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Pim de Voogt *Editor*

Reviews of Environmental Contamination and Toxicology

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Reviews of Environmental Contamination and Toxicology

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Pim de Voogt

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Coordinating Board of Editors

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Foreword

International concern in scientific, industrial, and governmental communities over traces of xenobiotics in foods and in both abiotic and biotic environments has justified the present triumvirate of specialized publications in this field: comprehensive reviews, rapidly published research papers and progress reports, and archival documentations. These three international publications are integrated and scheduled to provide the coherency essential for nonduplicative and current progress in a field as dynamic and complex as environmental contamination and toxicology. This series is reserved exclusively for the diversified literature on "toxic" chemicals in our food, our feeds, our homes, recreational and working surroundings, our domestic animals, our wildlife, and ourselves. Tremendous efforts worldwide have been mobilized to evaluate the nature, presence, magnitude, fate, and toxicology of the chemicals loosed upon the Earth. Among the sequelae of this broad new emphasis is an undeniable need for an articulated set of authoritative publications, where one can find the latest important world literature produced by these emerging areas of science together with documentation of pertinent ancillary legislation.

Research directors and legislative or administrative advisers do not have the time to scan the escalating number of technical publications that may contain articles important to current responsibility. Rather, these individuals need the background provided by detailed reviews and the assurance that the latest information is made available to them, all with minimal literature searching. Similarly, the scientist assigned or attracted to a new problem is required to glean all literature pertinent to the task, to publish new developments or important new experimental details quickly, to inform others of findings that might alter their own efforts, and eventually to publish all his/her supporting data and conclusions for archival purposes.

In the fields of environmental contamination and toxicology, the sum of these concerns and responsibilities is decisively addressed by the uniform, encompassing, and timely publication format of the Springer triumvirate:

Reviews of Environmental Contamination and Toxicology [Vol. 1 through 97 (1962–1986) as Residue Reviews] for detailed review articles concerned with

any aspects of chemical contaminants, including pesticides, in the total environment with toxicological considerations and consequences.

Bulletin of Environmental Contamination and Toxicology (Vol. 1 in 1966) for rapid publication of short reports of significant advances and discoveries in the fields of air, soil, water, and food contamination and pollution as well as methodology and other disciplines concerned with the introduction, presence, and effects of toxicants in the total environment.

Archives of Environmental Contamination and Toxicology (Vol. 1 in 1973) for important complete articles emphasizing and describing original experimental or theoretical research work pertaining to the scientific aspects of chemical contaminants in the environment.

The individual editors of these three publications comprise the joint Coordinating Board of Editors with referral within the board of manuscripts submitted to one publication but deemed by major emphasis or length more suitable for one of the others.

Coordinating Board of Editors

Preface

The role of *Reviews* is to publish detailed scientific review articles on all aspects of environmental contamination and associated (eco)toxicological consequences. Such articles facilitate the often complex task of accessing and interpreting cogent scientific data within the confines of one or more closely related research fields.

In the 50+ years since *Reviews of Environmental Contamination and Toxicology* (formerly *Residue Reviews*) was first published, the number, scope, and complexity of environmental pollution incidents have grown unabated. During this entire period, the emphasis has been on publishing articles that address the presence and toxicity of environmental contaminants. New research is published each year on a myriad of environmental pollution issues facing people worldwide. This fact, and the routine discovery and reporting of emerging contaminants and new environmental contamination cases, creates an increasingly important function for *Reviews*. The staggering volume of scientific literature demands remedy by which data can be synthesized and made available to readers in an abridged form. *Reviews* addresses this need and provides detailed reviews worldwide to key scientists and science or policy administrators, whether employed by government, universities, nongovernmental organizations, or the private sector.

There is a panoply of environmental issues and concerns on which many scientists have focused their research in past years. The scope of this list is quite broad, encompassing environmental events globally that affect marine and terrestrial ecosystems; biotic and abiotic environments; impacts on plants, humans, and wildlife; and pollutants, both chemical and radioactive; as well as the ravages of environmental disease in virtually all environmental media (soil, water, air). New or enhanced safety and environmental concerns have emerged in the last decade to be added to incidents covered by the media, studied by scientists, and addressed by governmental and private institutions. Among these are events so striking that they are creating a paradigm shift. Two in particular are at the center of ever increasing media as well as scientific attention: bioterrorism and global warming. Unfortunately, these very worrisome issues are now superimposed on the already extensive list of ongoing environmental challenges.

The ultimate role of publishing scientific environmental research is to enhance understanding of the environment in ways that allow the public to be better informed or, in other words, to enable the public to have access to sufficient information. Because the public gets most of its information on science and technology from internet, TV news, and reports, the role for scientists as interpreters and brokers of scientific information to the public will grow rather than diminish. Environmentalism is an important global political force, resulting in the emergence of multinational consortia to control pollution and the evolution of the environmental ethic. Will the new politics of the twenty-first century involve a consortium of technologists and environmentalists, or a progressive confrontation? These matters are of genuine concern to governmental agencies and legislative bodies around the world.

For those who make the decisions about how our planet is managed, there is an ongoing need for continual surveillance and intelligent controls to avoid endangering the environment, public health, and wildlife. Ensuring safety-in-use of the many chemicals involved in our highly industrialized culture is a dynamic challenge, because the old, established materials are continually being displaced by newly developed molecules more acceptable to federal and state regulatory agencies, public health officials, and environmentalists. New legislation that will deal in an appropriate manner with this challenge is currently in the making or has been implemented recently, such as the REACH legislation in Europe. These regulations demand scientifically sound and documented dossiers on new chemicals.

Reviews publishes synoptic articles designed to treat the presence, fate, and, if possible, the safety of xenobiotics in any segment of the environment. These reviews can be either general or specific, but properly lie in the domains of analytical chemistry and its methodology, biochemistry, human and animal medicine, legislation, pharmacology, physiology, (eco)toxicology, and regulation. Certain affairs in food technology concerned specifically with pesticide and other food-additive problems may also be appropriate.

Because manuscripts are published in the order in which they are received in final form, it may seem that some important aspects have been neglected at times. However, these apparent omissions are recognized, and pertinent manuscripts are likely in preparation or planned. The field is so very large and the interests in it are so varied that the editor and the editorial board earnestly solicit authors and suggestions of underrepresented topics to make this international book series yet more useful and worthwhile.

Justification for the preparation of any review for this book series is that it deals with some aspect of the many real problems arising from the presence of anthropogenic chemicals in our surroundings. Thus, manuscripts may encompass case studies from any country. Additionally, chemical contamination in any manner of air, water, soil, or plant or animal life is within these objectives and their scope.

Manuscripts are often contributed by invitation. However, nominations for new topics or topics in areas that are rapidly advancing are welcome. Preliminary communication with the Editor-in-Chief is recommended before volunteered review manuscripts are submitted. *Reviews* is registered in WebofScience™. Inclusion in the

Science Citation Index serves to encourage scientists in academia to contribute to the series. The impact factor in recent years has increased from 2.5 in 2009 to almost 4 in 2013. The Editor-in-Chief and the Editorial Board strive for a further increase of the journal impact factor by actively inviting authors to submit manuscripts.

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Caenorhabditis elegans, a Biological Model for Research in Toxicology

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1 Introduction

Caenorhabditis elegans is a non-parasitic nematode which, due to its many convenient features has become an important model in biology research; for example, it was the first animal whose genome was completely sequenced. This nematode was proposed as a model organism by Sydney Brenner in 1965 (Garcia-Sancho 2012). Since then, it has been used in cell biological, genetic and neurobiological studies of higher eukaryotes. Between 1970 and 1980, the complete cell lineage of this worm, from the fertilized egg to adult, was characterized by laser ablation and microscopy (Sulston et al. 1983). Electron microscopy and serial sectioning allowed for the reconstruction of the entire nervous system (White et al. 1986), together with the genetic and genomic data generated in the 1990s (Coulson et al. 1991). This organism has become a powerful tool for the discovery and functional characterization of eukaryotic genes (Dimitriadi and Hart 2010). Many aspects of *C. elegans* as a toxicological model have been reviewed in an excellent paper by Leung et al. (2008). In this state-of-the-art review, the authors present an update on that report, focusing on toxicity end points and assessments for many types of environmental pollutants.

2 Biological Features of *C. elegans*

The body of an adult *C. elegans* is approximately 1 mm long. Its transparency allows viewing of cell types in all stages of development. It has a simple nervous system of 302 neurons as an adult, where each neuron has a unique position (Dimitriadi and Hart 2010; Giles and Rankin 2009). Most organisms are hermaphrodites, with two ovaries, oviducts, a cavity for storing sperm called the spermatheca, and uterus (L'Hernault 2009). Hermaphrodites produce sperm as L4 larvae and oocytes during early adulthood; they reproduce by self-fertilization and therefore cannot fertilize other hermaphrodites. The males, which appear spontaneously with a frequency of less than 0.3 %, are able to fertilize hermaphrodites. The reproductive cycle of *C. elegans* lasts 2.5–4 days at room temperature, and with a usual lifespan of 12–20 days (Giles and Rankin 2009).

