dataset actually identified chemicals, and how the identified chemicals were distributed among chemicals with stereo-chemical information and those without. Further, the number of synonymous identifiers within each dataset was identified.

Table 4: Characterization of ecotoxicologically-relevant datasets using the MAGIC graph

Dataset	ID Type	Identifiers		Syno-nyms [4]	Chemicals	
		Chemical [1,2]	Other [2,3]		Stereo- [5]	Non-Stereo [5]
WQP	CAS RN, name	6384 (65%)	3374 (35%)	3133	385 (11%)	2987 (89%)
ECOTOX	CAS RN	11,550 (73%)	4214 (27%)	28	1454 (13%)	10,068 (87%)
USE	Name	451 (92%)	40 (8%)	2	58 (13%)	391 (87%)
PAN	CAS RN, name	10,399 (69%)	4640 (31%)	4453	707 (12%)	5388 (88%)
FOODTOX	CAS RN, name	4190 (75%)	1375 (25%)	752	636 (18%)	2802 (82%)

[1] Identifiers that were linked to specific structurally unique compounds using the CDDB. [2] Percentages refer to the entirety of chemical identifiers in the dataset. [3] Identifiers used by the respective dataset that could not be linked to a specific chemical using the CDDB. [4]Synonyms refer to the amount of additional chemical identifiers attributed to chemicals. [5] Percentages refer to the entirety of chemicals in the dataset.

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The considered databases vary in the absolute number of chemicals they cover and the proportion of identifiers for chemicals (Table 4). For instance, 35% of WQP identifiers are not categorized as a "chemical", because they refer to mixtures, physical attributes (e.g., temperature and flow velocity), biological parameters (e.g., algal density and toxicity endpoints) or other, non-chemical information. Lower proportions of chemical identifiers may primarily indicate that the respective database is not only focused on PEICs but also on other entities, such as formulations, mixtures, etc. However, lower proportions may also be a result of low-quality data reporting, such as non-adherence to standardized nomenclature.

The characterization of databases further reveals that PEICs with isomeric information constitute 11–18% of chemicals in all analyzed databases (Table 4). Integration of hierarchical structuring is therefore a graphs' valuable feature that not only allows for a more detailed

differentiation among chemicals but also enables transparent analyses over multiple levels of specificity. Synonym analysis shows that, for instance, in the ECOTOX database, synonymous relationships are rare ($n = 28$; <0.2%), which underlines the CAS RNs' suitability as identifiers. Nonetheless, while CAS RNs uniquely identify chemicals, there may be multiple CAS RNs (e.g., CAS RN vs. deprecated CAS RN) referring to the same chemical (e.g., cyfluthrin). This may produce spurious analysis results, if unaddressed. With the MAGIC graph, however, analyses are based on chemicals, instead of identifiers, and all data, related to a chemical, are considered equally, regardless of the chemical identifier used.

We also assessed to what extent the MAGIC graph allows linking more chemicals over the different datasets compared to a relational approach where only same-spelling identifiers were considered linkable (Figure 3). We found that linkage increased only marginally (1–2%) when using the graph in the case both merged databases used CAS RN (see Table 4). This increase, although only small, underlines that, even with CAS RN, nomenclature can be an issue for data linkage. Relational joins using same-spelling names were only successful for 0–63% compared to the graph approach (Figure 3), signifying that joins relying on names are substantially affected by differing nomenclatures. In contrast, the graph approach successfully linked 21–99% of entries. With relational joins, it was impossible to link CAS RN from one dataset and chemical names from another, whereas, with the graph approach, we successfully linked 98% of the data from ECOTOX and FOODTOX, a linkage that depends on using CAS RN and names simultaneously (Table 4). While this case may also be partially resolved using relational joins, prior manual harmonization of chemical identifiers would be required, which is time-intensive, yet unnecessary, when using the graph approach. Figure 3 provides further information, e.g., on fractions for individual database pairs or total linkage of chemical data. In large-scale ecotoxicological assessments, transcending national or continental boundaries, harmonization and subsequent linking of data may become unfeasible, while the MAGIC graph approach can provide better performance and coverage compared to traditional relational joins.

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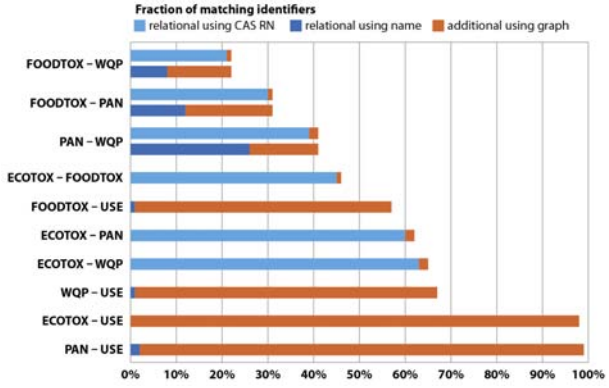


Figure 3: Linkage of chemical data relative to a theoretical maximum for different databases. Complete linkage presumes that all chemicals listed in the smaller database are contained in the larger one. Linkage with a relational approach, where only CAS RN (light blue) or same-spelling chemical identifiers match (dark blue), is compared to the additional gain with the graph approach (orange). See Table 4 for the types of identifiers that were available for each database.

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The use types and chemical classes of the PAN database give an example of how data, included in the MAGIC graph, can be used for characterizing datasets: the ECOTOX, FOODTOX and PAN databases cover a broad range of chemicals, including similar proportions of insecticides, herbicides, fungicides and microbiocides (Figure 4). In contrast, the USE dataset shows a higher proportion of insecticides, herbicides and fungicides, and a lower proportion of microbiocides, reflecting its focus on agricultural pesticide applications. Similarly, the WQP contains relatively fewer data of insecticides, herbicides and fungicides, since the number of chemicals being breakdown products (classified as other use type) in this environmental concentration dataset is rather high. The integration of the PAN database use types into the MAGIC graph thus enables an unprecedentedly comprehensive overview of the kind of PEICs that are contained in individual datasets (Figure 4). In addition, the successful data harmonization further demonstrates that ancillary chemical data can be readily incorporated into the MAGIC graph. For instance, supplementing regulatory information (e.g., regulatory status, environmental quality criteria) may now be added with only little effort.

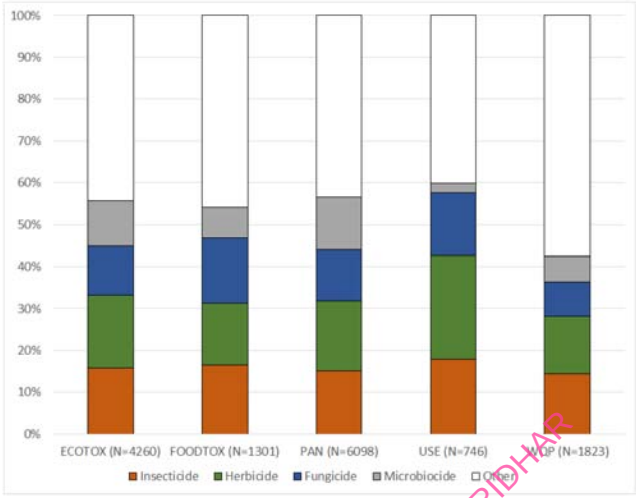


Figure 4: PAN use types of the chemicals in different datasets after linking them with the MAGIC graph. Chemicals may have multiple use types and are then included in several categories.

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METHODS

In contrast to relational database management systems, graph databases do not depend on predefined schemata. Briefly, nodes, relationships, labels, types and properties can be added, modified and removed ad hoc and as needed. While this tremendously facilitates the management of changing and growing heterogeneous datasets, it also complicates the usage of these data. Without a static and technically binding schema, the current semantics have to be discovered dynamically: it has to be found what kinds of nodes there are, what properties they have, how nodes are related to other nodes, etc. These concerns were addressed by specifying features of the data model informally outside the database and included semantics of node labels, relationships between nodes and restrictions of properties. To maintain consistency between this specification and the content of the database, as well as safeguard data integrity, 32 rules, checking specific aspects of the data model, were implemented (Appendix B, Table A1). Rules were iteratively formulated by expert judgment whenever new conceptual or technical requirements arose, while it was generally aimed at maintaining a small set of rules. Violations of the rules result in notifications that have to be resolved manually or semi-automatically (Figure 5). This rule-based

approach provides a balanced tradeoff between benefits of an agreed schema and flexibility of a graph database. Turning the a priori schema known from relational databases into a posteriori applied consistency rules also resulted in work-flows that resemble those of test-driven developments [24]. For instance, extension of the domain of the graph database application, e.g., by additionally linking taxonomic data to effect data, is achieved in the two following steps. First, one specifies and implements a set of additional rules, e.g., "species and genus are allowed labels", "an effect must be linked to a species", "a species belongs to a genus", etc., and afterwards modifies the database by adding nodes and relationships until all rules are fulfilled. Fulfillment of all rules then marks a new version of the database application that provides additional information.

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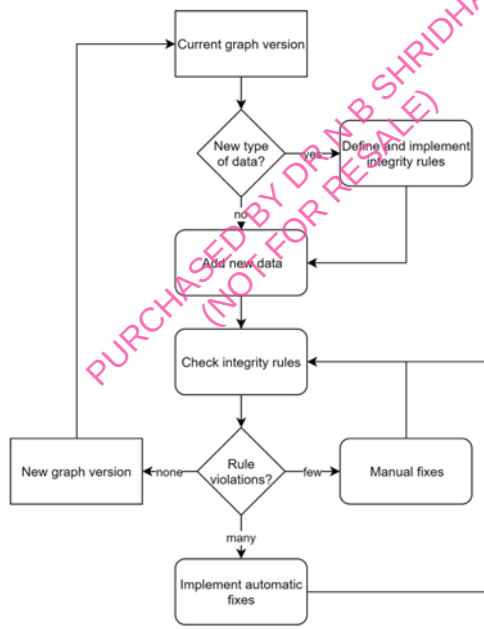


Figure 5: Workflow for adding data to the MAGIC graph while maintaining its integrity.

Initially, six publicly available chemical databases were identified and compared regarding quality of synonyms they provide for an array of organic pesticides ($n = 655$). After extensive quantity and quality assessments of the generated synonym links, the U.S. EPA Chemical Dashboard [11], containing approximately 765,000 chemical entries, was chosen as a synonym provider (see Appendix C).

In the first implementation, synonymous chemical identifiers (e.g., substance names) were interlinked directly (Figure 6a). However, following this concept, the number of steps necessary to collect all synonyms of a given identifier varied between queries, which resulted in complex queries. This concept also complicated the estimation of the quality of synonym relationships, as two distant identifiers could be linked over relationships of different certainty. A later refined representation distinguished between the chemical itself and its identifiers (Figure 6b), leading to a representation where the step sequence for collecting all synonyms of a chemical is well defined and only requires two steps. This adjustment improved the computational efficacy, at the same time allowing to add further chemical identifiers without increasing the maximum number of steps required.



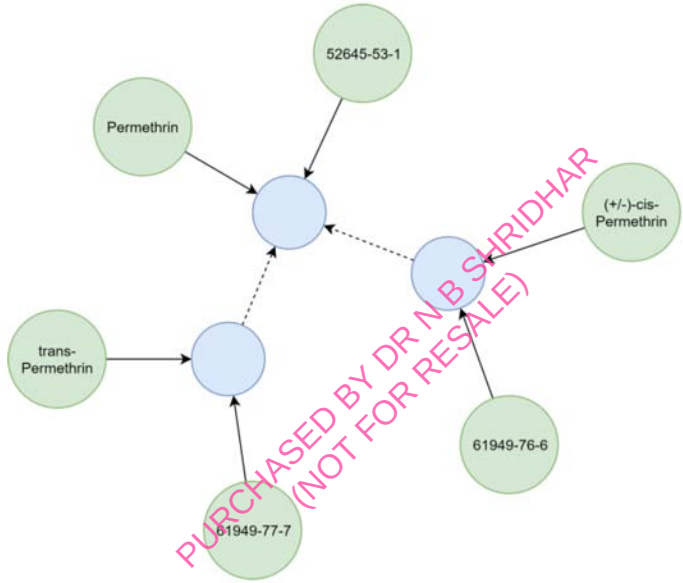
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Figure 6: Two representations of four synonym identifiers (1–4) in a graph. (a) Links between identifiers indicate known synonym relationships. Synonyms of an identifier are all directly or indirectly connected other identifiers. (b) Identifiers point to the identified chemical (blue circle). All identifiers pointing to the same chemical are synonyms. The chemical has a preferred identifier (double arrow).

Consistency between chemical query results was achieved by attributing each chemical a preferred identifier (used by the CDDB), so that chemicals can be identified in a default way. A descriptive property was attributed to relationships between identifiers and chemicals to reflect the identification type, e.g., CAS RN. Data output for chemicals can, thus, be restricted to specific types of relationships for identifying chemicals.

Substances relevant in ecotoxicological contexts can be described by varying detail of specificity (e.g., isomerism), which was addressed by creating hierarchical chemical sub-graphs. For example, permethrin (Figure 7), an insecticidal compound, represents a stereoisomeric mixture of cis- and

trans-permethrin isomers. The respective relationships between chemicals were resolved considering the presence of stereo-layers in their standard InChI strings [25], creating a hierarchical sub-graph (Figure 7). Further distinction of hierarchical levels (e.g., enantiomers and diastereomers) currently is not technically possible, as standard InChI strings do not support this operation [25]. However, it is also rarely needed for ecotoxicological assessments using field concentrations.



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Figure 7: Representation of some synonyms and different levels of specificity for permethrin. Identifiers (green) refer (solid arrows) to chemicals (blue). Chemicals with stereo-information refer (dashed arrow) to a structural identical chemical without stereo-information.

After evaluation of different solutions, the MAGIC graph was hosted using the Neo4j native graph database, a mature, actively developed and widespread graph database product that is available as an Open Source Community Edition (GPLv3 license) and as an extended Enterprise Edition. The MAGIC graph was implemented using versions 3.4.5 to 3.5.0 of the Community Edition (updates were applied as soon as available). Validation rules (Appendix B), as well as tools for automatic rule violation fixes, were implemented using PHP and integrated into an Apache 2 web server. For conducting manual fixes, a set of graphical tools was evaluated (Appendix D). The web server hosts a publicly available website (https://magic.eco)

that allows, among other functions, to access the data of the MAGIC graph (see user notes).

Currentness of data is accomplished by synchronization routines, which update the MAGIC graph when some external data sources, especially the synonym provider, change. To detect differences between the graph and external sources, we reapplied the rule-based approach by implementing a set of synchronization rules (Table A2). Violations of these rules indicated differences between databases, prompting a synchronization routine.

USER NOTES

The website https://magic.eco provides access to the most recent version of the MAGIC graph. It offers the possibility to visit individual chemical identifiers and to discover the synonyms and generalizations as well as the data that are currently connected to the chemical. The website also provides a user with an option to download an up-to-date version of the Microsoft® Excel worksheet published with this data descriptor.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.B. and J.W.; methodology, S.B. and J.W.; software, S.B.; validation, S.B. and J.W.; formal analysis, J.W.; investigation, J.W.; resources, R.S.; data curation, S.B. and J.W.; writing—original draft preparation, S.B., J.W., S.S., L.L.P. and R.S.; writing—review and editing, S.B., J.W., S.S., L.L.P. and R.S; visualization, S.B.; supervision, S.S. and R.S.; project administration, S.S. and R.S.; and funding acquisition, S.S. and R.S.

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APPENDIX A

A graph consists of vertices that are connected by edges. There is a variety of graph models that define the specifics of how vertices are connected

by edges, but we adhere to the conceptually simple, yet powerful, labeled property graph model. A graph consists, according to this model, of vertices named nodes that are interconnected by directed edges named relationships [19]. Relationships always connect two nodes but nodes may have an arbitrary number of out- or ingoing relationships, including relationships to itself or multiple relationships to the same other node. Nodes have zero to many labels that allow differentiating them functionally. Similarly, relationships have types, but the number of types per relationship is limited to one. Nodes and relationships can be enriched by attaching an arbitrary number of properties to them, each containing additional information to the node or relationship. In essence, the labeled property graph model offers a high degree of flexibility while it provides constructs such as labels, types, and properties that help in structuring the data.

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APPENDIX B

A rule-based approach has been chosen to maintain integrity of the MAGIC graph and to keep it synchronized with its synonym provider. This appendix sections lists the rules that have been defined and implemented in the MAGIC graph.

Table A1: Rules defining the MAGIC chemical graph

ID	"Rule" and Rule Description
G1	"All nodes must have a single label": Ensuring that each node has exactly one label results in a graph that is easier to maintain, as other rules can refer to specific sets of nodes without having to deal with possible labeling overlaps. If the graph gets more complex in the future, it might however become advantageous to allow multiple labels per node.
G2	"Only a set of predefined labels is allowed for nodes": Restricting labels to a predefined set prevents nodes in the graph that are not targeted by rules. The MAGIC graph may currently contain nodes with labels "ChemicalIdentifier", "Chemical" and "Dataset".
G3	"Nodes should have a label with an associated view": Making sure that each label has a defined way that it is represented by the front-end website makes the MAGIC graph completely navigable.
G4	"Only a set of predefined types is allowed for relationships": Predefining types assures that all relationships are addressed by rules. Currently, the relationship types "identifies", "prefers_identifier", "uses_identifier" and "specifies" are allowed in the MAGIC graph.

G5	"Every item in the MAGIC graph must have a name": Naming items provides an endpoint for visiting the item using the website front-end and facilitates modifications and synchronization of the graph by allowing identification of individual nodes. For many types of items, such as chemical identifiers, the name is a natural part of the data.
G6	"Labels should have an associated edit view": Edit views allow smaller modifications of items using the website front-end and the provision of such edit views helps in maintaining the graph database.
G7	"MAGIC graph items should have at least one ingoing relationship": Items, having no ingoing relationship, lack in significance because they are not navigable along the graph relationships and should not be part of the graph. Some items are considered as entry points to the graph (e.g., datasets) and are marked as globally available. The rule does not apply to these items.
CI1	"All chemical identifiers nodes must have a timestamp": Registering the date of item creation assists in synchronization chemical identifiers with external data sources and helps to resolve rule conflicts by indicating which item is more recent.
CI2	"All chemical identifiers that actually identify a chemical should be linked to exactly one chemical": A chemical identifier should identify a chemical, otherwise it is irrelevant for the chemical graph. However, there are two typical occasions when a chemical identifier does not identify a chemical: (1) when a data source, from which data were imported into the graph listed an identifier as a chemical identifier, but further investigation revealed that the identifier did not refer to a chemical in a strict sense (e.g., it identifies a mixture of chemicals); and (2) when a chemical identifier was not found by the synonym provider. Violating this rule gives the user a chance to recognize and mitigate the second occasion, e.g., by adding manual synonym relationships. The user also has a chance to mark a chemical identifier in such a way that it does not trigger this rule anymore (by marking it as a chemical identifier that does not actually identify a chemical), which also signifies that the rule violation was recognized and managed manually.
CI3	"All chemical identification relationships should have a timestamp": Registering the date of relationship creation assists in synchronization with external data sources and helps to resolve rule conflicts by indicating which relationship is more recent.
CI4	"All chemical identifications should have a type": Specifying the mode in which a chemical identifier identifies a chemical helps in estimating the quality and uncertainty of the relationship. It also helps to output specific sets of identifiers, e.g., only CAS RN. The types used for specifying the relationship of identification are currently not restricted but may be a predefined set in the future.

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CI5	"All chemical identifiers should be used by at least one dataset": The data sources where a chemical identifier is used should be given. If this is not the case, retracing the origin of chemical identifiers is not possible which decreases the overall quality of the MAGIC graph.
CI6	"All chemical identifiers should have an identifier from a predefined list showing what is actually identified": Other rules depend on the information that a chemical identifier actually identifies a chemical, that is, there applies a stricter meaning of chemical than in some other databases. To provide this information, a chemical identifier should describe what it actually identifies. Currently, the following possibilities are considered here: chemical, mixture, unmatched chemical (by no means a corresponding chemical could be identified), unspecific (is not specific enough to identify exactly one chemical) and ignored (for any reason).
CI7	"A chemical identifier, that does not actually identify a chemical, should not be linked with a chemical": Specifying that a chemical identifier identifies a chemical only does make sense in case the chemical identifier is marked as actually identifying a chemical. If this is not the case, but an identifying relationship exists nonetheless, a manual examination of the case is advised.
C1	"All chemicals must have a timestamp": Registering the date of item creation assists in resolving conflicts involving chemicals by indicating which item is more recent.
C2	"All chemicals should have exactly one preferred name": According to the MAGIC graph data model, a chemical is considered having many names. Making sure that every chemical has exactly one designated preferred name still allows it to be referred to in outputs in a harmonized way.
C3	"All chemicals should be identified by at least one chemical identifier": Chemicals that have no identifier cannot be related to actual chemicals and should be removed from the graph.
C4	"All identifier preferences should have a timestamp": Name preferences of chemicals, especially when taken from external sources, may change over time. In these occasions, timestamps help to identify the more recent preference.
C5	"All chemicals should indicate whether they have bond stereo-chemical information": To understand which level of specificity regarding stereo-chemistry a chemical has, presence or absence of stereo-information at double bonds should be indicated.
C6	"All chemicals should indicate whether they have tertrahedral stereo-chemical information": To understand which level of specificity regarding stereo-chemistry a chemical has, presence or absence of stereo-information at tetrahedral stereo centers should be indicated.

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C7	"Chemicals with stereo-information should specify other chemicals or indicate to not do so": The purpose of considering stereo-information is to distinguish two levels of specificity regarding stereo-chemistry: absence and presence of stereo-information. In the case of stereo-information presence, a chemical should specify a chemical without stereo-information but with the same chemical structure, so representing the two levels of specificity in the graph. For some chemicals, it is not reasonable to find a chemical with the same structure but without stereo-information. In this case, the more specific chemical should be marked such that this rule can be ignored.
C8	"Chemicals may not specify themselves": Violations of this rule may occur when stereo-information in external data sources changes.
D1	"All datasets must have a timestamp": Registering the date of item creation assists in resolving conflicts in datasets by indicating which item is more recent.
D2	"All datasets must have a title": A title provides a more extensive way for a short description of the dataset but is not, unlike its name, used as an identifier.
D3	"All datasets must have a description": A description is an even more extensive opportunity to characterize a dataset by text.
D4	"All datasets should haven an indicator of whether they are published": Distinction between published and non-published datasets allows to decide which datasets are accessible by the website front-end.
D5	"All datasets should be published": At least at later stages, after inserting a dataset into the graph and fixing possible rule violations, the dataset should be published to make its data available.
D6	"All datasets must have at least one author": Assigning authors to a dataset is an attribution to the persons who were responsible for inserting the dataset into the graph.
UTI1	"All Use Type Identifiers should be used by at least one dataset": Use type identifiers originate from datasets and attributions to these datasets should be given.
UTI2	"All Use Type Identifiers should be used by at least one chemical identifier": All use type identifiers should be linked to at least one chemical identifier, otherwise they are of limited use for assessments.
CCI1	"All Chem Class Identifiers should be used by at least one dataset": Chemical class identifiers originate from datasets and an attribution to this dataset should be given.
CCI2	"All Chem Class Identifiers should be used by at least one chemical identifier": All chemical class identifiers should be linked to at least one chemical identifier, otherwise they are of limited use for assessments.

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Table A2: Synchronization rules

ID	Rule and Description
SyncCDDB1	"All identifiers in the MAGIC graph that actually identify a chemical should have exactly one match in the CDDB": Identifiers that have no match in the CDDB have been removed from there and should also be removed from the MAGIC graph. In some (rare) cases, chemical identifiers have two or more matches in the CDDB. These cases should be resolved manually, e.g., by ignoring the chemical identifier.
SyncCDDB2	"All identifiers of a specific chemical in the MAGIC graph should have the same preferred name and DTXSID in the CDDB": Having different preferred names among the synonym identifiers of a chemical is a strong indicator that synonym relationships in the CDDB have changed. This should result in an update of synonym relationships in the MAGIC graph as well.
SyncCDDB3	"The preferred name of a chemical in the MAGIC graph should be the same as the preferred name in the CDDB": Preferred names of the CDDB may change. Making sure we use the same preferred name in the MAGIC graph as in the CDDB circumvents the need to establish a custom scheme for preferred names.
SyncCDDB4	"The stereo-information of a chemical in the MAGIC graph should be the same as the stereo-information of that chemical in the CDDB": This rule captures changes in the chemical structure stored in the CDDB. Again, these changes should be synchronized with the MAGIC graph to reflect the most recent specifying relationships.
SyncCDDB5	"The identifier type of the relationship between a chemical identifier and a chemical in the MAGIC graph should be the same as in the CDDB": Synchronizing the identifier type between CDDB and MAGIC graph eliminates the necessity to manage a custom set of identifier types while still allowing to use the benefits of typed identifiers.
SyncCDDB6	"All identifiers in the MAGIC graph that do not actually identify a chemical should have no match in the CDDB": On some occasions, new identifiers become recognized by the CDDB. This rule captures those instances where the newly recognized identifiers match identifiers in the MAGIC that previously have been marked as not actually identifying chemicals.

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APPENDIX C

In total, 655 substance names—categorized as organic contaminants—were obtained from the Water Quality Portal (https://www.waterqualitydata.us/) and used for benchmarking six databases. First, successful synonym attribution was compared quantitatively (Table A3) between databases, and then the quality of synonym relationships was manually assessed by validating correctness of generated links using assigned standard InChI-Keys.

Table A3: Comparison of chemical synonym providers regarding automated attribution of InChI-Keys for 655 organic contaminants

Database	Coverage (%)	Remarks
U.S. EPA Chemical Dashboard [1]	586 (89.5%)	correct links
PubChem [2]	645 (98.5%)	ambiguous response, incorrect links
PUG REST [3]	604 (92.2%)	ambiguous response, incorrect links
SRS [4]	601 (91.8%)	rarely incorrect links
ChemSpider [5]	346 (52.8%)	low coverage
Chemical Translation Service [6]	613 (93.6%)	ambiguous response, incorrect links

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[1]https://comptox.epa.gov/dashboard/. [2]https://pubchem.ncbi.nlm.nih.gov/. [3]https://pubchemdocs.ncbi.nlm.nih.gov/pug-rest. [4]http://www.exchangenetwork.net/data-exchange/srs/. [5]http://www.chemspider.com/. [6]http://cts.fiehnlab.ucdavis.edu/.

Attribution of synonyms and InChI-Keys was high for all six databases with the exception of one (ChemSpider), which was removed from subsequent analyses due to its comparatively low coverage (Table A3). Following this, manual validation of assigned InChI-Keys revealed that attribution was frequently incorrect or query responses were ambiguous, except for the U.S. EPA Chemical Dashboard (CDDB). Although multiple factors leading to misattribution of InChI-Keys or synonyms were identified, automatic aggregation of synonym lists from online-sources lacking expert curation was found the most prevalent factor that adversely affected attribution quality. The CDDB, that, unlike the other considered databases, curates records and gives quality indicators, was thus identified as the most

reliable source for synonyms and corresponding InChI-Keys (see below for sample data retrieved from the CDDB). Synonym quality provided by the CDDB was further assessed to sustain the robustness of the method. Although automated linking of Chemical Identifiers to chemicals was high (89.5%, n = 586) when using the CDDB (Table A3), 69 WQP entries could not be automatically assigned to Chemical Identifiers. Missing entries were manually assigned CAS RN and InChI-Keys via cross-validation using, among other databases, PubChem, PAN and PPDB. Attributed CAS RN and InChI-Keys were then used to link missing entries with the corresponding CDDB entry, which was successful in 75.4% (n = 52) of remaining cases. No manual links could be established in 24.6% (n= 17) of cases, because no corresponding entry was found in the CDDB. Failure to automatically establish synonym relationships was mostly due to chemical names in WQP being abbreviated, using wrong or uncommon identifiers, or referring to entities that are not chemicals in a strict sense (i.e., mixtures). Most importantly, a manual check revealed that no false synonym relationships (i.e., incorrect links) were generated automatically using the CDDB as synonym provider, which was manually checked. Thus, overall correctness and reliability of generated links were without any noticeable concern.

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Data were retrieved from the EPA Chemistry Dashboard (https://comptox.epa.gov/dashboard) using its batch search. The following is an excerpt of data retrieved:

- Input: Lindane, Found by: Approved Name, DTXSID: DTXSID2020686, Preferred name: Lindane, InChI key: JLYXXMFPNIAWKQ-GNIYUCBRSA-N, InChI string: InChI=1/C6H6Cl6/c7-1-2(8)4(10)6(12)5(11)3(1)9/h1-6H/t1-,2-,3-,4+,5+,6+
- Input: cis-Permethrin, Found by: Expert Validated Synonym, DTXSID: DTXSID0038338, Preferred name: (+/−)-cis-Permethrin, InChI key: RLLPVAHGXHCWKJ-HKUYNNGSSA-N, InChI string: InChI=1/C21H20Cl2O3/c1-21(2)17(12-18(22)23)19(21)20(24)25-13-14-7-6-10-16(11-14)26-15-8-4-3-5-9-15/h3-12,17,19H,13H2,1-2H3/t17-,19-/s2
- Input: lambda-Cyhalothrin, Found by: Synonym from Valid Source, DTXSID: DTXSID7032559, Preferred name: λ-Cyhalothrin, InChI key: ZXQYGBMAQZUVMI-GCMPRSNUSA-N, InChI string: InChI=1/C23H19ClF3NO3/c1-22(2)17(12-19(24)23(25,26)27)20(22)21(29)31-18(13-

28)14-7-6-10-16(11-14)30-15-8-4-3-5-9-15/h3-12,17-18,20H,1-2H3/b19-12-/t17-,18+,20-/s2

These data are sufficient: (1) to identify synonymous identifiers (Input, DTXSID, Preferred name, (InChI key, InChI string)); (2) to evaluate the quality of the synonym relationship (Found by); (3) to assess the presence of stereo-chemical information in a chemical and compare chemicals with the same structure (InChI string, InChI key); and (4) to assign a common preferred name to a chemical (Preferred name).

APPENDIX D

Various graphical user interfaces have been tested to identify a suitable tool for minor interactive modifications of the MAGIC graph, preferably without coding Cypher queries (Table A4).

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Table A4: Comparison of graphical user interfaces for Neo4j databases

Name	Version	License	Graph Edit	Last Update	Remarks
Bloom		commercial	+	recently	https://neo4j.com/bloom/
Cytoscape	3.7.0	GNU		10/2018	Does not support Neo4j natively, but possibly via (outdated) plug-in [1]; http://www.cytoscape.org/
Gephi	0.9.2	commercial (free edition)	–	9/2017	Does not support Neo4j natively, but possibly via plug-in[2]; https://gephi.org/
Graphexp		Apache	+	10/2018	Does not support Neo4j natively; https://github.com/bricaud/graphexp
Graphileon	2.0.0-beta	GNU	+	8/2018	Graphs with datetime properties (introduced in Neo4j v3.4) cannot be visualized or edited; https://graphileon.com/graphileon-personal-edition/
Keylines	5.0	commercial	–	11/2018	https://cambridge-intelligence.com/keylines
Linkurious	2.5.4	commercial	+	7/2018	Offers trial version, without price information; https://linkurio.us/solution/neo4j/
Neo4j Browser	3.2.5	GNU	–	11/2018	Shipped with Neo4j database; https://neo4j.com/developer/guide-neo4j-browser/
Neo4j Browser (forked)	3.2.7	GNU	+	11/2018	Extends Neo4j Browser by editing functionality; https://github.com/phdd/neo4j-browser
Neo4js	2	open source	+	5/2018	https://github.com/adadgio/neo4j-js-ng2
Neoclipse	1.9.5	open source	+	9/2014	Does not support current Neo4j version; https://github.com/neo4j-contrib/neoclipse

Structr	3.0.3	commercial	+	9/2018	https://structr.com/
Tom Sawyer	8.2.2	commercial	−	11/2018	https://www.tomsawyer.com/graph-database-browser/

[1.]https://apps.cytoscape.org/apps/cyneo4j. [2]https://tbgraph.wordpress.com/2017/04/01/neo4j-to-gephi.

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Chapter 14

Sand Goby—An Ecologically Relevant Species for Behavioural Ecotoxicology

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ABSTRACT

Locomotion-based behavioural endpoints have been suggested as suitable sublethal endpoints for human and environmental hazard assessment,

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as well as for biomonitoring applications. Larval stages of the sand goby (*Pomatoschistus minutus*) possess a number of attractive qualities for experimental testing that make it a promising species in behavioural ecotoxicology. Here, we present a study aimed at developing a toolkit for using the sand goby as novel species for ecotoxicological studies and using locomotion as an alternative endpoint in toxicity testing. Exposure to three contaminants (copper (Cu), di-butyl phthalate (DBP) and perfluorooctanoic acid (PFOA) was tested in the early life stages of the sand goby and the locomotion patterns of the larvae were quantified using an automatic tracking system. In a photo-motor test, sand goby larvae displayed substantially higher activity in light than in dark cycles. Furthermore, all tested compounds exerted behavioural alterations, such as hypo- and hyperactivity. Our experimental results show that sand goby larvae produce robust and quantifiable locomotive responses, which could be used within an ecotoxicological context for assessing the behavioural toxicity of environmental pollutants, with particular relevance in the Nordic region. This study thus suggests that sand goby larvae have potential as an environmentally relevant species for behavioural ecotoxicology, and as such offer an alternative to standard model species.

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Keywords: Locomotion, Gobiidae, fish larvae, behavior, ecotoxicology

INTRODUCTION

Chemical monitoring programs worldwide are documenting increasing levels of pollution in aquatic ecosystems. Coastal environments are continuously subjected to diverse chemical pollution, deriving from offshore oil spills, industrial and agricultural runoff, or domestic waste discharges. It has been estimated that more than 100,000 potentially hazardous substances could be leaking into the environment from anthropogenic activities [1]. With respect to increasing environmental pollution, it is important to investigate the effects of complex chemical mixtures on aquatic organisms and assess their overall impact on the health and functioning of ecosystems. Over the years, various biochemical and physiological approaches have been developed and deployed for assessing pollution-induced consequences on different aquatic organisms, and biomarkers have become an integral part of biomonitoring programs worldwide [2,3,4,5]. The prevalence and diversification of anthropogenic pollution raises demand for development of new techniques and approaches for pollution assessment and monitoring in

the aquatic environment. The increased availability of OMICS (genomics, transcriptomics, proteomics, and metabolomics) techniques has stimulated research and development of novel molecular endpoints for toxicity assessment of various environmental pollutants [6,7,8]. Such techniques are well suited as early warning signs of exposure, are highly valuable for detecting changes at molecular level and can provide mechanistic toxicological information, but do not implicitly relate to adverse effects on individuals or at the population level [9]. Therefore, the development of novel and robust tools, designed to detect sublethal effects of environmental contaminants at individual and population level is needed.

Recently, behaviour has been proposed as a sensitive and integrative tool for sublethal toxicity screening for various environmental chemicals [10,11,12]. Several studies have reported that analysis of the swimming activity of larval stages of fish could provide predictions of mechanisms of action of unknown or less known compounds [13,14,15,16,17]. Behavioural studies using automatic video tracking systems have been shown to be successful for toxicity screening of different contaminants, like endocrine disrupting chemicals (EDCs), nanoparticles and metals [18,19], including complex mixtures [20,21,22]. Increasing recognition and use of behavioural endpoints in ecotoxicological studies is promoting interest for inclusion of behavioural effects as an alternative for lethal endpoints in environmental hazard assessment [12,23,24]. The use of locomotive characteristics for toxicological applications in various animal models, such as fish, amphibians [25,26], crustaceans [27,28], insects [29], nematodes [10] or mollusc larvae [30], has been described. This not only highlights the usefulness of video tracking systems for ecotoxicological applications, but also reveals the versatility of locomotion tracking tools and emphasizes the need for translatability and validation of behavioural assays across different biological domains.