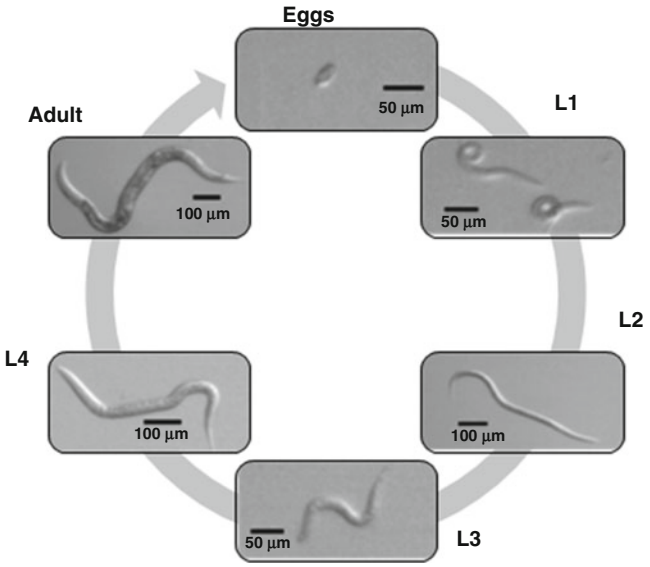

Fig. 1 Life cycle of *C. elegans.* Images were acquired using a dissection microscope Nikon smz 745T with 4× magnification

Embryonic development culminates in the generation of an L1 larva of 550 cells, after 113 cells have died by apoptosis. After four larval stages, the hermaphrodite worm becomes an adult organism with 959 cell nuclei (some syncytial), 302 of which are neurons. Males have 1031 cell nuclei. The mature adult is fertile for 4 days, and it can live between 10 and 15 additional days. Each adult hermaphrodite lays between 200 and 300 eggs, at intervals of about 20 min. Furthermore, the cycle time depends upon the temperature of incubation (Garcia-Sancho 2012). When environmental conditions are adverse, for example, during food shortages, high temperatures, or high population densities, successful reproduction is unlikely. Under such conditions, *C. elegans* can halt its development passing into an alternative L3 stage called *dauer*, which can survive for months. During this stage, the nematode does not feed and its cuticle is tougher. Nematodes can re-enter the reproductive life cycle at L4 when conditions are more favorable (Wang et al. 2010b). Figure 1 shows the complete cycle of *C. elegans.*

3 Advantages of Using *C. elegans* as a Biological Model

C. elegans is used as a model in genetic research because of its convenient features. First, its transparency allows for transgenic proteins fused to fluorescent markers to be visible in living animals in *in vivo* experiments (Giles and Rankin 2009). Its generation time is short (4 days) and it occurs by self-fertilization, ensuring rapid

reproduction in the laboratory (Zhuang et al. 2014) since each adult hermaphrodite produces 200–300 progeny (Megalou and Tavernarakis 2009).

Its excellent performance as a model in genetics has led to the development of many tools and resources, including thousands of characterized mutants and RNA interference libraries, useful for silencing gene expression (Giles and Rankin 2009; Megalou and Tavernarakis 2009). RNA interference (RNAi) with this organism is relatively simple, and therefore gene silencing is often used to dissect signaling pathways (Adam 2009).

C. elegans has been used in toxicological research, from the whole animal level to the level of individual cells (Zhuang et al. 2014). It is cultured in the laboratory in a nematode growth medium (NGM), which contains NaCl, agar, peptone, cholesterol, K_3PO_4, KH_2PO_4, K_2HPO_4 and $MgSO_4$. Another suitable culture medium is K agar, which also contains KCl (Meyer et al. 2010). The worms are maintained in an incubator at 20 °C and the bacteria *Escherichia coli* OP50 is utilized as a food source (Giles and Rankin 2009). The K medium prepared with KCl and NaCl is the liquid used to transfer worms to fresh dishes and to carry out bioassays (Williams and Dusenbery 1990).

4 Applications in Medicine

C. elegans is a model organism that has been important in the studies carried out to identify and understand the functioning of the machinery in nuclear transportation (Adam 2009). It has helped to elucidate biochemical pathways involved in diseases, such as obesity (Finley et al. 2013; MacNeil et al. 2013), diabetes (Estevez et al. 2014; Shi et al. 2012), and Alzheimer's disease (Diomede et al. 2014; Lublin and Link 2013). *C. elegans* is an excellent model to investigate aging because of its short lifespan, its susceptibility to oxidative stress and the similarities with the human aging process (Chatterjee et al. 2013; Pang and Curran 2014). This nematode has also been employed to identify biochemical pathways and mechanisms of action of new drugs, especially antihelmintics (Kumarasingha et al. 2014; Lublin and Link 2013; Wu et al. 2012b).

5 Toxicity Endpoints

Bioassays to assess the effects of a toxicant on *C. elegans* can be carried out through different endpoints. The normal procedure for acute exposure consists of the incubation of young adults in the K medium containing the toxicant at several concentrations, usually without food. In long term exposure assays, worms in the L1 stage are used; in this case *E. coli* OP50 is added as food (Zhuang et al. 2014). When worm reproduction is not required during an experiment, since brood size may affect the results, 5-fluorodesoxiuridine is used to inhibit DNA synthesis (Wu et al. 2012a). Endpoints can be grouped according to their effects on biological parameters, for

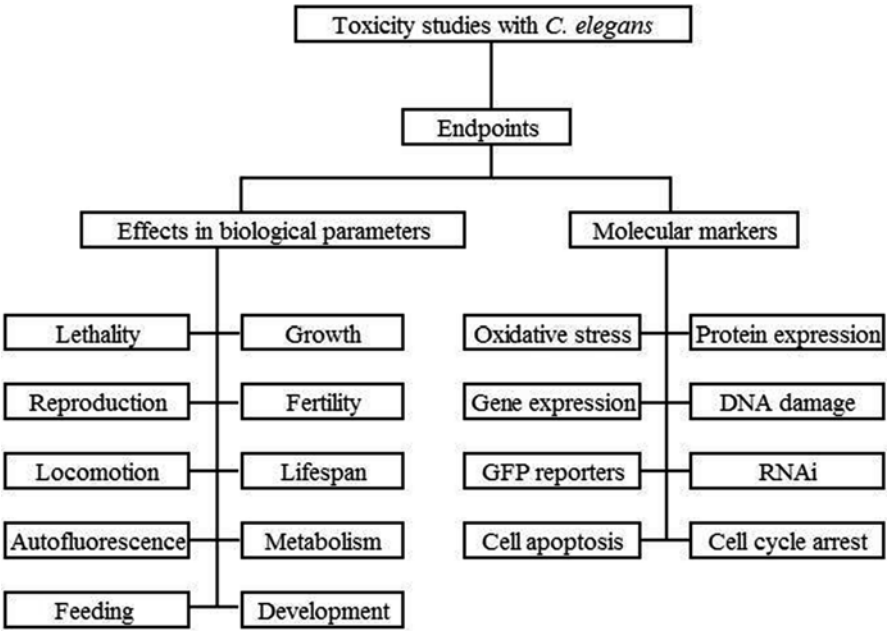

Fig. 2 End points toxicity on *C. elegans*. Toxicity studies with *C. elegans* could be carry out through two kinds of endpoints, those evaluate effects in nematode biology and those use molecular markers

instance, lethality, growth, locomotion, and reproduction. It is also possible to use molecular markers to determine oxidative stress, changes in gene or protein expression, DNA damage, or green fluorescence protein (GFP) expression. A classification of endpoints, commonly utilized in toxicity research using *C. elegans* as model is shown in Fig. 2.

Some of the frequently used endpoints related to toxicity assessment using *C. elegans* are presented below. These assays are usually performed employing concentration-response curves.

5.1 Lethality

This assay is performed to determine the death rate derived from acute toxicity in a concentration-response curve basis. 10 ± 1 young adults are transferred in microplates which contain different concentrations of the toxicant and a negative control. The exposure is carried out at 20 °C during 24 h in the absence of food. Then, the number of live and dead worms is counted through visual inspection using a dissecting microscope (Williams and Dusenbery 1990; Ellegaard et al. 2012; Helmcke and Aschner 2010; Kim et al. 2012; Wu et al. 2012a; Zhuang et al. 2014). Death is assumed when there is no movement during an observation period of 30 s (Rui et al. 2013; Shen et al. 2009; Wang et al. 2009a; Wu et al. 2013).

5.2 Growth

The effect of a toxicant in the development of the nematode can be evaluated by measuring the body length of synchronous worms before and after exposure, then comparing them to a vehicle-control. The bodies of the worms are observed employing a light microscope with 10X magnification and with image analysis software, such as Image-Pro® Express, ImageJ, or Fiji (Boyd et al. 2010; Cha et al. 2012; Höss et al. 2009b; Meyer et al. 2010; Roh and Choi 2011; Shen et al. 2009; Wang et al. 2010a; Yu et al. 2013a, b). Some authors have reported the warming of the worms to 50 °C in order to make them straight and ease the process of measuring their length (Wang et al. 2009a). The immobilization of the worms can also be achieved by using sodium azide (Turner et al. 2013). Growth can also be evaluated by registering the length of a curve, drawn from the tip of the head to the tip of the tail along the dorsal-ventral half of the animal intestine, using the reference line. Width measurements are taken in the vulva, drawing a line on the ventral side of the animal between the front edge and the posterior periphery of the vulva (Rudel et al. 2013). Other authors have proposed measuring the surface area for the flat worm (Rui et al. 2013; Wu et al. 2013; Zhuang et al. 2014). Currently, some laboratories have high-tech equipment, such as COPAS Biosort, which measures the optical density of the worm as an endpoint of growth (Hunt et al. 2012, 2013). The advantage is that the COPAS Biosort can analyze hundreds of nematodes per minute, and it can also evaluate mortality and fluorescence statistics (Hunt et al. 2012; Sprando et al. 2009). For growth assays, some authors perform 24 h exposure periods with *E. coli* OP50 as food (Boyd et al. 2010; Cha et al. 2012; Roh et al. 2009), whereas in other studies, *E. coli* uvrA, previously killed by UVA radiation is used; in this case, the exposure is carried out for 72 h and feeding is re-dosed every 24 h (Turner et al. 2013).