The early life stages of animals are known to be particularly susceptible to environmental stress and are affected by both biological factors and physical factors such as water transparency, temperature and salinity [31,32,33,34]. Animals at early developmental stages have been found to be more sensitive than other stages to contaminants [35]. Toxicity tests using early life stages of fish are already extensively used for regulatory purposes [36,37] and have proven useful for predicting long-term consequences on organisms [38,39]. Besides the use of fish early life stages for chemical screening of single compounds, this approach could potentially be used for assessing the effects of complex (environmental) mixtures or employed for monitoring

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water quality [13,40,41,42,43]. The sand goby (*Pomatoschistus minutus*) is a small fish of the Gobiidae family with a widespread distribution along European coasts [44]. The species inhabits soft or sandy bottoms, and can be found across different temperature and salinity ranges from marine to brackish water [45,46,47]. Its reproductive behaviour has been reviewed by Breder & Rosen [48] and Forsgren [49]. In short, spawning takes place during spring and early summer (April to July on the Swedish west coast). During this season, male sand gobies build nests by excavating a space underneath a bivalve shell, or similar hard substrate, and covering the top with sand [50]. The male then tries to attract females to the nest by visual and acoustic courtship, and, if accepted, the female will lay her eggs as a single layer on the inside of the nest. The male fertilizes the eggs and exhibits parental care by tending to the eggs, fanning and guarding them until they hatch [51,52,53]. Females may spawn with several partners over the reproductive period and nests typically contain eggs from more than one female [54,55,56].

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The sand goby is a valuable and ecologically relevant marine fish species for ecotoxicological studies [57,58,59,60,61,62,63,64] and has even been proposed as a potential sentinel species for environmental monitoring [65]. Disruption of adult behaviour after exposure to sewage effluent and xeno-estrogens has been shown, together with gene expression measurements of common ecotoxicological markers such as hepatic vitellogenin [59,62]. Because adult sand gobies display a number of well-documented reproductive behaviours, including courtship, male competition and parental care, this species has been a suitable species for studying endocrine disruptors that affect such behaviours [57,59,60,62,66]. In contrast to adult behaviour, the behaviour of sand goby larvae is still poorly understood. Because egg development is relatively slow, especially early in the breeding season when water temperatures are low, a parental male will normally stay with his eggs for a considerable time (approximately 2–4 weeks in 8–12 °C, 1-2 weeks in 12-18 °C; [67]). This means that both breeding males and developing embryos are likely to be exposed to any pollution in the sediment or water column.

In light of increasing and diverse pollution, there is an urgent need for the development of new approaches for monitoring environmental pollution in the aquatic environment and for the establishment of new protective measures to ensure the health of aquatic ecosystems. The aim of this study was to explore the potential of using early stages of sand goby as novel species for ecotoxicological studies. Here, we specifically examine the

possibility of using automatic locomotion tracking methods for behavioural testing of sand goby larvae, which have been proven to be a sensitive tool for toxicity screening for various environmental chemicals in model species. A pilot study focusing on behavioural toxicity assessment of three common environmental pollutants (copper (Cu), di-butyl phthalate (DBP) and perfluorooctanoic acid (PFOA)) on sand goby larvae was performed. Within the scope of this project, we also assessed the feasibility of rearing the species under laboratory conditions and attempted to develop a methodology suited for ecotoxicological testing.

RESULTS

Photo-Motor Response of Sand Goby Larvae

The locomotion of sand goby larvae was quantified in a behavioural assay using alternating light and darkness as a locomotion-inducing stimulus (Figure 1). Early larval stages of sand goby (at 13–14 days post fertilization, dpf) showed repeatable locomotive patterns: relatively high activity under light and low activity under dark conditions.

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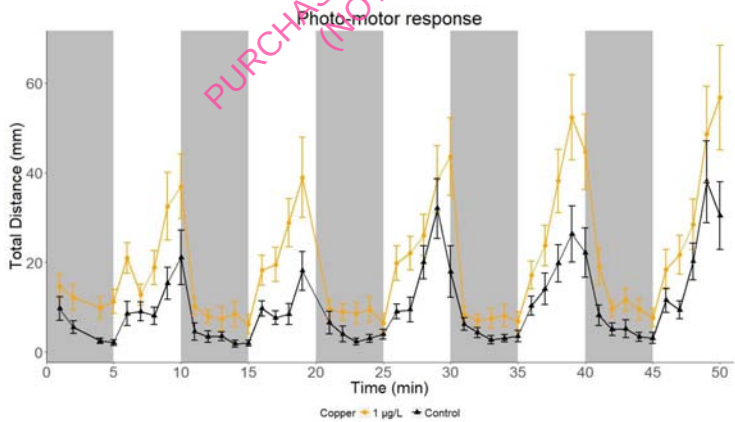


Figure 1: Photo-motor response of sand goby larvae exposed to copper (1 µg/L, n = 31) compared to control larvae (n = 38), tested under five cycles of light (white) and dark (shaded) conditions.

In each light cycle (5 min), the swimming activity of sand goby larvae consistently increased until the switch to darkness. After the onset of the dark cycle, the activity quickly (1 min) stabilized to a baseline level (5–10

mm/min). Such locomotive responses were consistently recorded in fish larvae, subjected to artificial light stimulation, for both control and exposed larvae (Figure 1). To exemplify the differential locomotive responses of sand goby larvae in a photo-motor test after toxicant exposure, a representation of swimming activity in five light:dark cycles of Cu (1 µg/L) in exposed and control goby larvae is displayed (Figure 1).

Locomotion-Based Behavioural Endpoints

Behavioural toxicity assessment consisted of analysis of three locomotion-based endpoints: activity (measured as counts of movements), duration and distance travelled that were analysed along the toxicant exposure gradient in light and dark cycles and compared to a control.

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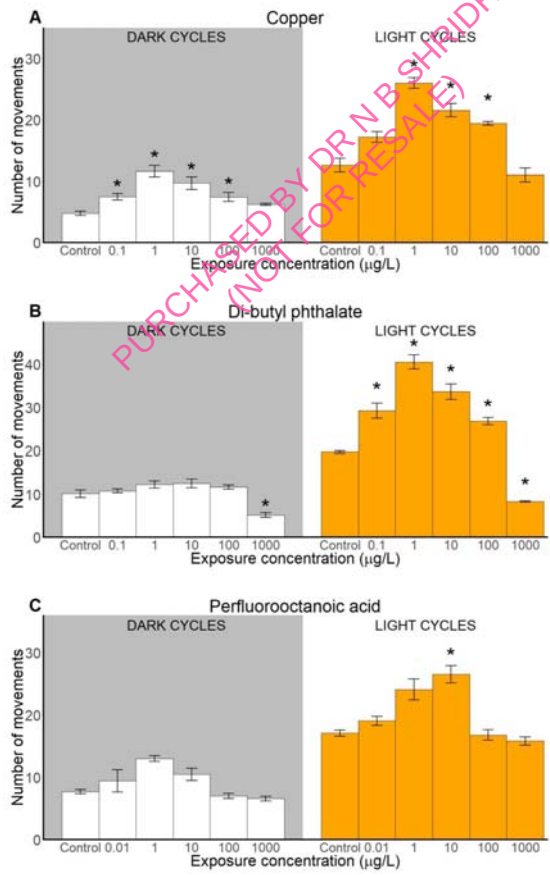
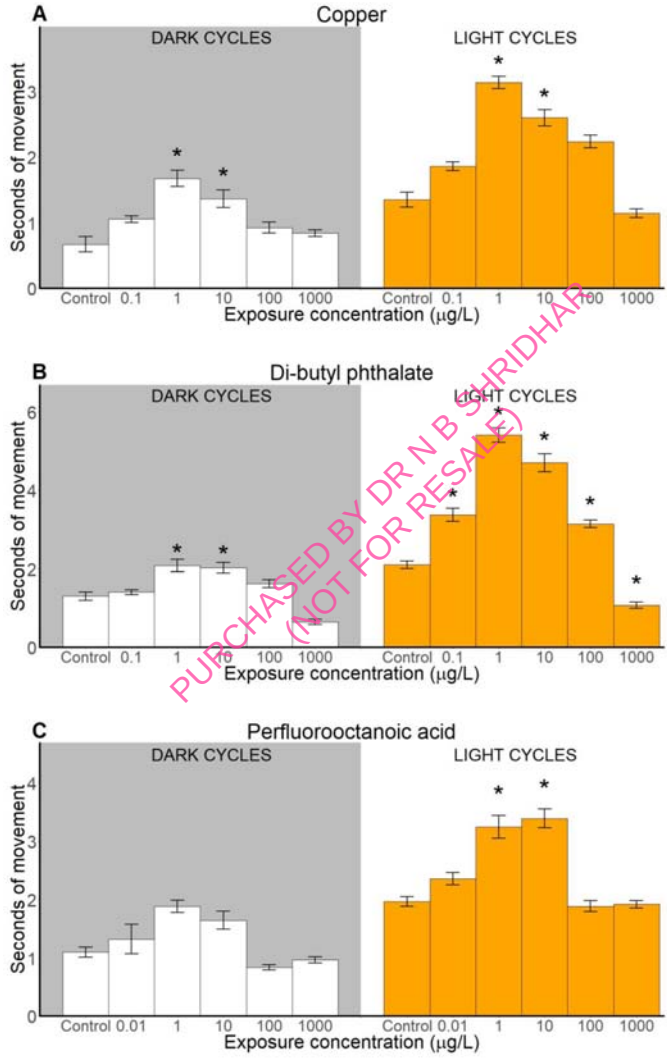


Figure 2: Locomotive activity (measured as number of movements) of sand goby larvae exposed to copper (A), di-butyl phthalate (B), and perfluorooctano-

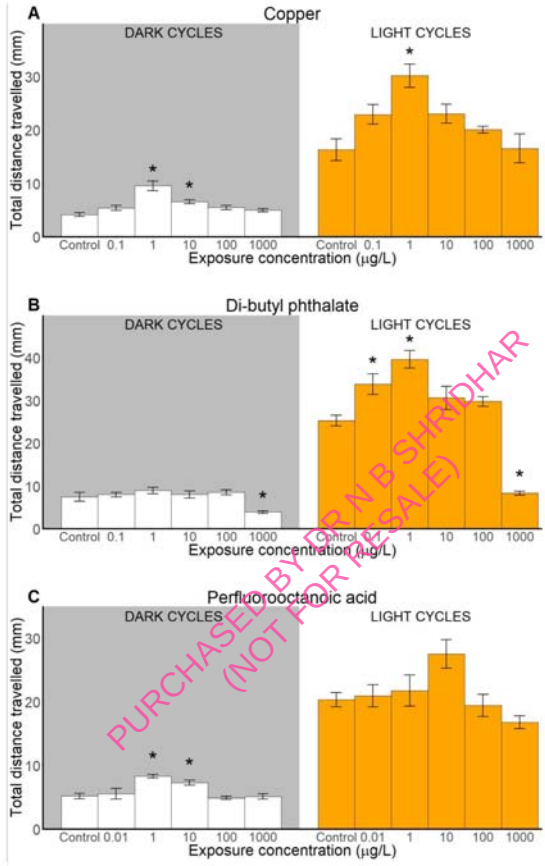
ic acid (C). Results presented as means ± SEM (standard error of the mean) for dark (left) and light (right) cycles. Statistically significant differences ($p < 0.05$) between control and other treatments are indicated with asterisks (*), ($n \geq 31$).



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Figure 3: Duration of locomotive activity (measured as seconds) of sand goby larvae exposed to copper (A), di-butyl phthalate (B), and perfluorooctanoic acid (C). Results presented as means ± SEM for dark (left) and light (right) cycles. Statistically significant differences ($p < 0.05$) between control and other treatments are indicated with asterisks (*), ($n \geq 31$).

All tested xenobiotic compounds caused behavioural alterations, including hyper- and hypoactivity (Figure 2, Figure 3 and Figure 4).



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Figure 4: Locomotive distance (measured as distance travelled) of sand goby larvae exposed to copper (A), di-butyl phthalate (B), and perfluorooctanoic acid (C). Results presented as means ± SEM for dark (left) and light (right) cycles. Statistically significant differences ($p < 0.05$) between control and other treatments are indicated with asterisks (*), ($n \geq 31$).

Number of Movements

Exposure to Cu, DBP and PFOA affected the locomotive activity (number of movements performed) of sand goby larvae in both dark cycles and in light cycles (Figure 2). Statistically significant differences (compared to control) were detected at the Cu concentrations 0.1, 1, 10 and 100 µg/L in dark cycles, and 1, 10 and 100 µg/L in light cycles (Figure 2A). For DBP exposure, a

significant effect was found only at the highest concentration in the dark cycles (1000 µg/L), and at all five concentrations tested in the light cycles (Figure 2B). PFOA seemed to affect sand goby larvae activity significantly only in light cycles, in particular at 10 µg/L (Figure 2C). However, the post-hoc analysis did not reveal any significant difference between the control and larvae treated with PFOA in terms of number of movements in the dark cycles.

Duration of Movements

Exposure to Cu significantly affected the duration of larval movements (measured in seconds), in both dark cycles and light cycles (Figure 3A). Statistical significances compared to control were detected at the Cu concentrations 1 and 10 µg/L in dark cycles and in light cycles. Exposure to DBP significantly increased duration of movement at 1 and 10 µg/L in dark cycles, and from 0.1 to 100 µg/L in light cycles (Figure 3B). For the highest concentration of DBP (1000 µg/L) in light cycles, the duration of movement was significantly lower than in the control. PFOA significantly increased the duration of movement in the light cycles at 1 and 10 µg/L (Figure 3C). The post-hoc analysis did not reveal any significant difference between the control and larvae treated with PFOA in terms of duration of movements in dark cycles.

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Total Distance Travelled

Results indicated that the distance parameter was also altered by toxicant exposure for all tested compounds (Figure 4). For example, Cu exposure significantly affected the total distance the larvae travelled, both in dark and light cycles (Figure 4A). Specifically, goby larvae exposed to 1 µg/L Cu travelled longer distances than control individuals in both light and dark cycles, whereas larvae exposed to 10 µg/L showed increased locomotion only in dark cycles. Moreover, after low DBP exposure (0.1–1 µg/L) larvae seemed to be more active and swam further in light than unexposed animals, whereas after exposure to the highest test concentration (1000 µg/L) the locomotive distance was significantly lower (Figure 4B). This locomotion suppression persisted in both light and dark cycles. Regarding PFOA exposure, statistically significant behavioural effects were detected only in the dark cycles for exposure to concentrations of 1 and 10 µg/L (Figure 4C). There was also a tendency for an increased activity in light at 10 µg/L.

DISCUSSION

Behavioural ecotoxicology is a growing discipline aimed at finding novel and sensitive behavioural endpoints that could be used for sublethal toxicity screening of environmental pollutants [11,68]. In the present study, we used sand goby larvae as an ecologically relevant species for the Nordic region and explored whether its locomotive behaviour could be used for screening environmental contaminants. This study is the first to describe the locomotive responses of sand goby larvae within a photo-motor test, thereby making use of a behavioural assay that is commonly used for pharmaceutical testing and chemical screening [15,16,69,70]. We demonstrated that sand goby larvae at 13–14 dpf are able to respond to light stimulation (19,000 lux) and display robust behavioural patterns. At this early stage of development, the goby larvae were able to detect an external signal and produce a consecutive response to the stimulation. The behavioural assay employed in the present study was directly adapted from zebrafish studies, which have previously been shown to be able to detect sublethal contaminant-induced behavioural changes [18,19].

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For all three tested compounds, we were able to establish dose–response relationships between each single locomotion variable and the toxicant exposure gradient. The observed behavioural changes were in line with previously published sublethal toxicity margins [71,72,73,74]. The observed toxicant-induced locomotive disturbances support the use of locomotion-based endpoints for sublethal toxicity screening. Two of the tested compounds (DBP and PFOA) are well-known EDCs [75,76,77,78], which may exert biological effects at low exposure levels and generate non-monotonic responses [79]. Interestingly, DBP demonstrated non-monotonic behavioural responses across the concentration gradient for all tested locomotion variables (Figure 2B, Figure 3B and Figure 4B) with significant elevation of locomotion parameters at low concentrations and decrease at the highest concentration. The locomotion assay used in this study may be useful for toxicity screening of endocrine disruptors, which is in agreement with previous studies that have suggested that behavioural endpoints are suitable for EDC screening [80]. Furthermore, a clear increase in swimming activity (in light cycles) was observed in sand goby larvae exposed to environmental levels of Cu (1 µg/L). A similar elevation in locomotive responses was previously documented using the same photo-motor test in zebrafish exposed to Cu ions [19]. On the basis of this comparison, we suggest that exposure to Cu induced similar behavioural disturbances in

two different fish species, which exemplifies the versatility of locomotion profiling approach and supports its applicability beyond the standard laboratory species. Moreover, this finding suggests that, despite the intrinsic differences in the behavioural repertoire of fish larvae from these species, changes in locomotion (intensity and/or patterns) could potentially be used as a pragmatic tool for toxicity screening.