5.3 Reproduction

Brood size is the end point used to evaluate whether a toxic environment affects reproduction of the nematodes, placing exposed adult or L4 worms onto fresh plates. The number of offspring at all stages is counted and compared with a control group (Cha et al. 2012; Gomez et al. 2009; Höss et al. 2009b; Höss et al. 2013; Kim et al. 2012; Leelaja and Rajini 2013; Menzel et al. 2009; Li et al. 2012b; Roh et al. 2009; Rui et al. 2013; Smith et al. 2013; Wang et al. 2009a, 2010a, b). Counting is facilitated by heating the worms to 50 °C and staining them with Bengal red (Höss et al. 2013). In several studies the fertility rate is calculated by counting the total number of larvae at the end of the test and dividing by the total progeny recovered to the total parents (Rudel et al. 2013). This assay may also be carried out using the COPAS Biosort by measuring optical density (Boyd et al. 2010). Moreover, the gonad size, obtained by image analysis under a microscope, has been utilized to evaluate the effects on reproductive organs (Wu et al. 2011). Toxic effects may also be seen as

changes in the egg-laying pattern and the number of eggs or larvae at different time intervals (Gomez et al. 2009; Smith et al. 2013). Finally, the rate of egg laying can be estimated by placing adult worms exposed to fresh plates and counting the number of eggs laid in 1 h (Jadhav and Rajini 2009; Shashikumar and Rajini 2010).

5.4 Fertility

Reproductive toxicity can also be assessed by calculating the percentage of L4 larvae that develop fertilized eggs after exposure. Gravid hermaphrodites are considered to have at least one egg inside their bodies (Höss et al. 2009a, b; Roh and Choi 2011; Wang et al. 2009a). To count the number of eggs in the uterus, nematodes can be transferred to a bleach solution, which dissolves the body of the worm, directly exposing the eggs and allowing them to be counted under a light microscope (Wu et al. 2011).

5.5 Lifespan

Healthy worms at the L4 larval stage are exposed to a toxic agent, for example 24 h and then placing them on NGM plates with *E. coli* OP50. To prevent the production of offspring, 5-fluorodeoxyuridine is added. Worms are transferred to new plates every 3 days. The number of survivors is recorded daily until all animals die. The survival rate is calculated by dividing the number of live nematodes by the total number of nematodes, including both live and dead worms. The lifespan is defined as the time period between the L4 larval stage and death (Cha et al. 2012; Li et al. 2009, 2012b; Shen et al. 2009; Wang et al. 2010a; Zhuang et al. 2014).

5.6 Intestinal Autofluorescence

The intestinal lysosomal lipofuscin deposits that accumulate over time in the nematodes generate autofluorescence, feature used as a marker of aging. Treated nematodes are placed on an agar pad on a glass slide, then the fluorescent signals are captured by a fluorescence microscope. A band filter of 525 nm is employed to detect the endogenous intestinal fluorescence, and images are analyzed using software such as Magnafire®. Lipofuscin levels can be measured using the software ImageJ, by determining the mean pixel intensity in the intestine of each animal. Adults need to be photographed on the same day to avoid the light variation related to the intensity of the fluorescence source (Boyd et al. 2010; Helmcke and Aschner 2010; Rui et al. 2013; Shen et al. 2009; Wang et al. 2010a; Wu et al. 2012a, c, 2013; Zhuang et al. 2014).

5.7 Locomotion

Effects on the locomotion of nematodes have been linked to a deterioration of the neural network which can be evaluated based on several criteria, such as head thrash, body bend frequency, and basic movements (Yu et al. 2013a). Each exposed nematode is transferred to a plate containing 60 μL of K medium on the top of the agar. After a recovery period of 1 min, the number of head trashes is counted for 1 min. A head trash is defined as a change in the direction of bending in the body. To test the body bend frequency, nematodes are collected in a second plate, and then the number of times that the body bends in a period of 20 s is recorded. The bend of the body is observed as a change in direction of the upper pharynx along the Y axis, assuming that the nematodes are moved along the X axis. To test the basic movements, the number of sinusoidal forward movements is counted at an interval of 20 s. Locomotion behavior of control and treated nematodes should be analyzed simultaneously to avoid possible influences of the light-darkness cycle (Giles and Rankin 2009; Li et al. 2009, 2012a, b; Matsuura et al. 2013; Roh and Choi 2011; Rui et al. 2013; Wu et al. 2012a, 2013; Xing et al. 2009a; Yu et al. 2013a, b; Zhuang et al. 2014). Alternatively, immobility is determined by counting the number of immobile worms, usually registering a response when touched by platinum wire (Jadhav and Rajini 2009; Leelaja and Rajini 2013; Roh and Choi 2011).

5.8 Metabolism

To assess the state of metabolism, the pharyngeal pumping speed and the average cycle length of defecation can be evaluated. For testing the pumping rate, the nematodes are placed on NGM agar plates with food. After a few minutes, the pumping movement of the pharynx is counted for a minute under a microscope (Jadhav and Rajini 2009). To test the average cycle length of defecation, every nematode is observed individually for a fixed number of cycles. A cycle is defined as the interval between initiation of two successive steps of muscle contraction (Liu et al. 2013; Wu et al. 2012a; Zhao et al. 2014a, b).

5.9 Development

The effects of toxicants on the development of nematodes can be investigated by counting the number of individuals in each stage of their life cycle: egg, L1, L2, L3, L4 and adults, at regular time intervals up to 96 h after treatment (Roh and Choi 2011). The development through the larval stages can be estimated using the following criteria: L1 if they have four or fewer gonadal cells; L2 if they possess over four gonadal cells which have begun to spread along the length of the animal; L3 if there is a further extension of the gonad, and vulval morphogenesis has started; L4 if there is a dorsal rotation of the gonad; and adults if they have observable eggs (Helmcke et al. 2009). Entrance into the *dauer* state can be used to analyze toxicity,

since *dauer* formation is induced by causing starvation in nematodes. Usually, treated nematodes in state of gravidity are placed on agar plates until laying eggs at 20 °C. This progeny is changed to 27 °C, and 72 h later. The organisms in the *dauer* stage are counted (Wang et al. 2010b).

5.10 Feeding Behavior

Some toxics can affect the feeding and foraging behavior in *C. elegans*. Jones and Candido (1999) described a procedure to assess feeding behavior by monitoring the decline in the density of the bacterial food in liquid cultures of nematodes by measuring absorbance at 550 nm. Another method consists of the use of agar with round holes located equidistant from the center of the dish. Each hole is filled with bacterial suspension in K medium. Toxic solutions are placed in different holes, and nematodes are inoculated in the center of the plate. The number of nematodes in the interior of each hole is counted at various intervals of time. This test shows whether the test nematodes try to avoid contaminated food (Monteiro et al. 2014).

5.11 Oxidative Stress

Several markers of oxidative stress can be determined in worms after exposure to the examined agent, both within the organism and the supernatant (Helmcke and Aschner 2010; Leelaja and Rajini 2012; Shashikumar and Rajini 2010). The production of reactive oxygen species (ROS) and oxidative damage may be determined by fluorescence measurements, usually labeling the nematodes with 5-(y-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (Eom et al. 2013; Leelaja and Rajini 2013; Li et al. 2012a; Rui et al. 2013; Wu et al. 2012a, 2013; Zhuang et al. 2014), and reading with a laser scanning confocal microscope (Eom et al. 2013; Helmcke and Aschner 2010; Rudgalvyte et al. 2013; Liu et al. 2012; Wang et al. 2010a; Wu et al. 2012a). Oxidative damage on macromolecules has been analyzed by detecting carbonylated proteins (Wang et al. 2010a, b; Wu et al. 2011). Moreover, oxidative stress can be evaluated by quantifying changes in gene expression of oxidative stress-related genes by Real Time PCR or GFP reporters, such as *sod-1, sod-2, sod-3, sod-4, sod-5, gst-4, gst-5, gst-8, gst-24* and *gst-42* (Rui et al. 2013).

5.12 Patterns of Gene Expression

One method used to investigate the change in expression of genes in *C. elegans* exposed to environmental pollutants is the use of DNA microarrays or Real Time PCR (Eom et al. 2013; Menzel et al. 2009; Li et al. 2012a; Roh et al. 2009; Roh and Choi 2011; Rudgalvyte et al. 2013). As reference genes, *act-1* (Zhuang et al. 2014; Wang et al. 2014a) and *ubq-1* (Wu et al. 2011) are commonly used. This method has been applied to evaluate the effect on *C. elegans* gene expression for various

environmental toxicants, such as river sediments (Menzel et al. 2009), veterinary drugs (Zhuang et al. 2014), sodium fluoride (Li et al. 2012b), nanoparticles (Eom et al. 2013), and metals (Roh et al. 2009; Wang et al. 2014b; Rudgalvyte et al. 2013) among others.

5.13 Protein Expression

Induction of proteins by exposure to pollutants can be evaluated by traditional techniques such as ELISA and Western Blot. The ELISA technique was used to determine HSP90 protein expression in wild type *C. elegans* exposure to zinc at different temperatures (Wang and Ezemaduka 2014). Western blots were used to evaluate the effect of lead on HSP90 expression (Wang et al. 2014b).

5.14 DNA Damage

C. elegans has been used to evaluate DNA damage through various techniques. One approach is the use of the qPCR technique to detect damage and DNA repair. This test works on the principle that DNA damage inhibits the progression of the polymerase used in qPCR (Roh et al. 2009; Li et al. 2012b). The amount of long PCR product provides a measure of the frequency of the injury (Leung et al. 2010). Another alternative is the comet assay, which was used to evaluate the genotoxic profile of river sediment (Menzel et al. 2009). More recently, the pathway of base excision repair has been proposed as a mechanism to assess the damage to DNA by specific qPCR (Hunter et al. 2012). Transgenic strains can also be used to assess DNA damage. The strain *xpa-1* is deficient in the mechanism of nucleotide excision repair, and its growth is significantly affected when there is damage to DNA. Therefore, the growth assay on this strain is an indicator of genotoxicity (Leung et al. 2010). The transgenic strain *hus-1::GFP* is utilized to assess DNA damage. HUS-1::GFP foci represents DNA double-strand breaks, allowing quantification by counting the number of bright foci per 20 pachytene gonadal germ cells (Hofmann et al. 2002) which can be observed and counted under a fluorescence microscope (Wang et al. 2014a).

5.15 GFP Reporters

Transgenic nematodes carrying the GFP gene fused to various stress-inducible gene promoters have been developed for the study of various biochemical pathways. GFP strains are placed in wells containing the sample solutions and suitable controls. The plates are incubated at 20 °C, performing fluorescence readings within 4–6, 8–20,

and 24–40 h for short, moderate and long exposures, respectively. GFP expression is quantified using a fluorometer with a wavelength of 485 nm excitation and 525 nm emission (Anbalagan et al. 2012; Anbalagan et al. 2013; De Pomerai et al. 2010; Roh et al. 2010; Roh and Choi 2011). Alternatively, the observation of fluorescence can be achieved under a light microscope, capturing images that are then analyzed by specialized software (Li et al. 2009; Shen et al. 2009; Wang et al. 2010a; Polak et al. 2014). The COPAS Biosort system has also been employed to measure fluorescence (Hunt et al. 2012; Turner et al. 2013). The transgenic strain F25B3.3::GFP with fluorescence expression in neurons has been utilized to study heavy metal (Du and Wang 2009; Helmcke et al. 2009) and pesticide toxicity (Negga et al. 2011).

5.16 RNA Interference (RNAi)

This technology has been widely used to study gene function. Bacterial RNAi is introduced for 48 h at room temperature for the expression of dsRNA. Double stranded (ds) RNA expression is induced in HT115 bacteria containing the gene-sequence of interest inserted in the L4440 vector, or else the empty vector as control (Kamath and Ahringer 2003). Approximately ten nematodes in stages L1–L3 are placed on the plate seeded with induced RNAi or vector-control bacteria and incubated at 20 °C. After 36–40 h, the worms are transferred to another plate seeded with the same bacteria and grown to adulthood, at which point cultures are synchronised by egg isolation, and the eggs transferred onto new plates with RNAi bacteria. To evaluate the efficiency of dsRNA feeding over 1000 worms are evaluated by using semiquantitative PCR (Cheng et al. 2014; Kumar et al. 2010; Roh and Choi 2011). RNAi can be used to evaluate genetic pathways involved in toxicant responses. For instance, RNAi has been involved in the transcription of the DAF-16 factor in an unpredicted upregulation of the *cyp-34A9* reporter gene by exposure to high levels of cadmium (De Pomerai et al. 2008). In another case, gene knockdown by RNAi was used to determine the effects on reproduction due to PCB52 exposure; several genes were identified as having a crucial role, being the most remarkable the cytochrome P450s group (Menzel et al. 2007).

5.17 Cell Apoptosis

To assess apoptosis in the cells of the nematode, acridine orange is used. After exposure to the toxicant for 24 h, the nematodes are immersed in mixed medium with acridine orange at 20 °C for 2 h. Then they are placed on top of agar allowing them to recover for 10 min. Finally, they are examined under an inverted fluorescence microscope with an excitation wavelength of 515 and 488 nm absorption. Apoptotic cells appear yellow or yellow-orange showing increased DNA fragmentation, whereas intact cells are uniformly green (Li et al. 2012b; Wang et al. 2009b, 2014a, c).

Another technique involves staining with SYTO 12 for 4 h at room temperature, followed by seeding with food for 30 min, washing with M9 buffer, and final observation under a fluorescence microscope with a red filter (Cha et al. 2012). Alternatively, the transgenic strain *ced-1::GFP* is used for visualization of apoptotic bodies in a fluorescence microscope (Cheng et al. 2014; Kumar et al. 2010).

5.18 Cell Cycle Arrest

To investigate whether the exposure to a toxicant causes cell cycle arrest in the germline, the number of cores of mitotic cells is determined by staining with 4′,6-diamidino-2-phenylindole. The number of mitotic nuclei present at the distal end of the germline is counted under a fluorescence microscope (Cheng et al. 2014; Kumar et al. 2010; Wang et al. 2014a).

5.19 Transgenerational Effects

Sublethal endpoints such as locomotion and growth can be evaluated in the offspring of exposed parents. Wild-type N2 nematodes at the L3 larval stage are exposed during the time when sperm, ova and eggs begin to form, providing a window of prenatal exposure (Yu et al. 2013b). Exposed worms are placed on several plates. Some of them are used for measuring the parents after 24 h of exposure, and the others, for obtaining the generations. This assay has been used to assess the effects of antibiotics (Yu et al. 2011) and heavy metals on the growth and locomotion of exposed parents and their first generation (Yu et al. 2013b).

6 Toxicity Assessments

Most currently known toxicants can be assessed using *C. elegans* as a model. The following are some research studies related to toxicity of environmental matrices, metals, pesticides, nanoparticles, and other chemicals.

6.1 Environmental Samples

C. elegans has been used as a model to assess the toxicity of environmental samples such as soils, sludges, and river sediment. The sediment of the Danube, the Rhine and the Elbe Rivers in Germany were studied by analyzing the changes in gene

expression profiling using DNA microarrays of the entire genome. At the same time, the reproduction and DNA damage were evaluated using the comet assay technique (Menzel et al. 2009). In a study of the toxicity of contaminated soils from Germany, fertility, growth, and reproduction were evaluated using the wild type Bristol N2 strain (Höss et al. 2009b). Organic extracts of contaminated soil from Spain were evaluated using transgenic strains of *C. elegans* carrying GFP reporter genes driven by promoters sequences from five stress-related genes, *hsp-16.2*, *gpx-6*, *hsp-6, gst-1*, and *cyp34A9*; allowing the identification of different mechanisms of toxicity (Anbalagan et al. 2012). Aqueous extracts of the same soils were evaluated using 24 similar GFP transgenic reporter strains, correlating this data with the concentrations of metals present in the soil (Anbalagan et al. 2013). A summary of the results generated from these investigations is shown in Table 1.

6.2 Pesticides

In the environment, *C. elegans* as a free-living nematode, is exposed to various pesticides used in agriculture as well as to persistent organic waste that can contaminate soil for long periods of time (Anbalagan et al. 2013). Some of the most recent studies relating to the toxicity of pesticides in *C. elegans* are summarized in Table 2. As many pesticides are neurotoxic, the well-defined nervous system of *C. elegans* is a suitable tool to assess the neurotoxicity induced by these chemicals (Gomez et al. 2009; Leelaja and Rajini 2012, 2013; Lewis et al. 2013; Negga et al. 2011; Roh and Choi 2008, 2011; Shashikumar and Rajini 2010; Meyer and Williams 2014). Fluorescence expression by GFP reporter genes has been employed to study the toxicity of pesticides such as Glyphosate, Paraquat, 2,4-days, Endosulfan, Cypermethrin, Carbendazim, Chlorpyrifos, Diuron, Rotenone, DDT, Deltamethrin, and Dichlorvos (Anbalagan et al. 2013). In another report, Chlorpyrifos was studied, and although it did not cause severe DNA damage, it inhibited growth of *xpa-1* deficient strain, whose mechanism of nucleotide excision repair is deficient (Leung et al. 2010). The herbicide Glyphosate and the fungicide dithiocarbamate have been studied to assess mortality and neurological damage in *C. elegans*. Neuronal damage by exposure to these pesticides was verified by using the transgenic strain *F25B3.3::GFP* (Negga et al. 2011). The effect of Paraquat, Diquat, and Parathion on brood size was evaluated with COPAS Biosort, with Paraquat showing the highest toxicity (Boyd et al. 2010). Acetylcholinesterase activity of pesticides has also been assessed in nematodes exposed to Fenitrothion and Monocrotophos (Leelaja and Rajini 2013; Roh and Choi 2011). Studies with tributyltin reported that this biocide caused cell apoptosis in *C. elegans* via DNA double-strand breaks (DSBs) (Wang et al. 2014a). Furthermore, tributyltin chloride caused increased sterility and embryonic lethality by DSBs and checkpoint activation in the germline (Cheng et al. 2014). Insecticidal proteins such as Cry, used in transgenic corn, were studied and showed dose-dependent inhibitory effects on *C. elegans* reproduction (Höss et al. 2013).