It is generally assumed that animals subjected to toxicant exposure experience stress and can enter new and potentially irreversible physiological states. According to the allostatic step-wise stress theory [81,82], organisms exposed to low levels of a stressor (not necessarily toxicant-induced) are capable of mediating the disturbances via homeostasis, while they may be irreversibly harmed when the stressor persists or exceeds the organisms' capacity to cope. Interestingly, we observed dual behavioural modalities, indicating the presence of low-level stimulatory effects of toxicants in low exposure concentrations, and suppressing (toxic) effects in high exposure levels (Figure 2B, Figure 3B and Figure 4B). We could speculate that fish larvae exposed to low levels of toxicants (Figure 2, Figure 3 and Figure 4) may have experienced metabolic stress (e.g., due to increased physiological needs for detoxification), which thereafter translated to hyperactivity and/or changes in susceptibility to light stimulation. For example, Cu and DBP exposure have been linked with oxidative stress [83,84], either from a direct toxicant effect or as a result of increased metabolism, which potentially causes observed behavioural disturbances. On the other hand, even though the underlying mechanism(s) of stimulatory (behavioural) swimming response remain largely unknown, a growing body of studies have documented toxicant-induced hyperactivity in fish larvae [18,85,86]. Hypothetically, hyperactivity may reflect an adaptive larval response originally evolved to allow avoidance of non-preferred micro-environments. However, in the context of macro-environmental pollutants, such a response is arguably not likely to be beneficial, and may even be harmful to the larvae. Furthermore, the use of high levels of solvents (e.g., dimethyl sulfoxide (DMSO) 0.01–0.1%) has been shown to increase locomotion in several aquatic model species [87], which should also be taken into consideration while designing experiments and interpreting the results from behavioural experiments with toxicants dissolved in a carrier medium. Then again, hypoactivity (reduced swimming capacity) may occur due to functional damage exerted on the motoric system, or overall homeostatic arrest, and in such context could be viewed as a behavioural disability. That said, explanation of observed behavioural disturbances, e.g., using stepwise-stress theory, validation of

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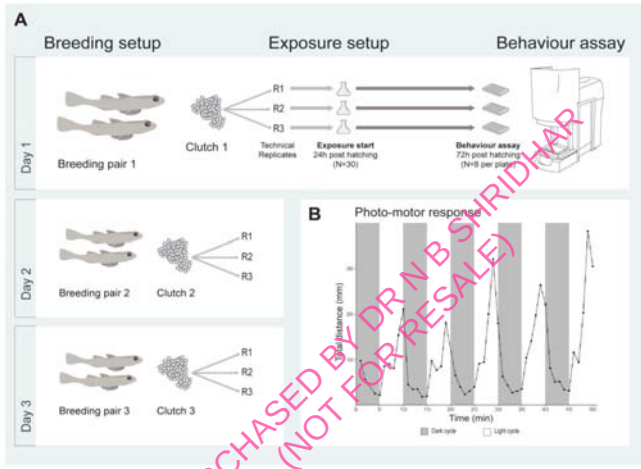
behavioural changes across contaminant exposure (concentration) gradients, as well as explanation underlying mechanisms remain prospects for the future. In the broader context of the newly emerging discipline of behavioural ecotoxicology, the interpretation of the adversity of "behavioural effects", as well as extrapolation of such effects on population level, are still subjects for discussion.

Even though our study was limited to three contaminants that are prevalent in coastal environments, we believe that this behavioural screening approach can extend beyond that. For instance, a proposed behavioural toxicity testing strategy could be used for testing complex environmental mixtures (e.g., solid phase extracts) for biomonitoring purposes. Furthermore, the sand goby behavioural assessment using larval stages used here could be further upgraded and potentially coupled with developmental toxicity assessment using traditional apical endpoints (mortality, incidence of malformations, as described in the Fish Embryo Toxicity Test [37]) or with various molecular techniques suited for obtaining comprehensive mechanistic insights of toxicity. Collection of additional toxicological endpoints could undoubtedly support results of behavioural assessment and could aid for data interpretation.

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As pointed out above, the locomotion of sand goby larvae has been shown to be suitable for sublethal toxicity screening of xenobiotics. Besides its robust photo-motor response, there are other attractive features of the sand goby species suggesting its applicability in ecotoxicological sciences. For example, a natural nest often contains 5000–10,000 eggs (2000–3000 eggs per female [44,88]), spawned by 2–6 females per nest [55], which ensures easy collection of fish embryos or larvae sufficient for execution of experiments. They also readily spawn in the laboratory. Notably, with experimental designs that include single female–male breeding (Figure 5A), both intrapopulation variability (within clutches) and interpopulation (between clutches) variability can be systematically controlled, which is highly important for making laboratory-based behavioural studies more ecologically relevant. Also, it is well known that offspring from fish sampled from populations in the field are expected to have higher level of genetic diversity (low inbreeding potential) compared to lab-reared populations. Sand goby larvae (10–14 dpf) that were used in the experiments did not initiate independent feeding, and therefore technically could be considered an in vitro system (according to European legislation), which represents another strength of using the species for toxicological exposures. Furthermore, the applicability of the species for ecotoxicological studies stretches beyond

its early life stages. Previously, it has been shown that other life stages of the species possess certain attributes that make them ecologically relevant in ecotoxicology. For example, the short generation time (1–2 years [89]) represents another advantage for the potential application of this species in transgenerational ecotoxicological studies. Moreover, due to interesting reproductive behaviours, the sand goby has been proven to be useful in studies assessing the impacts of compounds with an endocrine mode of action (e.g., 17α-ethinyl estradiol in [57,58]).



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Figure 5: Breeding setup and experimental design for sand goby larvae exposures (A) and behavioural toxicity assay utilizing alternating light and dark cycles (B). The locomotive response of unexposed sand goby larvae is presented.

However, the use of sand goby larvae as an alternative for behavioural ecotoxicology still presents some challenges and there are several methodological considerations for using sand goby as an ecologically relevant species for ecotoxicological experiments. In terms of practical prerequisites for species husbandry and breeding, there are some apparent limitations associated with seasonality. The sand goby normally has a breeding season that covers spring and early summer [49], and therefore exposure of larvae is limited to this season. Even though there is the potential for keeping sand goby and other species in the so-called sand goby group [90] in the laboratory and performing routine breeding in an artificial setup, it unfortunately remains a great uncertainty. Although they are unproblematic to keep in the laboratory all year around and they mature eggs and sperm in spring [91], raising the

pelagic larvae until settling has proven to be challenging [92]. Furthermore, depending on water temperature, it takes 1–4 weeks for sand goby larvae to hatch [67], and this period is considerably longer and more variable than for other standard laboratory species such as zebrafish (72 h post fertilization, hpf) and fathead minnow (5 dpf) [93]. For the Japanese medaka, another fish species commonly used in ecotoxicological studies, it takes nine days until hatching is completed [94]. The breeding and rearing conditions for the sand goby in the laboratory would need to be carefully controlled to ensure that embryos hatch at the same time and that larvae are at the same developmental stage at the onset of exposure. In addition, high baseline mortality was observed during our experiments, and is a challenge for the use of the sand goby as a model of chemical exposure. The mortality we observed varied between different clutches. Potential explanations for the intrinsically high mortality rates could be insufficient oxygenation, high embryo density on the plastic film, and handling stress (e.g., sensitivity due to transferring larvae). As the male would normally care for the eggs until they hatch, removal of unfit eggs from the nest before hatching may also have an effect on the mortality and quality of artificially reared clutches. Previous studies have also described high mortality rates for the rearing of sand goby larvae [92]. The behavioural toxicity assessment was conducted using an assay designed for zebrafish, and tracking parameters employed in the behavioural protocol may need further refinement to be suitable for sand goby larvae. Optimization of movement differentiation thresholds, as well as the selection of relevant lighting conditions (cycle length, acclimation duration, etc.), may be advised in future studies. For example, the level of light intensity may play an important role in (observed) behavioural responses. In the natural environment, the light intensity in the water column can vary greatly depending on different biotic and abiotic factors. Adult sand gobies used in this study were caught in shallow (0.5–1 m) coastal areas, with expected lower light intensity than in the experimental setup. It has been previously shown that adult *P. minutus* display nocturnal activity under low light conditions (high activity in darkness and low activity in light) [95]. However, this behaviour may well be ontogenetically flexible or undergo changes during seasons, affected by changes in light intensity. Overall, relatively little is known about the impact of light on sand goby activity patterns, especially how it changes from the early life stages to adulthood. Taken together, even though a solid knowledge base about the biology and behaviours of adults exists, information about the development and ontology of behavioural repertoire of the larvae is still largely lacking (see [92]).

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MATERIALS AND METHODS

Sand Goby Husbandry

Adult sand gobies (*P. minutus*) were caught during May 2016 by hand trawling in a clean bay near the Sven Lovén Center for Marine Infrastructure Kristineberg, University of Gothenburg, on the west coast of Sweden (58°14′53.8″ N 11°26′50.1″ E). Specimens were initially stored at the Kristineberg facility and fed ad libitum with live mysid shrimps (*Praunus flexuosus*) and mussel meat (*Mytilus* sp.). After three days the fish were moved to another aquarium facility, located in Gothenburg (Department of Biological and Environmental Sciences, University of Gothenburg), and kept in artificial seawater at 28.4 ± 1 practical salinity units (PSU) under 12:12 h light:dark cycle at 14 °C. Salinity was measured twice per week. Fish were fed ad libitum three times per week with frozen Chironomidae mosquito larvae (Imazo AB, Vara, Sweden). All fish were treated in accordance with the ethical practices defined by the Swedish Board of Agriculture (Permit number 39-2014).

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Breeding for Exposures

Prior to breeding, male and female fish were separated and kept in two separate tanks (200 L) filled with artificial seawater and a sand layer on the bottom. For breeding trials, a closed flow-through system equipped with mechanical, biological and ultra violet (UV) filters with smaller tanks containing 14 L water, an air stone and a 3 cm clean sand layer as substrate at the bottom was used. One male and one female were placed in each smaller flow through tank, together with a halved clay pot (7 cm Ø) that the male could use as a nest site. The inside of the clay pot was covered with a plastic film, which served as a substrate for the eggs. Once the females had laid eggs, they were removed from the tank in order to avoid possible egg predation. Larval development was monitored by visual inspection. Shortly before hatching, when the eyes of embryos were visible (after approximately 10–11 days), the plastic film with eggs was transferred from the clay pot to beakers with saline water and allowed to hatch.

Chemicals and Exposure Design

Three compounds, Cu, DBP and PFOA, were selected based on a list of commonly found chemicals from a sampling campaign in Swedish

west coast waters [96] and were tested as single compounds. Chemicals were purchased from Sigma Aldrich (Stockholm, Sweden). Exposure solutions were prepared in artificial seawater (28.4 PSU) to five nominal concentrations in the range of 0.01–1000 µg/L (PFOA) or 0.1–1000 µg/L (Cu and DBP). The lowest concentrations for DBP and PFOA were chosen based on the mean environmental concentrations found by Gustavsson et al. [96] and the concentration for Cu was 1 µg/L. For PFOA, a stock solution in DMSO (100%) was first prepared, and then diluted to final concentrations in artificial seawater containing a maximum of 0.1% DMSO, in compliance with OECD recommendations [97]. In the control treatment, artificial seawater was used.

The larval exposure was static; for each treatment, a total of nine technical replicates from three clutches (i.e., three per clutch) belonging to three different breeding pairs were used (Figure 5A). Approximately 24 h after hatching, 30 healthy larvae from each clutch were randomly selected and transferred to glass e-flasks with 90 mL exposure solution (for each compound and each concentration). Larvae were incubated at 14°C with a 12:12 h light:dark cycle. After 48 h of exposure (72 h post hatching), eight larvae per exposure condition were transferred to individual wells of 48-well plates containing 700 µL exposure solution. Mortality was not recorded during the exposure, but was estimated to be 20–40% prior to exposure. Natural, high baseline mortality for sand goby larvae has previously been described [92].

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Behavioural Toxicity Assay

Prior to assessing the behavioural toxicity of selected compounds, a pilot study was conducted to investigate and describe baseline activity patterns of sand goby larvae in a photo-motor test (Figure 5B). The swimming activity of unexposed goby larvae was quantified using a behavioural assay previously developed for zebrafish larvae [18]. The behavioural protocol was adapted for sand goby larvae with some minor adjustments. The locomotion of the sand goby larvae was quantified using the locomotion tracking with rotation angles module in the automatic behaviour tracking system ViewPoint (ViewPoint Life Science Inc., Montreal, QC, Canada). In order to artificially stress the animal and see its behavioural response in stress conditions, a protocol composed of 10 alternating light:dark cycles (5 min : 5 min) was used. Light saturation in light cycles was 100%, corresponding to 19,000 lux. The larvae were acclimated in light for 15 minutes before initiation of the locomotion assay. Three locomotion-based endpoints were assessed: (1)

number of movements; (2) duration of movements; and (3) total distance travelled. In this study we focused on the cumulative movement responses of fish larvae (during behavioural trials), without disseminating information on structural and dynamic components of locomotion. The positions of larvae within the 48 well-plate were randomly assigned to avoid potential plate effects. Also, for each behavioural plate, fish larvae exposed to all test concentrations for each single chemical exposure were included. Moreover, prepared plates were kept for at least 10–40 min before behavioural tracking to eliminate the potential impact of stress induced by handling. The temperature during behavioural experiments was kept constant (16 ± 1 °C). Behavioural experiments were consistently conducted between 08:30 and 18:30.

Data Analysis

For the statistical analysis, results for dark and light cycles from different technical and biological replicates were averaged. Results are presented as mean values for the three different locomotion endpoints ± standard error of the mean (SEM). Asterisks represent the statistical significance (i.e., p-value ≤ 0.05) between the condition of exposure and the relative control within dark or light cycles. From all the datasets, the values of the 3rd and the 20th min were deleted due to technical problems, probably caused by tracking software issues. These problems were found in all the datasets, without exception; therefore, it was decided to remove those data that could interfere with the further analysis. Moreover, all the data from larvae where movement was not recorded were removed.

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Data were checked for normal distribution with the Shapiro–Wilk test and for homoscedasticity with Levene's test. If these two assumptions of analysis of variance (ANOVA) were matched in the data, a one-way ANOVA was used, followed by pairwise t-test comparisons of each concentration against the control. This was done as a planned comparison post hoc testing to assess the effect of the tested compound on larval locomotion. Light and dark cycles were analysed separately. If the homoscedasticity assumption was not matched, a Welch's ANOVA test with the Games–Howell post-hoc test was used. If the normality assumption was not matched, a Kruskal–Wallis test followed by a Nemenyi post-hoc test was used. Statistical analysis was performed with the software R and RStudio [98].

CONCLUSIONS

The current study demonstrates that sand goby larvae can be used to screen for the behavioural toxicity of chemicals present in the marine environment and that the sand goby larval locomotion assay is a promising tool for single chemical hazard assessment on a species relevant for Nordic marine ecosystems. We adapted a behavioural assay developed for zebrafish with the sand goby as a more environmentally relevant species and were able to establish dose-response relationships for measured behavioural endpoints after exposure to single compounds. The early developmental stages of sand goby could also be used for more complex mixtures, as well as in future monitoring studies of complex mixtures, extracts, and water or sediment samples. Furthermore, it could be adapted for other species of fish. We conclude that locomotion of fish larval stages can be used as an indicator of sublethal effects of toxicants, and that sand goby larvae have potential for ecotoxicological behavioural studies. However, there are several unresolved challenges, including optimization of behaviour tracking, standardization, breeding limitations and embryo mortality, which should be addressed in future studies.

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AUTHOR CONTRIBUTIONS

D.A. performed the breeding, exposure and locomotion assays and analysed the data; G.A. designed the exposure setup and developed a framework for behavioural data analysis. G.A., L.B. and D.A. wrote the paper; L.B. and D.A. designed and edited the figures; C.K. and O.S. provided their expertise in sand goby husbandry and breeding, and contributed to discussions and critical reading of the manuscript; J.S. designed and supervised the study and contributed to discussions and critical reading of the manuscript.

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SECTION VIII: DIAGNOSTICS TOXICOLOGY AND THERAPEUTICS

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Chapter 15

Measured and Modeled Toxicokinetics in Cultured Fish Cells and Application to In Vitro - In Vivo Toxicity Extrapolation

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ABSTRACT

Effect concentrations in the toxicity assessment of chemicals with fish and fish cells are generally based on external exposure concentrations. External

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concentrations as dose metrics, may, however, hamper interpretation and extrapolation of toxicological effects because it is the internal concentration that gives rise to the biological effective dose. Thus, we need to understand the relationship between the external and internal concentrations of chemicals. The objectives of this study were to: (i) elucidate the time-course of the concentration of chemicals with a wide range of physicochemical properties in the compartments of an *in vitro* test system, (ii) derive a predictive model for toxicokinetics in the *in vitro* test system, (iii) test the hypothesis that internal effect concentrations in fish (*in vivo*) and fish cell lines (*in vitro*) correlate, and (iv) develop a quantitative *in vitro* to *in vivo* toxicity extrapolation method for fish acute toxicity. To achieve these goals, time-dependent amounts of organic chemicals were measured in medium, cells (RTgill-W1) and the plastic of exposure wells. Then, the relation between uptake, elimination rate constants, and log K_{OW} was investigated for cells in order to develop a toxicokinetic model. This model was used to predict internal effect concentrations in cells, which were compared with internal effect concentrations in fish gills predicted by a Physiologically Based Toxicokinetic model. Our model could predict concentrations of non-volatile organic chemicals with log K_{OW} between 0.5 and 7 in cells. The correlation of the log ratio of internal effect concentrations in fish gills and the fish gill cell line with the log K_{OW} was significant ($r>0.85$, $p=0.0008$, F-test). This ratio can be predicted from the log K_{OW} of the chemical (77% of variance explained), comprising a promising model to predict lethal effects on fish based on *in vitro* data.

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INTRODUCTION

Environmental regulations require comprehensive testing and risk assessment before a chemical can be approved for use. In assessing the environmental risk of chemicals, fish play a very important role, being the most frequently tested vertebrate representative for freshwater systems [1]. *In vitro* fish cell assays are considered to be a promising alternative to fish bioassays to replace or reduce the use of fish in toxicological testing [2], [3]._ENREF_3 Cells in culture plates or vials can be exposed to a large number of chemicals and toxicity after exposure to chemicals can be quickly analyzed_ENREF_3. In addition, few, if any, animals are used, little test substance is needed, and little toxic waste is produced_ENREF_4. For instance, *in vitro* fish liver cell assays, using freshly isolated hepatocytes, can be applied for *in vitro – in vivo* extrapolation of chemical biotransformation in fish [4], [5]. In addition,

permanent fish cell lines, which can be cultured indefinitely without further need of animals, provide another potential route for establishing *in vitro– in vivo* toxicity extrapolations. Tanneberger et al. [6]highlighted that, because gill epithelia are the primary uptake site of water-born contaminants into fish, they could also be a primary target for many toxicants in exposure scenarios where vital epithelial cell functions are destroyed, resulting in a toxic effect on the whole organism. Along these lines, Li et al. [7] noticed that in fish, gill tissue can be more sensitive to some chemicals than liver and muscle tissues. For these reasons, understanding the toxicokinetics in gill cells and the resulting improvement of *in vitro – in vivo* toxicity extrapolations is very important.

The quantification of chemical toxicity in cells is generally based on nominal (i.e. intended) chemical concentrations. However, recent studies show that measurements of external exposure are more appropriate than nominal concentrations due to the number of competing processes occurring in the culture well, like sorption to various compartments in a well or evaporation [3], [6], [8]. Yet, external concentrations as dose metric are still only a surrogate which may impede interpretation and extrapolation of toxicological effects because internal concentrations are thought to give rise to the biologically effective dose [9], [10]. In particular, the extrapolation of toxicity to other species, compounds and exposure patterns benefits from using dose metrics based on toxicokinetics (TK) [11], [12]. Toxicokinetics describes the time-course of a chemical concentration in a relevant biological matrix (e.g., cells in an *in vitro* assay or a tissue within the intact organism). For these reasons, we also need to understand the relationship between the external and internal concentration of chemicals in cells of *in vitro* cell line test systems. The quantification of the time course of internal concentrations in cells and whole organisms facilitates a better understanding of toxicity and may improve *in vitro* to *in vivo*toxicity extrapolation. Finally, following the tissue-residue approach, which proposes the use of tissue or total internal concentrations as the dose metric for characterizing a toxicant's potency [13]–[15], one can derive the hypothesis that, if the chemical acts by the same mode of action in cells and intact animal, the concentrations in an organism that cause toxicity must be similar to the concentrations that cause toxicity in a cell line. Support for this hypothesis was provided by research on surfactants, which elicited toxicity at fish cell residue levels corresponding closely to *in vivo* residue levels associated with surfactant toxicity [16]. The tissue residue hypothesis can be even further refined by using the free (unbound) internal concentration as dose metric. This is thought

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to be even closer to the biologically relevant dose metric than total internal concentrations [17], [18]; however, we here assume that a larger fraction of the uncertainty in *in vitro* to *in vivo* toxicity extrapolation originates from the difference between external and internal concentrations. Thus, we focus on improving quantification of total internal concentrations as a first step.

Thus far, existing modeling approaches for predicting chemical concentrations in cells assume equilibrium conditions [17], [19]._ENREF_16 However, it was shown, in particular for volatile and hydrophobic compounds, that obtaining stable exposure concentrations is difficult and that measured chemical concentrations in the test medium can differ significantly from nominal concentrations [8]. For this reason, partition-controlled dosing systems for *in vitro* cell assays, which allow stable exposures to be achieved, were developed [20], [21]. However, the applicability of these systems to a wide range of chemicals still needs to be addressed as only few chemicals were tested thus far and handling is still cumbersome. A TK model, which describes the chemical distribution in the experiment›s environment also under non equilibrium conditions, would be a useful alternative. Such a model, which takes into account not only chemical uptake by cells but also evaporation and binding to plastic, could support quantifying and understanding the toxicity of chemicals toward cells.

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Thus, the objectives of this study were as follows: (i) elucidate the time-course of the concentration of chemicals with a wide range of physicochemical properties in the compartments of an *in vitro* toxicity test system, (ii) derive a general, predictive model for toxicokinetics in the *in vitro* system, (iii) test the hypothesis that internal effect concentrations in fish (*in vivo*) and fish cell lines (*in vitro*) correlate, and (iv) develop a quantitative *in vitro* to *in vivo* toxicity extrapolation method for fish acute toxicity.

MATERIALS AND METHODS

Study Outline

A set of substances characterized by a wide range of Henry's Law Constants (log H), reflecting volatility, and by a wide range of octanol-water partition coefficients (log K_{OW}), reflecting hydrophobicity, was tested as these parameters can impact the toxicity to fish and cells [22] and determine the fate of the substance in the *in vitro* test system. The amount of each chemical in medium, cells and plastic of the well plate for various time

points was measured in order to study the chemicals' distribution. The relation between uptake and elimination rate constants and log K_{OW} was investigated in order to develop a multi-compartment TK model describing the chemical distribution in the exposure well. In addition, based on previous *in vitro* toxicity studies [6], [23], the empirically obtained multi-compartment TK model was used to predict internal effect concentrations in cells (IEC50), which were compared with internal effect concentrations in fish gills (ILC50) predicted by a Physiologically Based Toxicokinetic (PBTK) model [24].

Chemicals

For quantification, ^{14}C-labelled chemicals were used. The applied chemical concentrations as well as the chemicals' physicochemical properties are presented in Table 1. Chemical amounts were quantified based on total radioactivity. Eight substances with low volatility (log H<−6 atm·m^3/mol) and three substances with high volatility (log H>−4 atm·m^3/mol) were used. Stock solutions were prepared using methanol (Acros Organics, Geel, Belgium) with the exception of hexachlorobenzene (Sigma-Aldrich, Buchs, Switzerland), which was dissolved in DMSO (dimethyl sulfoxide). The final concentrations of solvents in the exposure system were 0.15% v/v for DMSO and ≤0.16% for methanol. Chemical concentrations added to the medium at the beginning of the experiments were chosen based on two criteria: to be high enough to ensure detection in test medium, cells and plastic using Liquid Scintillation Counting (LSC, Tri-Carb 2200CA, Packard, USA) and, if possible, to be below toxic levels. Chemical concentrations were considered as non-toxic if the number of cells in the exposed wells was within ±10% of the cell number in wells with no chemicals (control) for all time points as determined with the fluorescamine assay (see below). The above mentioned criteria could be fulfilled for all chemicals except for pentachlorophenol for which the limit of detection required us to use a concentration that caused death of 20–30% of cells (in agreement with Tanneberger et al. [6]).

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Table 1: Properties and concentrations of the test chemicals used for measuring and predicting chemical concentrations in the RTgill-W1 cell line

https://doi.org/10.1371/journal.pone.0092303.t001

Chemical	CAS	Molecular weight (g/mol)	Log K_{OW}[a]	Log H[a] (atm, m^3/mol)	Concentration in medium[b] (μg/L)
Imidacloprid	138261-41-3	255.66	0.57	−14.78	24.5
Dimethoate	60-51-5	229.26	0.78	−9.61	58.0
Carbendazim	10605-21-7	191.19	1.52	−10.67	17.5
Malathion	121-75-5	330.36	2.36	−8.31	64.0
Cyproconazole	94361-06-5	291.78	2.9	−9.15	23.5
Propiconazole	60207-90-1	342.22	3.72	−8.76	56.0
Pentachlorophenol	87-86-5	266.34	5.12	−7.61	5.0
Cypermethrin	52315-07-8	416.31	6.6	−6.38	1.3
1,2,3-Trichlorobenzene	87-61-6	181.45	4.05	−2.90	6.6
Naphtalene	91-20-3	128.18	3.3	−3.36	4.3
Hexachlorobenzene	118-74-1	284.78	5.73	−2.77	7.62

[a]- logK_{ow} and log H were taken from EPI Suite: experimental database.
[b]- nominal chemical concentration dosed at the beginning of the experiment
italic font– volatile compounds.
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Cell Culture

The RTgill-W1 cell line was obtained from rainbow trout (*Oncorhynchus mykiss*) gills [25].

One ml of Leibovitz (L15) medium (LuBio Science GmbH, Luzern, Switzerland), containing 350 000 cells determined based on electric field multi-channel cell counting (CASY1 TCC, Schärfe System, Germany), was added into the wells of 24-well plates (Greiner Bio-One, Frickenhausen, Germany) in order to seed cells for exposure. The incubation time after seeding cells and prior to exposure was between 24 and 30 hours to allow cells to form a confluent monolayer containing around 400 000 cells.

Exposure of Cells to Chemicals

At the beginning of the experiment and for each time point, the number of seeded cells was determined based on protein content measurements. The protein assay was chosen so that cell number could be determined directly in the cell culture wells. The rainbow trout gill cells were exposed to chemicals in 24-well tissue culture plates for 0, 1, 2, 4, 8, 16, 24 and 48 hours at 19°C in normal atmosphere. Dosing stocks were prepared in the exposure medium, L15/ex [26], in which the cells are viable but no longer proliferate, so the cell culture system remains stable. L15/ex is a modified Leibovitz medium and includes only galactose, sodium pyruvate and salts.

Cells from all well plates were washed with 1 ml of L15/ex before the chemical or control (solvent) was added. For each time point, triplicate wells were dosed in two culture plates: one plate was used to measure protein content (see "Determination of cell number") while the second plate was sampled to measure radioactivity in each compartment and derive a mass balance. During the whole experiment, all culture plates were covered with plastic foil (VWR International GmbH, Darmstadt, Germany) in order to reduce evaporation.

Chemical Extraction

On termination of exposure, first, 100 µl of medium were taken from all respective wells and added into 20 ml glass vials, each filled with 10 ml of Ecoscint A – liquid scintillation cocktail (ChemieBrunschwig, Basel, Switzerland). Then, all of the remaining medium was removed and replaced with 100 µl of versene (LuBioScience GmbH, Luzern, Switzerland). Next, 100 µl of trypsin (ChemieBrunschwig, Basel, Switzerland) was added to these wells to detach cells. After a few minutes, the detached cells, together with versene and trypsin, were pipetted into glass vials pre-filled with 10 ml of Ecoscint A.

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Two ml of methanol were added to empty wells (from which the cells and the chemical had been removed) in order to extract the chemical from plastic. Then, the wells were covered again with the same plastic foil (VWR International GmbH, Darmstadt, Germany) and the whole well plate was wrapped with aluminum foil and shaken for 10 minutes. Methanol with the extracted chemical was taken from the wells and each added into a glass vial, pre-filled with 10 ml of Hionic Fluor (liquid scintillation cocktail for organic solvents; Perkin, Elmer, Massachusetts, USA).

For a mass balance approach, concentrations were converted to % of chemical added to the medium (three technical replicates). As shown in this Table, exposures and chemical analysis were performed for two independent biological replicates (i.e. using cells from different passages) for four of the tested compounds (pentachlorophenol, malathion, propiconazole and hexachlorobenzene), giving very similar results. The other chemicals were then tested in a single experiment.

Determination of Cell Number

The cell number in each well was determined based on total protein content of the cells using the fluorescamine assay method. Cell number was calculated

based on a standard curve depicting the relationship between cell number, protein content and fluorescence.

For each time point, L15/ex medium was removed from wells, and cells were rinsed with 500 µl of PBS (Dulbecco's Phosphate Buffered Saline w/o calcium and magnesium). Then PBS was replaced by 500 µl of nanopure water, and the well plate was stored at −80°C for at least one hour to disrupt cells. After thawing, 1 ml of PBS and then 0.5 ml of fluorescamine (Sigma-Aldrich, Buchs, Switzerland) diluted in acetone (3 mg of fluorescamine per 10 ml of acetone), was added to each well containing cells and to one additional, cell free, well per plate as a control. Then, each plate was covered with aluminum foil and shaken for 5 minutes. The fluorescence was measured in each well using the Infinite M200 microplate reader (TECAN, Männedorf, Switzerland; excitation: 360 nm, emission: 460 nm).

Modeling Chemical Distribution in a Well

The time course of chemical concentrations was modeled in three compartments of the *in vitro*test system: cells, plastic and medium. For volatile compounds also a fourth compartment, headspace, was modeled. For this, uptake and elimination rate constants were fitted based on the total loss of a chemical deduced from the mass balance. The model and measurements did not include biotransformation nor transformation products at this point. The simple exposure medium L15/ex does not favor metabolic processes in general; for example, cells in this medium do not proliferate [26]. However, physiological metabolism is not necessarily correlated with the ability to biotransform xenobiotics and dedicated research, beyond the scope of this study, is needed to characterize biotransformation processes in cultured fish cells.

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In each compartment, chemical concentrations were simulated based on the following equation:

$$\frac{d}{dt}C_{int}(t) = k_{in} \cdot C_m(t) - k_{out}(t) \cdot C_{int}(t) \quad \textbf{(eq.1)}$$

where $C_{int}(t)$ is the chemical concentration in the compartment, i.e. internal concentration (cells: amount × mass^{-1}, plastic: amount × surface^{-1}, headspace: amount × volume^{-1}), $C_m(t)$ is the chemical concentration in the medium (amount × volume^{-1}), k_{in} is the uptake rate constant (cells: volume × mass^{-1} × time^{-1}, plastic: volume × surface^{-1} × time^{-1}, headspace: volume × volume^{-1} × time^{-1}) and k_{out} is the elimination rate constant (time^{-1}).

Uptake and elimination rate constants were fitted to measured concentrations of each chemical in the respective compartment both separately and simultaneously by minimizing the sum of squares between measured and modeled concentrations using the Levenberg–Marquardt algorithm. Optimized parameter values were similar for both methods. Because fitting rate constants separately for each compartment yields more accurate predictions of chemical concentrations in cells, results and discussion are presented for rate constants which were fitted by modeling concentrations in each compartment separately.

Time to steady-state conditions was calculated for each chemical, taking into account the fluctuation of chemical concentrations in medium over time, based on the elimination rate constant and assuming 90% attainment of steady-state before the compound was judged to be at steady-state [27].

The model was implemented and solved using ModelMaker (version 4.0, Cherwell Scientific Ltd., Oxford, UK).

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Internal Effect Concentrations of Chemicals: Cells and Fish

Fish toxicity values, for example those taken from the U.S. EPA fathead minnow toxicity database [28], are usually expressed as lethal concentrations for 50% of the test population (LC50), where concentration refers to that measured in the exposure medium. Similarly, Tanneberger et al. [6] expressed the concentration in the test medium that causes effects in 50% of the cell population (EC50 values) based on measured concentrations. Thus, the comparison between internal effect concentrations in fish and in fish cells (ILC50 and IEC50) was performed based on LC50 and EC50 data from the CEllSens database [6]. The CEllSens database includes EC50 values measured for the RTgill-W1 cell line in the CEllSens project and LC50 values for fathead minnow, taken from the U.S. EPA fathead minnow database [28]. EC50 values were calculated based on the assumption that toxicity is a function of the chemical concentrations available during the entire 24 hours of exposure, even if the steady-state conditions may not have been reached within this time range. From this database, two groups of chemicals were excluded: polar compounds (because their partitioning behavior cannot be well characterized by means of the octanol-water partition coefficients [29]) and highly volatile compounds (due to possible experimental artifacts, see "Modeling sorption to plastic"). In addition, only chemicals with log K_{OW} between 0.5 and 7 were chosen, as the *in vitro* TK model (this study) was calibrated for chemicals with log K_{OW} between 0.58

and 6.6 (see Table 1). Based on these criteria, 13 chemicals (Table 2) were used for the *in vitro* and *in vivo*comparison based on modeled internal effect concentrations.

Table 2: Properties and concentrations of chemicals selected from CEllSens project

https://doi.org/10.1371/journal.pone.0092303.t002

Chemical	CAS	Mode of Action[a]	Log K_{ow}[b]	Log H[b]	LC50[c] µg/L	EC50[d] µg/L
2,2,2-Trichloroethanol	115-20-8	NPN	1.42	−6.81	298100	102100
Diethylphthalate	84-66-2	NPN	2.42	−6.21	32100	63900
Di-n-butylphthalate	84-74-2	NPN	4.5	−5.74	831.8	250
Menadione	58-27-5	reactive	2.2	−8.51	109.6	120
Dichlorophene	97-23-4	reactive	4.26	−11.94	309	50
4-Fluoroaniline	371-40-4	reactive	1.15	−5.65	16800	200000
2,4-Dinitrophenol	51-28-5	uncoupler	1.67	−7.07	13489.6	770
Pentachlorophenol	87-86-5	uncoupler	5.12	−7.61	218.8	10
Malathion	121-75-5	AChE	2.36	−8.31	14100	12900
Disulfoton	298-04-4	AChE	4.02	−5.67	4000	1900
Parathion ethyl	56-38-2	AChE	3.83	−6.53	1584.9	810
Permethrin	52645-53-1	neurotoxic	6.50	−5.73	20	3760
Lindane	58-89-9	neurotoxic	4.14	−5.29	100	6900

[a]- according to Russom et al.[28] _ENREF_2_ENREF_2_ENREF_2_ENREF_2; NPN: non-polar narcosis; AChE: Acetylcholine Esterase inhibition; uncoupler: uncoupler of oxidative phosphorylation.
[b]- logK_{ow} and log H (atm, m^3, mol^{-1}) were taken from EPI Suite: experimental database.
[c]- LC50 values taken from the U.S EPA fathead minnow toxicity database [28].
[d]- measured EC50 values for the endpoint metabolic activity of cells [6].
doi:10.1371/journal.pone.0092303.t002

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Internal Effect Concentrations (IEC50) in the rainbow trout gill cell line (*in vitro*) were simulated using equation (1) and the TK parameters obtained in this study. The exposure concentration (C_m) was set to the EC50 value and the internal concentration after 24 hours was taken as the *in vitro* IEC50. Internal Lethal Concentrations (ILC50) in the fathead minnow gill compartment were calculated using the Physiologically Based Toxicokinetic (PBTK) model developed for fish by Nichols et al. [30]–[32] and adapted for fathead minnow in our previous study [24]. The exposure was set to the 96 h-LC50 concentration (taken from the U.S. EPA fathead minnow database [28]) and the concentration in gills (richly perfused tissue) after 96 hours was taken as the ILC50 (*in vivo*). Fathead minnow weight was assumed to be 1.326 g (weight corresponding to the recommended length of fathead minnow used for LC50 tests by the OECD 203 guideline [33]; for the impact of body weight on toxicokinetics in fish see [24]).