Table 1 Evaluation of environmental samples using *C. elegans* as a model

Environmental sample	Strain	End point	Result	Reference
Sediments from three rivers in Germany: Danube, Rhine and Elbe	N2	Reproduction, DNA damage, changes in gene expression	Disaccharide and glycogen metabolism and functional pathways were affected	Menzel et al. (2009)
Water and sediment basins affected by coal mining in Virginia (United States)	N2, *gpdh-1, gpdh-2, mtl-2, mtl-2::GFP, pcs-1, smf-2, sod-3*	Growth and GFP expression	Inhibition of growth.	Turner et al. (2013)
Contaminated soils from Germany	N2	Fertility, growth and reproduction	Toxicity was correlated with the organic fraction	Höss et al. (2009a)
Organic extracts of contaminated soils in Southeast Spain	*hsp-16.2::GFP, gpx-6::GFP, hsp-6::GFP, gst-1::GFP, cyp34A9::GFP*	GFP expression	Induction of expression of transgenes	Anbalagan et al. (2013)
Aqueous extracts of soil	*hsp-16.1::GFP:lacZ, cep-1::GFP, hsp-16.2::GFP, sod-3::GFP, cyp-35a2::GFP, daf-16::GFP, gpx-6::GFP, hsp-6::GFP, gpx-4::GFP, cyp34A9::GFP, hsp-3::GFP, mtl-1::GFP, elt-2::GFP, gst-1::GFP, skn-1::GFP, gst-4::GFP, sod-4::GFP, cyp-29A2::GFP, ctl-2::GFP, hsf-1::GFP, mtl-2::GFP, hsp-60::GFP, hsp-70::GFP* y *sod-1::GFP*	GFP expression	Correlation between metals and expressed transgenes	Anbalagan et al. (2012)
Wastewater from recycled paper plant	N2, *daf-2*	Life span, *dauer* formation	Life span decreased	Wang et al. (2010b)
Particulate matter	N2, *unc-47::GFP*	Lethality, lifespan, growth, reproduction, locomotion, intestinal autofluorescence, oxidative stress, defecation behavior, gene expression	Effects on lifespan, reproduction, locomotion behavior, and intestinal development exposed and their progeny	Zhao et al. (2014a,b)
Surface water containing brominated organic compounds	N2	Locomotion, pharyngeal pumping, defecation, mechanical sensory stimulus, chemotaxis, thermotaxis	The surface water was neurostimulatory	Ju et al. (2014)
Dispersed oil crude	N2, (*bcIs39* [*(lim-7)ced-1p::GFP + lin-15(+)]), and TJ1(cep-1(gk138) I*)	Cell apoptosis, gene expression	Increasing of germ cell apoptosis following a CEP-1-dependent pathway	Polli et al. (2014)
Soil-derived Fe oxide colloids	N2, *sod-2(ok1030)*	Uptake of Fe, growth, reproduction	The toxicity of ferrihydrite, goethite and akaganeite depends on aggregate size and specific surface area	Höss et al. (2015)

Table 2 Evaluation of pesticide toxicity

Pesticide	Strain	End point	Result	Reference
Phosphine	N2	Oxidative stress	Delayed development and oxidative stress	Leelaja and Rajini (2012)
Fenitrothion	N2, *cyp35a2*	Paralysis, growth, fertility, development, AC activity	*Cyp35a2* involved in toxicity	Roh and Choi (2011)
Monocrotophos	N2	Fertility, oxidative stress, AC activity, paralysis	Decreased oxidative stress, paralysis and brood size. AC inhibition	Leelaja and Rajini (2013)
Cypermethrin	N2	Expression of *hsp-16*, rate of egg laying, life span, brood size, oxidative stress	Brood size, egg laying and life span decrease in a dose-dependent fashion Increased ROS and carbonylated proteins	Shashikumar and Rajini (2010)
Dithiocarbamate and glyphosate	N2, *F25B3.3::GFP*	Lethality and neuronal damage	Neurotoxicity was verified by fluorescent expression at neuronal level	Negga et al. (2011)
Cry proteins	N2, *bre-5*	Reproduction, gene expression	Reproduction inhibition MAPK pathway upregulated	Höss et al. (2013)
Gliphosate, Paraquat, 2,4-D, Endosulfan, Cypermethrin, Carbendazim, Chlorpyrifos, Diuron, Rotenone, DDT, Deltamethrin, Dichlorvos	*hsp-16.1::GFP:lacZ, cep-1, hsp-16.2, sod-3, cyp-35a2, daf-16, gpx-6, hsp-6, gpx-4::GFP, cyp34A9, hsp-3, mtl-1, elt-2, gst-1, skn-1, gst-4, sod-4, cyp-29A2, ctl-2, hsf-1, mtl-2, hsp-60, hsp-70, and sod-1*	GFP expression	Dichlorvos and Rotenone induced several stress response genes in a dose- dependent manner Endosulfan, DDT, Carbendazim, Deltamethrin, Cypermethrin, 2,4-D and Chlorpyrifos induced few genes	Anbalagan et al. (2013)
Chlorpyrifos	N2, *emb-8, glp-1, xpa-1*	Growth and DNA damage	No DNA damage was detected, but the *xpa-1* mutant was more sensitive than the wild type	Leung et al. (2010)

(continued)

Table 2 (continued)

Pesticide	Strain	End point	Result	Reference
Chlorpyrifos	N2	Gene expression, changes of free concentration of chlorpyrifos in the medium	The free concentration of chlorpyrifos quickly diminished and the expression of *cyp* genes varied with the volume of exposure medium and the test duration	Roh et al. (2014)
Diquat, Paraquat, Parathion	*N2, myo-2::GFP*	Body length, optical density, GFP expression, reproduction	Parathion > Diquat > Paraquat	Boyd et al. (2010)
Tributyltin	N2, *ced-3*, *ced-4*, *egl-1*, *ced-9*, *cep-1*, *cep-1*, *clk-2*, *hus-1*, *lin-45*,*mek-2*, *ksr-1*, *mpk-1*, *nsy-1*, *mek-1*, *jkk-1*, *mkk-4*, *jnk-1*, *sek-1*, *pmk-1*	Cell apoptosis, Cell cycle arrest, gene expression, DNA damage	Induction of apoptosis in germline via DNA damage	Wang et al. (2014a)
Imidacloprid and Tiacloprid	*N2*	Reproduction	Synergistic effect after exposure to the mixture	Gomez et al. (2009)
Dichlorvos	*N2*	Pharynx pumping, contraction of the nose, paralysis, egg laying rate	Correlation between the biochemical effects and behavior parameters	Jadhav and Rajini (2009)
Monocrotophos	N2	Locomotion, lifespan, egg laying, growth, AC activity	Decreased locomotion, lifespan, egg laying, and brood size	Salim and Rajini (2014)

6.3 Metals

Metals, in particular those named as heavy metals, constitute one of the most important groups of environmental toxicants, and reach the ecosystems from sources such as oil refineries, mining, and industrial effluents, causing severe toxic effects on living systems. This group has been one of the most studied using *C. elegans* as a biological model. Several of the reports related to the effects of heavy metals on *C. elegans* are presented in Table 3. The effects of different metals such as Ag, As, Cr, Cd, Cu, Hg, Mn, Pb, Ni and Zn have been studied for several end points such as lethality (Williams and Dusenbery 1990), lifespan, fertility, growth, intestinal autofluorescence, GFP expression, morphology changes (Shen et al. 2009; Rudel et al. 2013; Hunt et al. 2012), neuronal damage, neurodegeneration, neuronal loss, and axonal degradation (Du and Wang 2009; Xing et al. 2009a, b). On the other hand, exposure to Zn, Cd, Hg, Cu, Fe, Cr, and As has also been monitored using GFP transgenic reporter strains (De Pomerai et al. 2010).

6.4 Nanoparticles

The toxicological potential of nanoparticles (NPs) is receiving increased attention because of their massive release into the environment. Although a number of manufactured NPs are employed for medical and clinical purposes, the interaction between nanomaterials and biological systems remains unknown. For this reason, the NPs have joined the group of Emerging Contaminants and every year more studies on the subject are performed with *C. elegans* (Table 4). It has been considered that the main mechanism of nanotoxicity is oxidative stress (Zhao et al. 2014a). Toxicity of hydroxylated fullerene nanoparticles was studied using *C. elegans*, and it was demonstrated that water-soluble fullerol NPs have a potential for inducing apoptotic cell death (Cha et al. 2012). The study of TiO_2 NPs was carried out by analyzing different toxicity endpoints such as lethality, reproduction, growth, locomotion, intestinal autofluorescence, and oxidative stress. TiO_2 NPs caused severe deficits in gut development, defecation behavior, and changes in gene expression (Rui et al. 2013; Zhao et al. 2014a). The toxicity of TiO_2, ZnO, and SiO_2 NPs has been compared using endpoints including lethality, locomotion, growth, reproduction, and production of ROS. The order of toxicity was $ZnO > TiO_2 > SiO_2$ (Wu et al. 2013). In a study of the intake of silver NPs by image analysis, there was absorption of silver NPs in the body, transgenerational transfer, and inhibition of growth (Meyer et al. 2010). The lethal effects of AgNPs on *C. elegans* are increased if the exposure is through *E. coli OP50* (Ellegaard et al. 2012). In other research, reduction was observed in survival and reproduction and there was interaction of Ag NPs with biological surfaces of *C. elegans*, causing severe edema (Kim et al. 2012).