Statistical Evaluation

Trend lines in graphs were obtained by linear or polynomial (second or third

order) fitting to measured data. Coefficient of determination (R^2) refers to the square of the coefficient between measured and modeled values, and quantifies the fraction of the variability in the data that is explained by the model (based on the FOCUS guidance document [34]).

Coefficient of correlation (r) refers to the Pearson correlation coefficient which describes the strength of a linear correlation (association) between two variables. r=−1 represents a perfect negative correlation, while r=1 means a perfect positive correlation.

RESULTS AND DISCUSSION

Experiments with Cells

For all non-volatile chemicals, mass balances were around 100%. Hydrophobic chemicals (higher log K_{OW}) were accumulated by cells more strongly than less hydrophobic chemicals (lower log K_{OW}, Figure 1). This is consistent with the observation that hydrophobic chemicals generally accumulate more in organisms (and their cells) than hydrophilic compounds.

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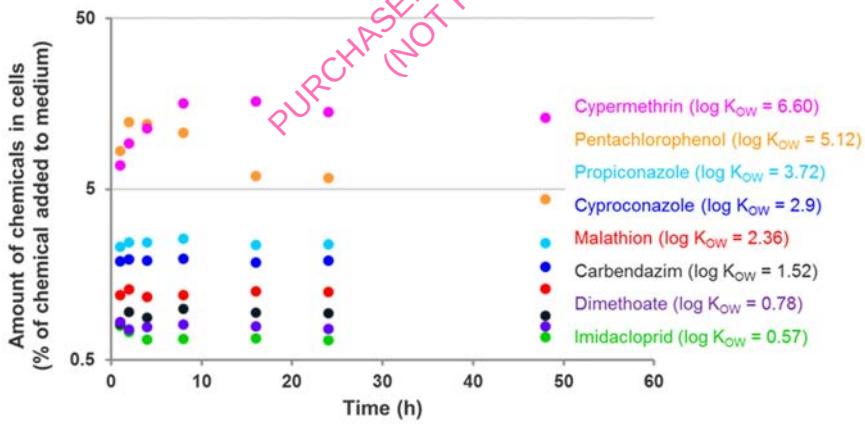


Figure 1: The average accumulation of chemicals in cells over time, expressed as percentage of chemical added to the medium at the beginning of the experiment.

Symbols: measured values.

https://doi.org/10.1371/journal.pone.0092303.g001

Figure 1 shows that amounts of chemicals characterized by low log K_{OW} values stabilized in cells within 4 hours (i.e. toxicokinetics reached equilibrium). However, due to the fact that neither cell number nor chemical concentration in medium was exactly the same at every time point, stabilization of the chemical amount in cells does not necessarily mean the stabilization of chemical concentrations in cells. Calculated times to steady-state conditions ranged from 0.6 days for compounds with low log K_{OW} to 20 days for cypermethrin. The 95% confidence intervals of all rate constants were reasonably small and indicate reliable calibration for all chemicals. Amounts of pentachlorophenol and cypermethrin in cells started to decrease after a few hours. For pentachlorophenol this phenomenon was attributed to its toxicity to cells. A few hours after adding pentachlorophenol to the well plates, cells started to die and detach (confirmed by microscopic observation) which resulted in the decrease of the measured total amount of chemical in the remaining cells.

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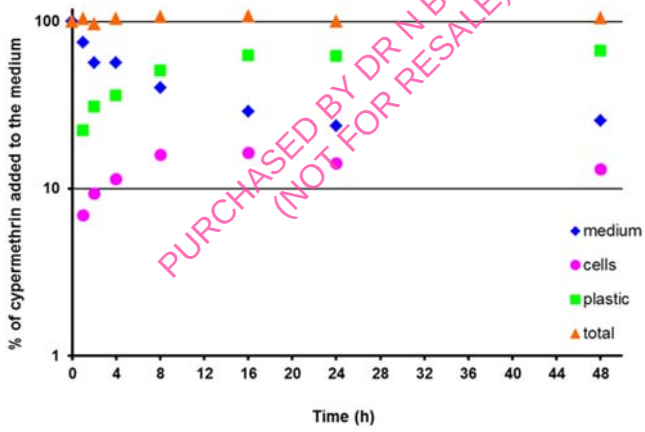


Figure 2: Distribution of cypermethrin in the well presented as average percentages of the chemical in each compartment over time.

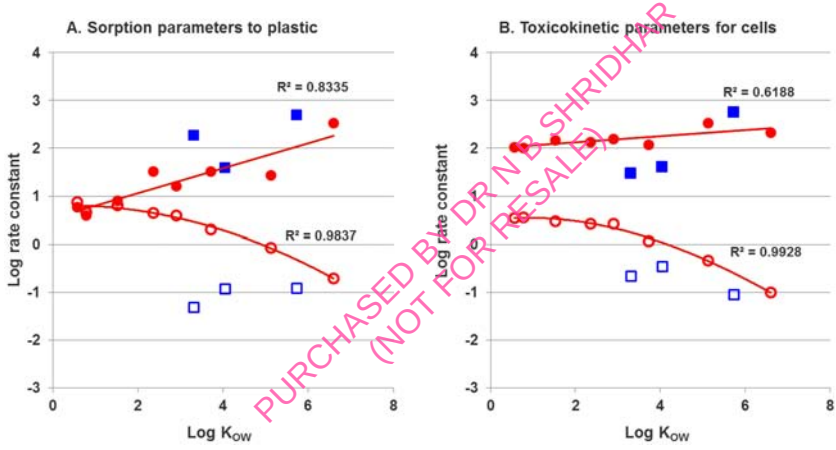
https://doi.org/10.1371/journal.pone.0092303.g002

Cypermethrin is very hydrophobic (log K_{OW}=6.6) and most of the cypermethrin added to the medium was adsorbed to the plastic well (more than 50% was found in plastic and around 15% was absorbed by cells within 8 hours of exposure, Figure 2). For later time points, even though sorption of all tested chemicals to plastic was a faster process than to cells, the cypermethrin amount in the plastic was still increasing. Thus, we assume that the increase in the plastic compartment caused the decrease of the

amount of cypermethrin in the medium which in turn caused the decrease of cypermethrin in the cells at late time points. This observation is in accordance with the fugacity theory which explains diffusive fluxes between matrices. In equilibrium, all matrices (compartments) have equal fugacities but until then, net fluxes occur along fugacity gradients [35].

Modeling Sorption to Plastic

Uptake and elimination rate constants of non-volatile chemicals for the plastic compartment were fitted to time series of measured concentrations of each chemical in plastic. They show a strong correlation with log K_{OW} values (Figure 3A).



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Figure 3: Regression of model parameters and log K_{OW} **for non-volatile (red •– log k_{in},** red ^{O} **– log k_{out} and volatile (blue ▪ – log k_{in},** blue □ – log k_{out}) compounds.

Data for volatile compounds were not used for fitting the model and trend lines. Red — – trend lines for log k_{in} and log k_{out} (A – plastic: log k_{in}=0.2602 · log K_{OW}+0.5385; log k_{out}=−0.0388 ·(log K_{OW})^2+0.0272 · log K_{OW}+0.7982, B – cells: log k_{in}=0.0641 · log K_{OW}+1.9898, log k_{out}=−0.0447 · (log K_{OW})^2+0.0619· log K_{OW}+0.525).

https://doi.org/10.1371/journal.pone.0092303.g003

The relationship between logarithm of uptake rate constant (log k_{in}) and log K_{OW} was linear (coefficient of determination R^2=0.834) while the relationship between elimination rate constant (log k_{out}) and log K_{OW} could be well described by a second-order polynomial (R^2=0.984); however,

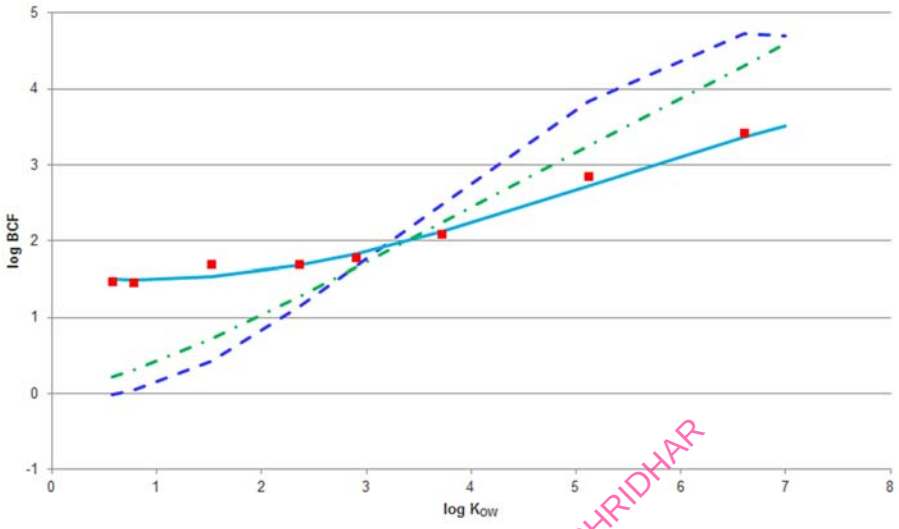
exact values of parameters might differ for different test conditions. The strong binding to plastic was shown previously and it can be influenced by serum content (serum was not present in the medium used in our study) [19], [26], [36].

For volatile compounds, uptake rate constants are similar to these for non-volatiles, while elimination rate constants are much lower for volatile chemicals (Figure 3A). This results in much higher concentrations of volatile compounds in plastic. One of the explanations for these differences could be related to the experimental procedures. As each culture well plate was covered with the plastic foil during the whole experiment, it is possible that some of the chemical, which had evaporated from medium, was adsorbed to that foil [37]. Then, during the shaking process to extract the chemical from plastic, methanol in the wells could have extracted the chemical also from this plastic foil which resulted in an apparent higher amount of the chemical measured in plastic. Thus, the measured distribution to the plastic and the rate constants derived from those are possibly influenced by an experimental artifact that leads to larger measurement errors for volatile compounds. We therefore excluded uptake and elimination rate constants of volatile compounds from our model and expect that measurement and modeling of the fate of volatile compounds in *in vitro* test systems requires a different test design, possibly including sampling of the headspace, use of a different foil material as cover for the well plates, reducing headspace volume or passive dosing.

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Modeling Toxicokinetics in Cells

Uptake and elimination rate constants for non-volatile compounds in cells were fitted to time series of measured concentrations of each chemical in the cells. The relationships between the *in vitro* toxicokinetic parameters and log K_{OW} (Figure 3B) were linear for log k_{in} (R^2=0.619) and well described by a second-order polynomial for log k_{out} (R^2=0.993). Kinetic bioconcentration factors (BCFs) were calculated as the ratio of uptake and elimination rate constants ($BCF=k_{in}/k_{out}$) and compared with measured values (BCF=internal concentration / external concentration after 24 hour exposure) and those predicted in fish by the Arnot and Gobas BCF model and by the PBTK model [38] (Figure 4).



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Figure 4: Comparison between measured and modeled bioconcentration factors (log BCF) in fish and fish cells.

Average BCF measured in cells (red •), kinetic BCF calculated from our empirical model for rate constants (light blue —), equation: log BCF=−0.0078·(log K_{OW})^3 +0.1236·(log K_{OW})^2 −0.2073·log K_{OW}+1.5872, predicted BCF in fish by the Arnot & Gobas model [38](dark blue - -) and BCF predicted in rainbow trout by the PBTK model (green - •); model parameters: fish weight - 2 g, lipid fraction - 5%, water fraction - 75%). For propiconazole (log K_{OW}=3.72) and pentachlorophenol (log K_{OW}=5.12), steady-state condition was not reached within 24 hours.

https://doi.org/10.1371/journal.pone.0092303.g004

The measured BCF value could not be derived for cypermethrin (characterized by the highest log K_{OW} value in our study) due to the decrease of this chemical amount in exposure medium over time. Furthermore, the measured BCF value for pentachlorophenol (log K_{OW} 5.12) had to be corrected for the decrease of cell number, caused by the toxicity of this chemical.

Our data and model indicate a different pattern of accumulation from what could be expected based on the assumption that the log BCF vs. log K_{OW} relationship is dominated by chemical partitioning to cell protein. According to commonly obtained chemical partitioning for tissues like plasma or muscle, which like the gill cells do not contain much lipid [39], this correlation should have a slope between 0.6–0.8 and BCF for chemicals

characterized by low log K_{OW} values should be much lower than those measured in the fish cell line. To exclude the possibility of experimental artifacts caused by the influence of remaining medium on the chemical concentration in cells, additional experiments on the chemical concentration in versene and trypsine were carried out. Experiments confirmed however, that using versene and trypsin for cleaning and detaching cells did not influence chemical concentrations measured in cells.

On the other hand, common BCF models (e.g. Arnot and Gobas model) are based on lipid partitioning and log K_{OW} only. Inclusion of proteins and other tissue components could shift the fish BCF upwards by about one order of magnitude [40]. In addition, DeBruyn and Gobas [41]noticed that for chemicals characterized by low log K_{OW} values (below 2), octanol is a rather poor predictor of the sorptive capacity of proteins. Furthermore, the inaccuracy of K_{OW} and lipid based partitioning models might be greater for H-bond donor compounds [40] and based on chemical structures, all of our compounds with log K_{OW} values below 2 (i.e. imidacloprid, dimethoate and carbendazim) can be H-bond donors. For instance, the log BCF of carbendazim measured in fish varies between 1.36 and 2.2 [42], while the log BCF predicted in fish was equal to 0.42 and the log BCF measured in cells was around 1.7 (Figure 4). However, in order to understand chemical bioconcentration in fish cells better, experiments on a larger set of chemicals, characterized by an even wider range of log K_{OW} values, and with different fish cells should be carried out.

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Internal Effect Concentrations of Chemicals: Cells and Fish

We used our empirically obtained toxicokinetic model to predict internal effect concentrations in cells (IEC50). These internal concentrations were then compared with internal effect concentrations in fish gills (ILC50) predicted by the Physiologically Based Toxicokinetic (PBTK) model. The basis for calculating IEC50s and ILC50s were the measured external concentrations obtained from the CEllSens project [6], [23].

Comparing internal effect concentrations reduces a wide range of external effect concentrations (covering more than four orders of magnitude) to a narrower range of internal effect concentrations (around three orders of magnitude, Figure 5). It is important to notice that, due to the small number of data, the analysis is very sensitive to a few chemicals. However, a significant negative correlation was found between log LC50 and log K_{OW} values (Figure 5A: $r=-0.78$, $p=0.02$, F-test), while external effect

concentrations for cells (Figure 5C), as well as internal effect concentrations in fish and cells (Figure 5 B and D) were not significantly correlated with log K_{OW} ($p>0.05$). In addition, the lack of a relation between predicted internal effect concentrations in fish gills and a fish gill cell line (ILC50 and IEC50) did not support the hypothesis that if chemicals are characterized by the same mechanism of action in fish and fish cells, the concentrations of chemicals in a fish that cause toxicity must be similar to these concentrations in cells that cause toxicity in a fish cell line. These differences result from the pattern that chemical bioconcentration in fish is more sensitive to changes of log K_{OW} values than concentrations in cells (Figure 4). Fish BCFs increase substantially with log K_{OW}. For a given toxic waterborne concentration, therefore, we can expect the internal concentration at steady-state to increase with log K_{OW}. Thus, the decreasing trend in log LC50 with log K_{OW} tends to be offset by a strongly increasing trend of higher accumulation. The result is that the ILC50 is relatively constant with log K_{OW} (Figure 5B). On the other hand, for cells, BCFs do not change as much with log K_{OW} (Figure 4). For this reason, there was no significant correlation between effect concentrations in cells and log K_{OW} (Figures 5C and 5D). Our observation that internal effect concentrations do not depend on log K_{OW} - for both fish and fish cells - is in agreement with the "critical residue" approach that is used to predict waterborne concentrations likely to cause simple narcosis.

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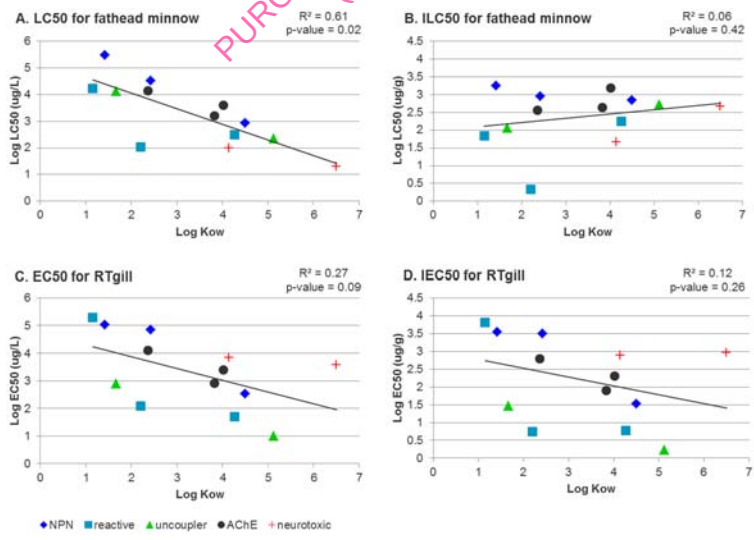
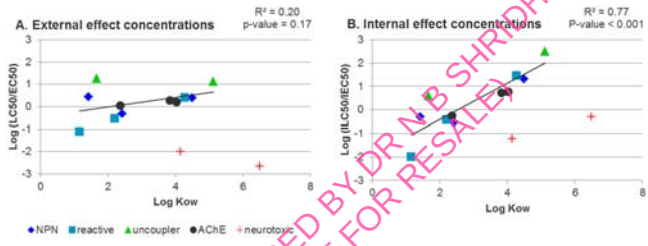


Figure 5: External and internal effect concentrations for fathead minnow gills (A, B) and rainbow trout gill cells (C, D).