Table 3 Evaluation of metals and their derivatives

Metal	Strain	End point	Result	Reference
Zn, Cd, Hg, Cu, Fe, Cr, As	*mtl-1::GFP, mtl-2::GFP, hsp-16.1::GFP, hsp-16.2::GFP, hsp-3::GFP, hsp-6::GFP, hsp-60::GFP, hsp-70::GFP, hsf-1::GFP, elt-2::GFP, sod-1::GFP, sod-3::GFP, sod-4::GFP, ctl-2::GFP, skn-1::GFP, gst-1::GFP, gst-4::GFP, cyp-35a2::GFP, cyp34A9::GFP, cyp-29A2::GFP, daf-16::GFP, gpx-6::GFP, gpx-4::GFP, cep-1::GFP*	GFP expression	Pattern of gene expression induced by heavy metals	De Pomerai et al. (2010)
Cd	N2, *age-1,mtl-2, sod-3, daf-21, cyp35a2, skn-1, daf-12, hsp-16.2, daf-18, ctl-2, sod-1, daf-16, cep-1, cdr-2*	LC50, growth, reproduction	*mtl-2* gene expression upregulated	Roh et al. (2009)
Ag, Cr, Cd, Cu, Hg, Mn, Pb, Zn	N2, *hsp-16::2-GFP*	Lethality, life span, intestinal autofluorescence, GFP expression	Ag, Cr, Pb, Cu, Hg and Cd induced a stress response in the intestine; Pb, Hg, Cr, Zn and Mn in the neurons of the head	Shen et al. (2009)
Cd, Cr, Hg, Pb	N2	Brood size, generation time	Brood size was significantly correlated with the concentrations of the Metals. Hg produced severe reproductive toxicity	Guo et al. (2009)
Cd	*N2, myo-2::GFP*	Body length, optical density, GFP expression, reproduction	Brood size decreased in a concentration-dependent manner	Boyd et al. (2010)
Cr (VI)	N2	Lethality, growth, gene expression	Alterations in growth and modified gene expression were observed	Saikia et al. (2014)
Cr (VI)	N2	Lethality, locomotion, metabolism, intestinal autofluorescence, oxidative stress	Lethality, locomotion, metabolism, intestinal autofluorescence and ROS production were severely altered	Wu et al. (2012a)
Ca, Cd	*N2, mev-1*, *daf-16*, *daf-2*, *daf-16, hsp-16.2::GFP*	Life span, autofluorescence, GFP expression, oxidative stress, brood size, body length	The combined exposure to Cd and Ca increased *hsp-16.2::GFP* expression and oxidative damage	Wang et al. (2010a, b)

Metal	Strain	End point	Result	Reference
$H_3C\text{-}Hg^{(+)}$	*N2, F25B3.3*::*GFP, unc-17*::*GFP, unc-25*::*GFP, unc-47*::*GFP, cat-1*::*GFP, tph-1*::*GFP, eat-4*::*GFP, dat-1*::*GFP, F49H12.4*::*GFP*	Lethality, life span, growth, reproduction, development, pharynx pumping, GFP expression	Induction of developmental delay and reduced pharynx pumping	Helmcke et al. (2009)
$H_3C\text{-}Hg^{(+)}$	N2, *gst-4::GFP, hsp-4::GFP, mtl-1::GFP, mtl-2::GFP, gst-4, mtl-2, mtl-1*	Lethality, intestinal autofluorescence, GFP expression, oxidative stress	The methyl mercury induced hormesis.	Helmcke and Aschner (2010)
Cd, Pb, Cu, Zn	N2	Locomotion, growth, generational effects	Inhibition of growth and locomotion was more severe in the offspring than in exposed parents	Yu et al. (2013b)
Pb, Zn, Ni	N2	Reproduction, growth, feeding behavior	In reproduction Pb > Ni > Zn. In growth Ni > Pb > Zn	Monteiro et al. (2014)
Mn	N2, *hsp12.2::GFP*	Life span, growth, reproduction, GFP expression, oxidative stress	Defects in life span, development and reproduction. Increase in GFP expression and genes responsive to oxidative stress	Xiao et al. (2009)
Ni	N2, JK574	Growth, life span, fertility	Decreased larval survival and adult longevity depending upon concentration. Aqueous Ni inhibits fertility	Rudel et al. (2013)
$NiCl_2$	N2, MD701, KX84, DJR1	DNA damage, gene expression, fluorescence microscopy, apoptosis	Apoptosis is a result of exposure at high concentrations	Huffnagle et al. (2014)
Cd, Cu, Hg, K, As	N2	Lethality, fluorescence using COPAS Biosort	Changes in the morphology of the vulva and gonad, the thickness and internal intestinal integrity and retained eggs	Hunt et al. (2012)
$CdCl_2$, $CrCl_2$, $HgCl_2$, $Pb(NO_3)_2$	N2	Locomotion	Younger larvae showed more severe deficits in neurobehavioral phenotypes	Xing et al. (2009a)
$CrCl_2$, $CuSO_4$, $HgCl_2$, $AgNO_3$	N2, *lin-15, gcy-8::GFP*	Thermotaxis, GFP expression, gene expression	Severe deficits in thermotaxis	Xing et al. (2009b)

(continued)

Table 3 (continued)

Metal	Strain	End point	Result	Reference
Zn	N2, *daf-21*	Lethality, protein expression	High temperatures increase the toxicity of zinc. *daf-21* was more sensitive.	Wang and Ezemaduka 2014
Hg	N2, *hsp-16.2::GFP*	Reproduction, fertility, gonad size, GFP expression	Altered expression patterns of genes regulating oxidative stress causing deficiencies in the reproduction	Wu et al. 2011
Pb, Hg, Cu, Cd	N2, *unc-47*::*GFP*	Neuronal loss and neuronal degradation by fluorescence	Axonal degeneration and loss of neurons	Du and Wang (2009)
Cu	N2, *ced-3*, *ced-4*, *egl-1*, *ced-9*, *cep-1*, *clk-2*, *hus-1*, *abl-1*, *lin-45*, *mek-2*, *mpk-1*, *nsy-1*, *mek-1*, *jkk-1*, *mkk-4*, *jnk-1*, *sek-1*, *pmk-1*, *pmk-3*	Cell apoptosis	Caspases and Apaf-1 are required for the apoptosis germline	Wang et al. (2009b)
Cu	N2, *unc-2*	Changes in cuticle, chemotaxis, reproduction, development, and development	Body surface from vulva to tail was wrinkled and folded. Vulva size decreased. Development was delayed, egg-laying was reduced	Song et al. (2014)
Pb	N2, *daf*-2	Lethality, HSP 90 expression, pharyngeal pumping, reproduction, longevity	Temperatures affected lead toxicity	Wang et al. (2014b)

Table 4 Assessment of nanotoxicity

Nanoparticle	Strain	End point	Result	Reference
AgNPs	N2, *nth-1, sod-2, xpa-1, mtl-2, mev-1*	Growth, intake of silver NPs by image analysis	There was absorption of silver NPs in the body, transgenerational transfer and inhibition of growth	Meyer et al. (2010)
AgNPs	N2, *pmk-1, hif-1, egl-9, vhl-1, fmo-2, cyp35a2*	Oxidative stress and gene expression	Toxicity depends on size of NPs and ions in solution	Eom et al. (2013)
AgNPs	N2	Lethality and reproduction.	Reduction in survival and reproduction. Interaction of Ag NPs with biological surfaces	Kim et al. (2012)
AgNPs	*pha-1*	Lethality	Lethality is increased if the exposure is through food	Ellegaard et al. (2012)
AgNPs	N2, *cep-1*	Lethality, oxidative stress, DNA damage, mitochondrial membrane potential	Induction of oxidative stress-related mitochondrial DNA damage	Ahn et al. (2014)
AgNPs	N2	Lethality, hiperspectral image analysis	The presence of natural organic matter decreased toxicity and intestinal matter	Yang et al. (2014)
AgNPs	N2, pmk-1, ndx-4, nth-1.	RNAi, gene expression, DNA damage, survival	p38 MAPK/PMK-1 plays an important protective role in AgNP-induced oxidative DNA damage-repair	Chatterjee et al. (2014a)
AgNPs	N2	Growth, locomotion, gene expression, uptake of AgNPs	Results with *C. elegans* correlated with outcomes in rodents for AgNP size vs. uptake and toxicity	Hunt et al. (2014)
AgNPs	N2	Lethality, growth, reproduction	Sulfidation of AgNPs decreased toxicity. Reproduction was the most sensitive endpoint	Starnes et al. (2015)
Al_2O_3 and AgNPs	N2	Growth, survival, reproduction	In NPs-treated soils no acute toxic effects were found	Fajardo et al. (2014)
Al_2O_3NPs	*N2, sod-3*, *sod-2*, *hsp-16.2::GFP*	Locomotion, gene expression, oxidative stress, oxidative damage	Effects on locomotion by induction of ROS production	Li et al. (2012a)
CeO_2 and TiO_2NPs	N2	Lethality, Growth, fertility, gene expression, RNAi	Induction of *cyp35a2* gene expression, reduction of fertility and life span	Roh et al. (2010)
TiO_2NPs	N2, *mtl-1, mtl-2, sod-1, sod-2, sod-3, sod-4, sod-5, mev-1, aak-2, xpa-1, pcm-1, hsp-16.48, hsp-16.2, gst-4, gst-8, gst-24, gst-5, gst-42, isp-1*	Lethality, growth, reproduction, locomotion, intestinal autofluorescence, oxidative stress, gene expression	*sod-2*, *sod-3*, *mtl-2*, and *hsp-16.48* genes were susceptible to exposure.	Rui et al. (2013)
TiO_2NPs	N2	Lethality, growth, locomotion, autofluorescence intestinal, oxidative stress	Toxicity in oversized NPs was detected in the range of mg L^{-1} and small sizes were in the range of ng L^{-1}	Wu et al. (2012c)