Significant correlation between effect concentrations and log K_{OW} were found only for LC50 values (log LC50=−0.5856·log K_{OW}+5.2237).

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To further explore possible relationships between effect concentrations, we developed ratios (log LC50/EC50 and log ILC50/IEC50) that compare the effect concentrations *in vivo* and *in vitro*, and plotted these as functions of chemical log K_{OW} (Figure 6). For these calculations, lindane and permethrin were excluded from statistical analysis. These neurotoxic compounds act on sodium or chloride channels in the brain of fish, respectively, and previous studies had demonstrated that, as opposite to other chemicals from this study, their toxicity to cells [6] and even to fish embryos [43] does not reflect toxicity to fish.



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Figure 6: Relationship between (A) external effect concentrations in fish (LC50) and cells (EC50) vs. log K_{OW} **and (B) between internal effect concentrations in fish gills (ILC50) and cells (IEC50) vs. log K_{OW}.**

The correlation between the ratio of internal effect concentrations and log K_{OW} was significant (r=0.86, p<0.001 F-test). Neurotoxic compounds (lindane, permethrin) are not considered in the regression line. Log (ILC50/IEC50)=0.7736·log K_{OW}−1.9537.

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The ratio between cell EC50 and fish LC50 does not depend on log K_{OW} (Figure 6A, R^2=0.2, p=0.17, F-test), which is in accordance with Tanneberger et al. [6]. However, our analysis of internal effect concentrations revealed a significant correlation between the ratio of ILC50 and IEC50 values and log K_{OW} (Figure 6B, r=0.86, R^2=0.77, p<0.001, F-test; log (ILC50/IEC50)=0.7736·log K_{OW} - 1.9537). This correlation could result from the fact that internal effect concentrations predicted in gills of fathead minnow and a gill cell line of rainbow trout show different patterns due to the differences in toxicokinetics between cells and organism (see Figure 5). It is possible that the toxicity of chemicals was caused by effects in

tissues other than gills; however, since most tissue "targets" reside in the well-perfused compartment, concentrations in other non-gill tissues could be expected to change in direct proportion to those in the gill. This means that even if the site of chemical action is located in tissues other than gills, it could be expected that the slopes of the log EC50 vs. log K_{OW} or log IEC50 vs. log K_{OW} for a tissue relationship are similar, although the intercepts might somewhat change. It therefore might be possible to predict effects on fish based on internal effect concentrations in cells; however, experiments on more chemicals and quantification in cells and fish tissue would be needed to either confirm or reject that hypothesis.

In conclusion, the significant correlation ($r=0.86$, $p<0.001$, F-test) between the ratio of log ILC50 and IEC50 with log Kow values is a very promising model to predict lethal effects on fish based on *in vitro* data. Essentially, IEC50 values can be measured *in vitro* and, using the above relationship, the corresponding ILC50 value can be calculated. Then, the ILC50 value can be converted to an LC50 value using the PBTK model for fish [24]. In our study we derived an empirical prediction model so that internal concentrations in cells can now be predicted for non-volatile organic chemicals with log K_{ow} between 0.5 and 7. In the future, chemical concentrations in cells could also be linked with sublethal toxic effects, thereby providing many other potential applications in *in vitro – in vivo* toxicity extrapolation.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JSM KT KS RA. Performed the experiments: JSM. Analyzed the data: JSM KT KS RA. Contributed reagents/materials/analysis tools: JSM KT KS RA. Wrote the paper: JSM KT KS RA.

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Chapter 16

Are Pharmaceuticals with Evolutionary Conserved Molecular Drug Targets More Potent to Cause Toxic Effects in Non-Target Organisms?

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ABSTRACT

The ubiquitous use of pharmaceuticals has resulted in a continuous discharge into wastewater and pharmaceuticals and their metabolites are found in the environment. Due to their design towards specific drug targets, pharmaceuticals may be therapeutically active already at low environmental concentrations. Several human drug targets are evolutionary conserved in aquatic organisms, raising concerns about effects of these pharmaceuticals

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in non-target organisms. In this study, we hypothesized that the toxicity of a pharmaceutical towards a non-target invertebrate depends on the presence of the human drug target orthologs in this species. This was tested by assessing toxicity of pharmaceuticals with (miconazole and promethazine) and without (levonorgestrel) identified drug target orthologs in the cladoceran *Daphnia magna*. The toxicity was evaluated using general toxicity endpoints at individual (immobility, reproduction and development), biochemical (RNA and DNA content) and molecular (gene expression) levels. The results provide evidence for higher toxicity of miconazole and promethazine, i.e. the drugs with identified drug target orthologs. At the individual level, miconazole had the lowest effect concentrations for immobility and reproduction (0.3 and 0.022 mg L^{-1}, respectively) followed by promethazine (1.6 and 0.18 mg L^{-1}, respectively). At the biochemical level, individual RNA content was affected by miconazole and promethazine already at 0.0023 and 0.059 mg L^{-1}, respectively. At the molecular level, gene expression for cuticle protein was significantly suppressed by exposure to both miconazole and promethazine; moreover, daphnids exposed to miconazole had significantly lower vitellogenin expression. Levonorgestrel did not have any effects on any endpoints in the concentrations tested. These results highlight the importance of considering drug target conservation in environmental risk assessments of pharmaceuticals.

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INTRODUCTION

Pharmaceutical residues are ubiquitous in the environment due to extensive human and veterinary use. Sewage treatment plants cannot successfully eliminate all pharmaceuticals that remain in the effluent either in their original form or as metabolites with residual activities [1]. To achieve optimal therapeutic function, pharmaceuticals are chemically designed to fit specific molecular targets [2], [3], which are often evolutionary conserved and have orthologs in a variety of organisms [4]. This raises concerns regarding potential impacts of pharmaceutical pollution on non-target organisms that have high similarity of molecular targets, such as receptors and enzymes, with humans (so called, the read-across hypothesis [5]). Adverse effects on these organisms may occur already at environmentally relevant concentrations that are well below LC_{50} (i.e., concentration at which 50% mortality occurs) determined in acute tests. The synthetic estrogen 17α-ethinyl estradiol (EE2) is a high-profile case when acute assays were unable to provide sufficient toxicological information [6]. The EE2 enters

the environment through sewage treatment plants and at concentrations in the ng L^{-1} range causes feminization in fish, where estrogen receptors are present [7]–[9]. However, the LC_{50}values obtained in the acute mortality tests on fish are in the range of mg L^{-1} [10]. In contrast to fish, no receptors for EE2 have been found in crustaceans, in which the observed effects of EE2 are minor [11], [12]. Compared to vertebrates, invertebrates have fewer identified orthologs to human drug targets. However, many biochemical and physiological systems are evolutionarily conserved among mammals and invertebrate non-target species, including crustaceans [4]. For instance, the pharmacological target of serotonin reuptake inhibitors is conserved in invertebrates and have consequences for the toxicity of this group of pharmaceuticals [13]. Therefore, knowledge of drug target conservation status could assist in study design, selection of appropriate test species and endpoints as well as data interpretation; this approach is currently referred to as "intelligent testing" in environmental risk assessment [4], [14]. According to the read-across hypothesis, a pharmacological effect in non-target species will occur if the drug target is conserved and the plasma concentration of a drug is similar to the human therapeutic concentrations [5]. However, detecting pharmacological response before a toxicological one is challenging, particularly in small invertebrates, in which drug target orthologs may be involved in other metabolic pathways than their counterparts in vertebrates and the effective dose may differ [15]. Therefore, testing read-across hypothesis *stricto sensu*, i.e. linking haemolymph (analogous to plasma concentrations in fish) and mode of action based effects [5], is not readily applicable to microcrustaceans used in ecotoxicological testing. Yet, it should be possible to use information on various downstream toxicological effects derived from measuring multiple endpoints across different levels of biological organization for drug screening.

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In this study, we hypothesized that the capacity of a pharmaceutical to cause an effect in non-target organisms depends on the presence of human drug target orthologs in the organism. This hypothesis was tested using pharmaceuticals selected to represent those with and without an identified human drug target ortholog in the cladoceran *Daphnia magna*, a common model test species in ecotoxicology. For two of the pharmaceuticals, miconazole and promethazine, an ortholog for the human target calmodulin (CaM) has been identified in *Daphnia*, whereas the third pharmaceutical, levonorgestrel, does not have an identified target ortholog for progesterone or estrogen in this genus [4]. Miconazole belongs to the imidazoles, a family of drugs that deplete ergosterol and thus alter membrane structure in fungi

[16], but it has also been suggested that imidazoles act as inhibitors of CaM activity [17]. Although promethazine's therapeutic action is to inactivate the H1-receptor [18], it has also been found to be a CaM antagonist [19]. Levonorgestrel is a second generation synthetic progestogen that regulates female reproductive cycle and is used in human contraceptive drugs [20]; to date, no target ortholog has been reported for this drug in invertebrates [4]. More specifically, we hypothesized that miconazole and promethazine, i.e. pharmaceuticals for which a drug target ortholog is present in the test species, will cause toxic effects at lower concentrations compared to levonorgestrel.

To address this hypothesis, we applied a battery of ecotoxicological endpoints at individual, biochemical and molecular levels. Immobility, reproduction and development were measured as individual endpoints since they represent crucial life events in the crustacean life cycle [21]. Feeding inhibition, a commonly used and sensitive endpoint [22] that could have consequences for energy intake, growth and metabolism, was also assessed [23]. Individual RNA and DNA contents were used as biochemical endpoints, as RNA content is a practical and relatively sensitive tool for assessing changes in bulk protein synthesis rate, metabolic performance and growth [24]. Gene expression of vitellogenin and cuticle protein were used as molecular endpoints since they are relevant as indicators of reproductive and developmental effects [25]. In *Daphnia*, expression of these genes has been used to diagnose endocrine disruption as a result of exposure to various contaminants, including pharmaceuticals [25], [26] and metals [27], but also as a part of phototoxicity and oxidative stress responses [25]. Alterations in gene expression can be a direct response or a downstream result of a toxic exposure [28] and could facilitate identification of toxic mechanisms and affected pathways within the adverse outcome pathway concept [29].

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MATERIAL AND METHODS

Pharmaceuticals

All pharmaceuticals (98% purity) were purchased from Sigma-Aldrich. They were dissolved in dimethyl sulfoxide (DMSO) for use in the bioassays. DMSO volume in test medium corresponded to 0.1‰ of the total volume and solvent control was used in all tests. Water samples were collected for chemical analysis (Tables A and B in Text S1 and Table S1) and stored at −20°C.

Test Organisms

In all bioassays, the neonates (24-h) of *D. magna* used originated from a single clone (environmental pollution test strain Klon 5, the State office for nature, environment, and customer protection North-Rhine Westfalia, Bonn, Germany; originally from the Federal Environment Agency, Berlin, Germany). The animals were cultured in groups of ~20 individuals in 3-L beakers with M7 medium (OECD standard 202 and 211), and fed a mixture of the green algae *Pseudokirchneriella subcapitata* and *Scenedesmus subspicatus* three times a week.

Acute and Reproduction Test

For all three pharmaceuticals, acute toxicity (OECD 202) [30] and reproduction (21-d, OECD 211) [31] tests were conducted, using glass beakers with 50 mL test volume, at a light:dark cycle of 16:8 h and a temperature of 20±1°C. Immobility tests (48-h, OECD 202) were conducted for miconazole (0.11–0.56 mg L^{-1}), promethazine (0.12–9.4 mg L^{-1}) and levonorgestrel (0.11–1.7 mg L^{-1}), with 4 replicates (5 neonates each) per concentration, and observations every 24 h. Reproduction tests for miconazole (0.00078–0.064 mg L^{-1}), promethazine (0.0062–0.53 mg L^{-1}) and levonorgestrel (0.013–1.02 mg L^{-1}), with 10 replicates per concentration, were conducted using individual daphnids fed *P. subcapitata* (0.1 mg C d^{-1}during the first week and 0.2 mg C d^{-1} during the following two weeks). Test beakers were checked every 24 h and new neonates were noted and removed. Test medium was changed three times per week.

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Exposure for Development, RNA/DNA Measurements and Gene Expression Analysis

Due to logistic constraints, exposure of animals for assessment of development rate, gene expression and RNA content was conducted on several occasions. For promethazine, these endpoints were measured in a single experiment, whereas exposure for miconazole and levonorgestrel were repeated twice, first for the RNA measurements and then for the development rate and gene expression analyses. Daphnids were exposed individually in 24-well microplates, with 2 mL test solution per well, at the same light and temperature conditions as in the acute and reproduction tests. The test solution and food (*P. subcapitata*, 0.025 mgC $animal^{-1}$) were renewed every second day. For the development rate assessment, the wells (n=130–150) were examined for shed exoskeletons every 24 h, and the

current instar was noted. The mean instar at the check point was used as a proxy of development rate. The experiment was terminated when ≥50% of the animals reached instar 3, this occurred on day 2 for promethazine and on day 3 for miconazole and levonorgestrel. Dissolved oxygen saturation and pH were recorded at the start and at the end of the test (Table S1).

Test concentrations for incubations used for RNA and DNA measurements and gene expression analysis were 0.0023 mg L^{-1} for miconazole and 0.059 mg L^{-1} for promethazine corresponding to a factor nine and three below their respective LOEC (lowest observed effect concentration) obtained in the reproduction tests. As no significant reproduction effects were found for levonorgestrel within the concentration range tested, the test concentration for biochemical and molecular endpoints was set to 1.02 mg L^{-1}.

All water samples (except accidentally lost samples from immobility and reproduction tests with levonorgestrel) were sent for chemical analysis (see Text S1 for details on the analytical methods).

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Feeding Inhibition Test

Feeding inhibition tests (24-h) with promethazine (0.12–1.04 mg L^{-1}) and levonorgestrel (0.11–1.7 mg L^{-1}) were performed according to Barata et al. [22] with some modifications. Exposure was conducted in glass vessels with green alga *P. subcapitata* as a food source (1.5 µg C mL^{-1}) in darkness at 20±1°C. Algae concentration (mg L^{-1}) was measured at the start and end with Fluorometer Turner designs model 10-AU-000. Feeding rate (mg C $animal^{-1}$ h^{-1}) was calculated according to Allen et al. [32].

RNA and DNA Measurement

Ten daphnids per treatment and control were used for the RNA and DNA content measurements. The body length (BL, mm; defined as the distance between the eye and the base of the tail spine) of these animals was measured and used as a measure of body size. Individual RNA content was used as a proxy for protein synthetic capacity, whereas DNA content was determined to detect treatment effects on the relationship between BL and cell number in the animals. The length-measured daphnids were preserved individually in 30 µL RNAlater and stored at −20°C until analysis. Measurements of RNA and DNA followed the method described by Gorokhova and Kyle [33] using FLUOstar Optima (filters: excitation 485 nm, emission 520 nm) and black flat-bottom microplates.

Gene Expression

Real-Time quantitative PCR was used to measure relative difference in mRNA expression of vitellogenin and cuticle protein 12 between the exposed and control animals; as a reference gene, β-actin was used. At the termination of the experiment, three replicates for each treatment and control with 20–25 animals per replicate were sampled in Eppendorf tubes and immediately frozen at −80°C until the RNA extraction. Total RNA was extracted using the RNeasy Mini kit with on-column DNase treatment (Qiagen, UK), following the manufacturer's instructions. RNA concentrations were determined on a NanoPhotometer (Implen, Germany). Reverse transcription and amplification were conducted using Power SYBR Green RNA-to-CT 1-Step Kit (Applied. Biosystems, Foster, CA, USA) in a 20 µL reaction volume according to the protocol recommended by the manufacturer. The primers for a 77 bp fragment of vitellogenin (GenBank accession number: DY037239; forward: 5′-GCGGACGAGGTTGCAAAG-3′ and reverse: 5′-AGGAGCAGGAAGATGTCGTTCT-3′), a 199 bp fragment for cuticle protein 12 (DW985490; forward: 5′-AGCCAGTGGAACTACG-3′ and reverse: 5′-TCCAGCATCATCAGCG-3′), and a 71 bp fragment for β-actin (AJ292554; forward: 5′-CCACACTGTCCCCATTTATGAAG-3′ and reverse: 5′-CGCGACCAGCCAAATCC-3′) were adopted from Soetaert et al. [26] and Kim et al. [25]. The reaction plate was subjected in StepOne (Applied BioSystems) real time PCR with reverse transcription. The thermal profile consisted of 30 min of reverse transcription at 48°C one cycle and 10 min of polymerase activation at 95°C, followed by 40 cycles of PCR at 95°C for 15 s and 60°C for 60 s. Following amplification, a melting curve analysis was performed to verify the authenticity of the amplified product by its specific melting temperature. Validation experiments confirmed that the efficiencies of the target and endogenous control (β-actin) amplifications were similar (95–98%). The comparative threshold cycle method [34] was used to assess the relative levels of target gene mRNAs normalized to those of β-actin.

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Statistics

In acute tests, LC_{50} values were calculated using Probit (version 2.3). Reproduction effects, i.e. mean number of offspring, and mortality were analyzed with ANOVA and LSD post hoc using SPSS (PASW Statistics 18); Levene's test was used to assess the equality of variances. General linear models were run in R 2.13.2 to evaluate treatment effects on the

relationships between RNA content and BL and between DNA content and BL; all data for BL, RNA and DNA content were log transformed. If the interaction between BL and treatment was non-significant ($p>0.05$), it was removed to test treatment effect on RNA content. To evaluate differences in mRNA expressions and development between the exposed and controls animals, unpaired t-test was applied.

RESULTS

Acute and Reproduction Toxicity

Both promethazine and levonorgestrel were present in the water phase. By contrast, miconazole was not found in the water phase; yet the exposure effects on the immobility and reproduction were evident from the dose-response curve. Moreover, miconazole was the most toxic among the three pharmaceuticals tested, with the lowest LC_{50} and LOEC values for immobility and reproduction, respectively (Table 1). Effects of promethazine were observed at five- (acute toxicity) and eight-fold (reproductive toxicity) higher concentrations than for miconazole. No effects of levonorgestrel were observed within the range of the concentrations tested. The pH and oxygen levels were all within acceptable limits throughout all exposures.

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Table 1: Effect concentrations obtained in acute toxicity test (OECD 202) (LC_{50}; mg L^{-1}), reproduction test (OECD 211) (LOEC reproduction and mortality; mg L^{-1}) and feeding inhibition test (LOEC; mg L^{-1}), and concentration range (Range, mg L^{-1}) for the pharmaceuticals tested

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Pharmaceutical	Acute toxicity		Reproduction			Feeding	
	LC_{50}	Range	LOEC offspring production	LOEC mortality	Range	LOEC	Range
Miconazole	0.3	0.11–0.56	0.022	0.064	0.00078–0.064	-	-
Promethazine	1.6	0.12–9.40	0.18	>0.53	0.0062–0.53	1.04	0.12–1.04
Levonorgestrel	>1.7	0.11–1.70	>1.02	>1.02	0.013–1.02	>1.7	0.11–1.70

Feeding inhibition test with miconazole could not be conducted due to high toxic effects on the algae.
doi:10.1371/journal.pone.0105028.t001

Development

Daphnids exposed to promethazine developed significantly faster than the control animals ($t=-2.14$, $p=0.034$; Figure 1). In the 2-d incubations, no significant effects on development of either miconazole ($t=0.23$, $p=0.82$) or levonorgestrel ($t=-1.72$, $p=0.087$) were observed (Figure 1).

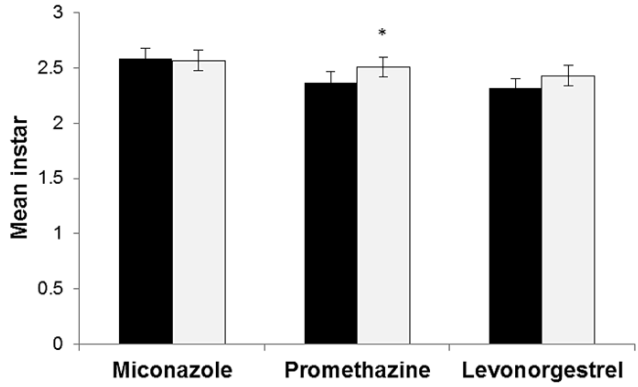


Figure 1: Development and mean instar.

Mean instar (with 95% confidence intervals) for the 2-d incubation (n=10). Test concentrations were 0.0023 mg L^{-1} for miconazole, 0.059 mg L^{-1} for promethazine and 1.02 mg L^{-1} for levonorgestrel. Black bars represent controls and white are the treatments. Asterisk indicates significant level: $p \leq 0.05$ (*) determined by an unpaired t-test.

https://doi.org/10.1371/journal.pone.0105028.g001

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Feeding Inhibition

No effect on feeding rate was observed for levonorgestrel, whereas promethazine had a LOEC of 1.04 mg L^{-1} (Table 1). Feeding inhibition test on miconazole was not possible to conduct due to toxic effects on the algae used as a food source.

RNA and DNA Content

As expected, BL was a significant positive predictor of the individual RNA and DNA contents (Table 2), moreover both miconazole and promethazine had significant effects on RNA allocation. The RNA allocation per BL unit was significantly elevated in animals exposed to miconazole compared to the control, as indicated by the significant treatment × BL interaction (Table 2). In the promethazine treatment, the overall RNA content in the animals was elevated compared to the control, as indicated by significantly positive treatment effect (Table 2). No significant treatment effects were observed in animals exposed to levonorgestrel. No significant treatment effects on DNA content were observed for any of the substances (Table 2).

Table 2: General linear models testing treatment effects on the RNA – body length (BL) and DNA-BL relationships

https://doi.org/10.1371/journal.pone.0105028.t002

Pharmaceutical	Variable	RNA				DNA			
		Estimate	Df	F	p	Estimate	Df	F	p
Miconazole	Treatment	0.27	1	0.56	0.47	0.0089	1	1.88	0.19
	BL	0.92	1	35.27	<0.001***	0.13	1	9.00	0.0081**
	Treatment*BL	1.82	1	9.34	0.0075**	-	-	-	-
Promethazine	Treatment	0.12	1	4.62	0.047*	0.014	1	2.99	0.10
	BL	1.45	1	14.34	0.0016**	0.111	1	44.2	0.001***
Levonorgestrel	Treatment	0.019	1	1.62	0.22	0.010	1	1.83	0.19
	BL	1.33	1	18.34	<0.001***	0.12	1	5.86	0.027*

Tested concentrations were for miconazole 0.0023 mg L^{-1}, for promethazine 0.059 mg L^{-1} and for levonorgestrel 1.02 mg L^{-1}. Asterisks indicate significant level: p≤0.05 (*); p≤0.01 (**); p≤0.001 (***). All treatments were compared against the control.
doi:10.1371/journal.pone.0105028.t002

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Gene Expression

A three-fold decrease was observed for cuticle protein 12 expression in daphnids exposed to promethazine (t_4=4.935, p<0.0078; Figure 2) and miconazole (t_4=6.114, p<0.0036). Additionally, in the latter treatment, there was more than a two-fold decrease in vitellogenin expression (t_4=6.763, p<0.0025), whereas this effect in promethazine was positive, although not quite significant (t_4=2.511, p>0.066). By contrast, no significant changes were observed in the animals exposed to levonorgestrel (vitellogenin: t_4=1.135, p>0.32; cuticle protein: t_4=1.997, p>0.12; Figure 2).

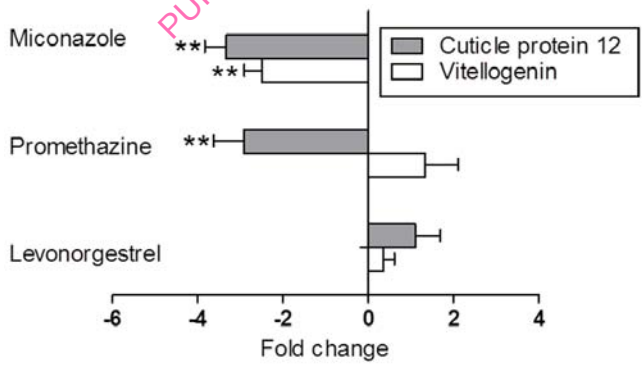


Figure 2: Gene expression changes.

Change in gene expression of cuticle protein 12 and vitellogenin for *D. magna*, instar 3, exposed to miconazole (0.0023 mg L^{-1}), promethazine (0.059 mg L^{-1}) or levonorgestrel (1.02 mg L^{-1}). The fold change (mean ± SD; *n*=3) is shown in relation to the respective controls. Asterisks indicate significance level: p≤0.01 (**) determined by an unpaired t-test.

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Chemical Analysis

The results from the chemical quantification are presented in Table 3. Miconazole is a highly hydrophobic substance (log K_{ow}=6.25); it was therefore not recovered from the aqueous phase. However, the dose-response relationships for mortality and reproduction indicate that the substance was present in the system and caused effects on the test animals.

Table 3: Measured concentrations (mg L^{-1}) in the aqueous phase of the test medium before (Start) and after (End) exposures

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	Acute		Reproduction		RNA/Gene expression	
	Start	End	Start	End	Start	End
Miconazole	n.d. (9.3)		n.d. (0.022)	n.d. (0.022)	n.d. (0.0023)	n.d. (0.0023)
Promethazine	2.9 (3.1)		0.61 (0.53)	0.19 (0.53)	0.044 (0.059)	0.0053 (0.059)
Levonorgestrel					0.76 (1.02)	0.91 (1.02)

Water samples from acute and reproduction tests as well as for the incubations providing material for RNA/gene expression assays were analyzed. The nominal concentrations are presented in brackets. Measured concentrations for reproduction tests are mean concentrations from water samples taken at three time points during the 21-d exposure in connection to water change.
doi:10.1371/journal.pone.0105028.t003

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DISCUSSION

As hypothesized, the presence of drug target orthologs in *D. magna* was positively associated with the responses to the pharmaceuticals tested. In the animals exposed to miconazole and promethazine, which have identified drug targets in daphnids, significant effects on reproduction, mortality, RNA content and gene expression were observed. Moreover, promethazine had additional effect on developmental rate. By contrast, we found no effects of levonorgestrel, which lacks any identified drug target in this species. These findings indicate that drug target conservation is an important parameter for predicting toxic capacity and possible environmental effects of pharmaceuticals on non-target organisms.

For the standardized endpoints, we found miconazole to be the most toxic of the three pharmaceuticals tested with *D. magna*, with the lowest LC_{50} and LOEC values for mortality and reproduction, respectively. Even though the most toxic pharmaceutical could be identified by these tests, the effects at the individual level are as expected first observed when the molecular and

biochemical effects are already severe [35]. Exposure to both miconazole and promethazine had positive effects on the RNA content in the exposed daphnids. This suggest an increased protein synthesis since individual RNA content is positively related to protein production capacity, which should ultimately lead to accelerated development and growth. Indeed, the number of ribosomes consisting largely of rRNA increases with increased protein synthesis rate [36]. Moreover, promethazine had a significantly positive effect on development, which agrees with the increase in RNA and probable increase in protein synthetic capacity. Although elevated RNA and protein synthesis levels may indicate increased growth rate and, hence, potentially higher fitness, they can also reflect increased synthesis of stress related proteins [37]. Heat shock proteins (hsps) belong to a family of evolutionary well-conserved proteins that are found in all living organisms [38]. Their function is to protect proteins and important cell structures from denaturation during stress situations. It is unlikely that the positive effects on RNA content were related to the stimulating effects on somatic growth and associated protein synthesis given the suppressed expression of cuticle protein and vitellogenin mRNA, i.e., mRNA coding for proteins involved in moulting and egg production processes, in the miconazole and promethazine treatments. One can speculate that the observed increase in RNA levels is a consequence of enhanced production of hsps induced by the pharmaceuticals. Moreover, exposure to miconazole resulted in increased RNA allocation per unit of mass as suggested by the increased slope of the relationship between RNA content and BL in the exposed daphnids. However, this increase was not related to any alterations in the allometric relationship between body mass and length as indicated by the homogenous slopes of the DNA-BL regressions (Table 2), which reflects an uniform increase of the cell number with BL. Rather, the observed increase in the slope of the RNA-BL regression following the miconazole exposure was a result of increased rRNA allocation to metabolic maintenance and synthesis of stress proteins that are not involved in routine metabolism. Even though protein synthesis is positively linked to RNA content, it also depends on the ribosomal activity, moreover, the relative importance of the ribosome number and activity may vary depending on the growth conditions [39]. Indeed, in fed rats, the rate of protein synthesis in liver tissue was entirely dependent on ribosomal activity, whereas in starved animals, ribosome number becomes an important controlling factor [39]. This indicates that under our experimental conditions, with food provided at relatively high levels (Table 1), the regulation of protein synthesis may largely rely on

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ribosomal activity and that the increase in RNA content observed in this study is not related to the increased synthesis of structural proteins needed for biomass build-up, but to the increased maintenance costs due to the stress.

Both miconazole and promethazine negatively affected the gene expression of the cuticle protein; moreover, miconazole also inhibited the vitellogenin expression. These two proteins are functionally related to reproduction and development [25]. Both miconazole and promethazine are known to target the calcium-binding CaM [17], [19], a highly conserved protein that in *D. pulex* has a predicted sequence similarity of 98% with the human CaM sequence [4]. CaM controls many physiological functions, among them ecdysteroid-production in crustaceans [40]. Hence, it is possible that the observed decrease in gene expression for cuticle protein is related to an inhibition of CaM activity. Also, the miconazole-induced suppression of vitellogenin expression may be related to the inhibition of CaM, similar to a decreased activity of CaM and concomitant effects on vitellogenin observed in frogs [41]. To establish whether CaM is involved in the observed responses, a controlled experiment with CaM gene expression and the protein activity measurements would be decisive. Further, the decreased vitellogenin expression is likely to be associated with the reduced reproductive potential as observed in *D. magna*exposed to fungicides propiconazole and feranimol [26], [42]. In these studies, decreased expression of genes associated with reproduction and development, among them vitellogenin and cuticle protein, was observed in the exposed animals. Our results support these findings and, together with the low effect concentration for reproduction, show that vitellogenin expression is a sensitive endpoint reflecting changes in reproduction.

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In agreement with our hypothesis, no toxic effects for any of the endpoints investigated were observed at the tested concentrations for levonorgestrel, which lacks any identified drug target in *Daphnia*. Levonorgestrel has been found to cause adverse toxic effects on other vertebrates than humans, such as frogs and fish [20], [43] that have identified drug target orthologs [4] and binding receptors [3] for this drug. Estrogen receptor has not been found in *Daphnia spp.* [44]and Hannas et al. [45] found that vitellogenin gene expression in *D. pulex* was non-responsive to estrogenic activity of chemicals, which is in agreement with our findings.

By studying endpoints across different levels of biological organization, we observed complex responses to the pharmaceuticals tested. Acute tests

rapidly provide information about the general toxicity; this, however, may be grossly underestimated [46], [47]. Reproduction tests provide more ecotoxicologically relevant information, as longer exposure time to lower concentrations is a more likely scenario in the environment. Biochemical and molecular responses may become manifested over shorter exposure times and effects can be observed at even lower concentrations. Even though the tested concentrations were much higher than commonly reported environmental concentrations (miconazole ~8 ng L^{-1} [48], promethazine ~2 ng L^{-1} [49] and levonorgestrel ~7 ng L^{-1} [50]), this study demonstrates the principal feasibility to predict how potent a pharmaceutical may be based on the presence of a drug target ortholog.

As many other types of pollutants, pharmaceuticals may also act via narcosis [51]. The hydrophobic properties of miconazole [52] and promethazine [52], [53] (logP of 6.1 and 4.8, respectively; compared to levonorgestrel with logP: 3.8) as well as the observed toxic responses at relatively high concentrations indicate that narcosis could have been involved in some of the individual-level responses (immobility in particular) observed in this study. Ankley et al. [54] pointed out the importance of using endpoints and biomarkers associated with the MoA when assessing the toxicity of pharmaceuticals. However, for many pharmaceuticals the MoA in non-target species is not necessarily the same as in target species. Our results show that low-level endpoints, representing subcellular aberrations, such as RNA allocation and expression of genes related to egg production and cuticle building, can detect toxic effects at low concentrations when ortholog is present in the test species. Taken together, our results show that the increased toxic capacity of pharmaceuticals with conserved drug targets is detectable by endpoints of general toxicity.

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Several authors [4], [14] have stressed the importance of incorporating the knowledge of drug target conservation in risk assessments of pharmaceuticals. Our results provide experimental evidence for the importance of this approach when applying endpoints of general toxicity and invertebrates as test organisms in risk assessments, although these species have fewer identified human orthologs than vertebrate species [4] and great uncertainties regarding their functionality. The drug target conservation approach could, and should, be strengthened by using adverse outcome pathway approach and molecular docking as essential tools to predict the affinity of pharmaceuticals to molecular targets and their toxic capacity towards non-target organisms [3]. Risk assessments of pharmaceuticals should focus on species that are at risk due to presence of

drug target orthologs and thus facilitate selection of relevant test species for risk evaluation. This knowledge and increasing use of genomic tools would make risk assessments of pharmaceuticals more efficient.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: SF ELB MB EG. Performed the experiments: SF AF ELB. Analyzed the data: SF EG. Wrote the paper: SF MB EG.

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Veterinary Toxicology

Veterinary Toxicology refers to the study of environmental and food hazards that affect animals both domestic and wild.

This book is a useful source of information for the veterinary clinician, student, and toxicologist. The book has 8 sections. The book is divided into many sections, including sections on basic toxicological concepts (section 1), neurotoxicity (section 2), imunotoxicology (section 3), radiation and radioactive materials toxicity (Section 4), and toxicity of metals (section 5). Other sections cover pesticides, insecticides, rodenticides and herbicides (section 6); and environmental toxicology (section 7); The book ends with sections on diagnostic toxicology and therapeutics (section 8).

Sections 1 discuss the general concepts in Veterinary science, like the one-health concept and veterinary pathogens; Translational and application of toxicology studies.

Section 2 concentrates on the study of Neurotoxicity, a form of toxicity in which a biological, chemical, or physical agent produces an adverse effect on the structure or function of the central and/or peripheral nervous system.

Section 3 is dedicated to Immunotoxicity related with nanoparticles. This section includes general chapters dedicated to nanoparticles of different materials, metallic nanoparticles, zebrafish as a model for nanoparticles toxicity and reduce quantity of nanoparticles to reduce toxicity.

Another important subject in veterinary toxicology is the one caused by radiation and radioactive materials (section 4), due to exposer during nuclear radiation accidents or due to radiation treatments.

Metals toxicity is the subject of section 5, which is separated in chapters that analyse different metals (zinc oxide, aluminium, arsenic, chromium, cadmium, copper, mercury, selerium and gold) in different animals.

Section 6 studies the toxicity of pesticides, insecticides, rodenticides and herbicides.

Environmemtal toxicology (section 7) which includes ecotoxicology is the next subject. Ecotoxicology is the study of the effects of toxic chemicals on biological organisms, especially at the population, community, ecosystem and biosphere levels. Ecotoxicology is a multidisciplinary field, which integrates toxicology and ecology.

The last section (section 7) is about diagnostic and therapeutics.

The strengths of this book comprise the inclusion of large and small animal species; the broad range of topics encompassing regulatory, diagnostic, pathologic, and clinical toxicology.

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