(continued)

Table 4 (continued)

Nanoparticle	Strain	End point	Result	Reference
TiO_2 NPs	*mtl-1*, *mtl-2*, *sod-1*, *sod-2*, *sod-3*, *sod-4*, *sod-5*, *mev-1*, *aak-2*, *xpa-1*, *pcm-1*, *hsp-16.48*, *hsp-16.2, gst-4*, *gst-8*, *gst-24*, *gst-5*, *gst-42*, *isp-1*	Lethality, reproduction, locomotion, lifespan, development, growth, oxidative stress, intestinal autofluorescence, pharyngeal pumping and defecation	*sod-2*, *sod-3*, *mtl-2*, and *hsp-16.48* were susceptible strains for reproduction and locomotion, *sod-2*, *sod-3* and *mtl-2* for survival and *mtl-2* for development	Wu et al. (2014b)
TiO_2NPs	N2	Intestinal autofluorescence, oxidative stress, defecation cycle	Severe deficits in gut development and defecation behavior	Zhao et al. (2014a)
ZnONPs	N2	Lethality	Fototoxicity of NPs was correlated with ROS generation	Ma et al. (2011)
ZnO, Al_2O_3 and TiO_2NPs	N2	Lethality, fertility, growth and reproduction	Inhibition of growth and reproduction	Wang et al. (2009a)
TiO_2, ZnO and SiO_2NPs	N2	Lethality, locomotion, growth, reproduction and oxidative stress	ZnO > TiO_2 > SiO_2	Wu et al. (2013)
CeNPs	N2	Lethality, synchrotron X-ray analysis	The positively charged CeO_2-NPs were more toxic. The addition of humic acid decreased the toxicity	Collin et al. (2014)
Carbon nanotubes	N2	Reproduction, growth, life span, gene expression	Retarded growth, lower life span and defective embryogenesis	Chen et al. (2013)
Graphene oxide	N2, *mir-231,mir-235,mir-244, mir-73, mir-74,mir-247, mir-797,mir-246, mir-81-82,mir-360,mir-259*	Lifespan, oxidative stress, intestinal autofluorescence, locomotion, miRNA expression	23 up-regulated and eight down-regulated miRNAs, and correlation with lifespan	Wu et al. (2014a)
Nanosized zero-valent iron	N2	Growth, survival and reproduction	There were toxic effects at 5 and 10 mg mL^{-1}	Sacca et al. (2014)
Hydroxylated fullerene	N2, *ced-3, ced-4, elt-2::GFP*	Life span, reproduction, body length, cell apoptosis	Induction of death by cell apoptosis	Cha et al. (2012)
CdTe quantum dots	N2, *oxIs12[Is(*P*unc-47::GFP)], Ex(*P*unc-25-unc-30), Ex(*P*unc-25-sod-2),* and *Ex(*P*unc-25-sod-3)*	Foraging behavior, defecation behavior, reproduction, fluorescence	Neurotoxicity in the range of µg/L via oxidative stress and cell identity	Zhao et al. (2015)

6.5 Drugs

Drugs and their metabolites have been classified within the group of emerging contaminants because of increased concentrations in the environment. The impact of several drugs on organisms has been evaluated using *C. elegans* as a model (Table 5).

Table 5 Evaluation of the toxicity of drugs

Drug	Strain	End point	Result	Reference
Sulphamethoxazol	N2	Generational effects on locomotion and growth	Defects in locomotion of exposed parents and first generation were dose dependent	Yu et al. (2011)
Sulphamethoxazol	N2	Reproduction, growth, life span, pharynx pumping, lipid peroxidation and gene expression	Reduction in reproduction	Liu et al. (2013)
Sulfonamides	N2	Lethality, life span, growth, behavior inhibition	Concentration-dependent toxicity interactions	Yu et al. (2015)
Ethyl methanesulfonate	N2, *unc-58(e665)*	Mutagenesis, gene expression, DNA damage	Elevated mutation frequencies because embryonic cell cycles are rapid and DNA damage checkpoints are muted in embryos	Hartman et al. (2014)
Clenbuterol and Ractopamin	N2, *daf-2, daf-15, daf-16, sgk-1, skn-1, aak-1, age-1, sod-2, pdk-1, rict-1, act-1*	Lethality, reproduction, growth, locomotion, intestinal autofluorescence, oxidative stress, life span, gene expression	Toxicity by different mechanisms	Zhuang et al. (2014)
Caffeine and methadone	*N2, myo-2::GFP*	Growth, optical density, fluorescence and reproduction	Alteration in reproduction	Boyd et al. (2010)
Nicotine	N2	MicroRNA expression	There was alteration of microRNA expression profiles during post-embryonic stages	Taki et al. (2014)

(continued)

Table 5 (continued)

Drug	Strain	End point	Result	Reference
Nicotine	N2	Reproduction and gene expression	Loss of response to stimuli, early egg laying and alterations in genes related to reproduction and neuronal development	Smith et al. (2013)
Nicotine	N2, *lev-1*, *unc-29*, *bas-1*, *cat-2*, *tph-1*	Nicotine preference, taste plasticity, locomotion	Nicotine preference increased and taste plasticity inhibited	Matsuura et al. (2013)
Nicotine	N2	Locomotion	Reduction in velocity of basic movements and paralysis	Sobkowiak et al. (2011)
5-Fluorouracil	N2, *rrf-3*	Cell cycle arrest, apoptosis, RNAi, growth, development, gene expression	Induction of cell cycle arrest and germline apoptosis. Alteration in vulva development and egg laying	Kumar et al. (2010)
Genkwa Flos (traditional Chinese medicine)	N2, *daf-16*, *skn-1*, *mdt-15*, *oxIs12*	Lethality, growth, reproduction, locomotion, oxidative stress, defecation, gene expression	Toxicity effects on lifespan, development, reproduction, and locomotion. There was formation of abnormal vulva	Qiao et al. (2014)
Acrylamide	*gst-4::GFP::NLS, dop-3::RFP*	GFP and RFP expression	GSTs and other phase II enzymes were down regulated by XREP-1	Leung et al. (2011)

For instance, the effects of nicotine in plasticity and locomotion (Matsuura et al. 2013; Sobkowiak et al. 2011), changes in gene expression (Smith et al. 2013), and changes in microRNA expression (Taki et al. 2014); other studies show the effects of caffeine and methadone on reproduction (Boyd et al. 2010); and the effects of sulphamethoxazol on locomotion and growth of offspring of exposed parents (Yu et al. 2011), and on reproduction, growth, lifespan, pharynx pumping, lipid peroxidation, and gene expression (Liu et al. 2013); and the effect of 5-fluorouracil on reproduction and development (Kumar et al. 2010) among others.

6.6 Toxins

Natural toxins have also been studied using *C. elegans* as a biological model (Table 6). Microcystin, a toxin produced by toxic blooms of cyanobacteria on eutrophic waters, produced changes in the behavior of locomotion and GFP expression in *C. elegans* (Moore et al. 2014; Li et al. 2009; Saul et al. 2014). Aflatoxin β1, generated by *Aspergillus fungi*, inhibited growth and formed adducts with DNA by activation of the Cytochrome P system (Leung et al. 2010).

6.7 Other Chemicals

In addition to the above groups, *C. elegans* has been reported as a biological model for assessing the toxicity induced by other chemicals (Table 7) such as sodium fluoride (Li et al. 2012b), vinyl chloride (Nam and An 2010), benzo pyrene [a] and β-naphthoflavone (Leung et al. 2010), ethyl methanesulfonate and DMSO (Boyd et al. 2010), $NaAsO_2$, NaF, $Na_2B_4O_7$, valproic acid, caffeine and DMSO (Sprando et al. 2009); and acrylamide which has been shown to induce a *gst-4::GFP* transgene (Leung et al. 2011).

Table 6 Evaluation of toxicity of toxins

Toxin	Strain	End point	Result	Reference
Microcystin	N2, *hsp-16-2::GFP*	Life span, development, generation time, brood size, locomotion and gene expression	Life span reduced, development retarded, generation time lengthened, brood size decreased, locomotion inhibited	Li et al. (2009)
Microcystin	N2	Chemotaxis	Alteration of chemotactic behavior	Moore et al. (2014)
Microcystin	N2	Life span, reproduction, growth, gene expression	Deficiencies in lifespan, reproduction and growth. Changes in gene expression were dominated by neuromodulation	Saul et al. (2014)
Aflatoxin β1	N2, *emb-8, glp-1, xpa-1*	DNA damage and growth	DNA damage, DNA adducts by CYP activation	Leung et al. (2010)
Bioactive and probiotic marine bacteria	N2	Survival	*V. coralliilyticus* S2052 caused decreased survival after 72 h	Neu et al. (2014)

Table 7 Evaluation of the toxicity of other chemicals

Compound	Strain	End point	Result	Reference
Vinyl chloride	N2	Lethality, *cyp35s* expression.	Alterations in reproduction and gene expression	Nam and An (2010)
NaF	N2	Growth, life span, locomotion, cell apoptosis oxidative stress, gene expression	Growth and locomotion inhibited, life span decreased, ROS production increased in a dose-dependent relationship	Li et al. (2012b)
$NaAsO_2$, NaF, $Na_2B_4O_7$, valproic acid, caffeine and DMSO	N2	Growth and development	$NaAsO_2$ > NaF > $Na_2B_4O_7$ > valproic acid > caffeine > DMSO	Sprando et al. (2009)
PCB 52	N2, *cyp-14A2, cyp-14A5, cyp-23A1, cyp-34A9*	RNAi, gene expression	CYP-14A and CYP-34A6 contribute to the metabolism of PCB52	Schäfer et al. (2009)
Benzo[a]piren and β-naftoflavona	N2, *emb-8, glp-1, xpa-1*	DNA damage and growth	There was no DNA damage, but there was inhibited growth	Leung et al. (2010)
Ethyl methanesulfonate and DMSO	*N2, myo-2::GFP*	Body length, optical density, GFP expression, reproduction	DMSO inhibited brood size in a concentration-dependent manner	Boyd et al. (2010)
Dibromoacetic acid and tetrabromobisphenol-A	N2	Locomotion, pharyngeal pumping, defecation, mechanical sensory stimulus, chemotaxis, thermotaxis	TBBP-A had an impact on locomotion DBAA led to neurostimulation	Ju et al. (2014)
$NaAsO_2$	N2, *daf-16*, *daf-2*, *age-1*, *sqt-1*, *dpy-10*, *unc-52*, *akt-1*, *pdk-1*, *akt-2*, *sgk-1*	Cell apoptosis, oxidative stress	DAF-2, AGE-1 and AKT-1 negatively regulate arsenite-induced apoptosis	Wang et al. (2014c)

Se	N2	Growth, lifespan, reproduction, and hatching rate	Survival decreased significantly at all stages. Growth in the L1 stage declined	Li et al. (2014)
Se	N2	Gene expression	Increase in ROS and stress responses, visible by amplified expression of oxidoreductases and reduced expression of cuticle-associated genes	Boehler et al. (2014)
7-Ketocholesterol	N2	Lethality, growth, lifespan, thermotolerance, reproduction, oxidative stress, germline apoptosis	Reduction in reproduction, decrease in lifespan in a concentration-dependent manner, impairment in thermotolerance, induction of germline apoptosis and increase in ROS	Zong et al. (2014)
Na_2SiF_6, H_2SiF_6 and NaF	N2	Growth, feeding behavior, reproduction	Silicofluorides showed similar toxicity to NaF	Rice et al. (2014)
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	N2, PE255	Growth, mitochondrial and nuclear DNA damage, ATP level, O_2 consumption	Reduced oxygen consumption. Damage to nuclear and mitochondrial genomes	Bodhicharla et al. (2014)
Bio oils from biomass pyrolysis	N2, *pmk-1, daf-16, sir-2.1, hif-1, ctl-2, cyp35a2, mtl-2, sod-3, hsp16.2, ced-3, cep-1*	Survival	Dose-dependent decrease in survival	Chatterjee et al. (2014b)

7 Conclusion

C. elegans is a powerful, suitable and robust model for toxicological studies due to the transparency of its body, its short life cycle, easy fertilization, economical maintenance in the laboratory, large numbers of offspring and easy genetic manipulation. The use of this nematode has enabled the understanding of many biochemical pathways activated by environmental toxicants, allowing the study of multiple endpoints including lethality, growth, reproduction, fertility, and locomotion among others. Additionally, the ease of obtaining transgenic nematodes allows the possibility of studying direct changes in gene expression induced by toxicants or mixtures. Finally, this model may be used in the assessment of toxicity of several pollutants such as environmental samples, metals, pesticides, nanoparticles, drugs, and toxins, among others.

8 Summary

Caenorhabditis elegans is a nematode of microscopic size which, due to its biological characteristics, has been used since the 1970s as a model for research in molecular biology, medicine, pharmacology, and toxicology. It was the first animal whose genome was completely sequenced and has played a key role in the understanding of apoptosis and RNA interference. The transparency of its body, short lifespan, ability to self-fertilize and ease of culture are advantages that make it ideal as a model in toxicology. Due to the fact that some of its biochemical pathways are similar to those of humans, it has been employed in research in several fields.

C. elegans' use as a biological model in environmental toxicological assessments allows the determination of multiple endpoints. Some of these utilize the effects on the biological functions of the nematode and others use molecular markers. Endpoints such as lethality, growth, reproduction, and locomotion are the most studied, and usually employ the wild type Bristol N2 strain. Other endpoints use reporter genes, such as green fluorescence protein, driven by regulatory sequences from other genes related to different mechanisms of toxicity, such as heat shock, oxidative stress, CYP system, and metallothioneins among others, allowing the study of gene expression in a manner both rapid and easy. These transgenic strains of *C. elegans* represent a powerful tool to assess toxicity pathways for mixtures and environmental samples, and their numbers are growing in diversity and selectivity. However, other molecular biology techniques, including DNA microarrays and MicroRNAs have been explored to assess the effects of different toxicants and samples.

C. elegans has allowed the assessment of neurotoxic effects for heavy metals and pesticides, among those more frequently studied, as the nematode has a very well defined nervous system. More recently, nanoparticles are emergent pollutants whose toxicity can be explored using this nematode. Overall, almost every type of known toxicant has been tested with this animal model. In the near future, the available knowledge on the life cycle of *C. elegans* should allow more studies on reproduction

and transgenerational toxicity for newly developed chemicals and materials, facilitating their introduction in the market. The great diversity of endpoints and possibilities of this animal makes it an easy first-choice for rapid toxicity screening or to detail signaling pathways involved in mechanisms of toxicity.

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Pore Water Collection, Analysis and Evolution: The Need for Standardization

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Abbreviations

AVS	Acid volatile sulfide
BLM	Biotic ligand model
COPEC	Contaminants of potential ecological concern
DGT	Diffusive gradient thin-film
IWBU	Interstitial water benchmark units
IWTU	Interstitial water toxic units
MCL	Maximum contaminant level
NRWQC	National recommended water quality criteria
SEM	Simultaneously-extracted metal
SEP	Sequential extraction procedure
TVA	Tennessee Valley Authority
USEPA	United States Environmental Protection Agency

1 Introduction

The United States Environmental Protection Agency (USEPA) estimates that approximately 10 % of the sediment underlying waters in the United States are contaminated to the point of posing a risk to human health or ecological receptors (US EPA 1998). Investigating potential human health and ecological risks of contaminated sediment requires integrating information from multiple lines of evidence. Concentration and speciation data on chemicals in interstitial water can be important lines of evidence for assessing risks of contaminated sediment in aquatic ecosystems. In many depositional sediments, interstitial waters are relatively static (Adams et al. 2000) and many investigators assume that contaminants in the particulate phase of sediment and the pore water are in equilibrium (e.g., Di Toro et al. 1991). Although not the only exposure medium in a sediment environment, pore water is a key route of exposure for aquatic organisms (Chapman et al. 2002). Measuring contaminant levels in interstitial waters is useful for estimating aquatic toxicity (Ankley et al. 1996).

Appropriately sampled pore water can provide useful information on contaminant movement and toxicity in the aquatic environment (Chapman et al. 2002). Unfortunately, many collection techniques inadequately consider chemical transformations of contaminants of potential ecological concern (COPECs) during sediment sampling and subsequent pore water extraction.

Relevant ecological risk-based benchmarks against which to evaluate pore water chemistry results are lacking and there are no relevant regulatory limits for constituents in pore water, and reported pore water data analyses for risk assessments vary considerably. Sediment pore water does not meet the definition of surface waters (Fish 2011) and it cannot reasonably be considered as groundwater. The lack of recognized criteria often leads to comparisons to established, but inappropriate, sediment, groundwater, surface water and drinking water benchmarks. This topic is evaluated in greater detail in Sect. 5 below.

This review paper is meant to present the important considerations necessary to properly collect pore water samples and appropriately evaluate the analytical data in a concise and useable format. The author's intent is that the information presented herein will be used, in conjunction with other published research and project specific objectives, to design comprehensive, quality driven, scientific pore water studies. Additionally, the authors hope that by highlighting the inconsistencies in data usage researchers will intensify efforts towards establishing specific benchmarks for pore water.

2 Uses of Pore Water Data

Pore water data collection has been part of scientific and remedial investigations since at least the 1930s (e.g., Reid 1930, 1932). Since then, the practice of pore water collection has advanced significantly beyond digging holes to collect seep water, but the wide variation in current pore water collection methods illustrates a need for further understanding. No single collection method is suitable for all situations and project objectives, but fundamentally pore water extraction should preserve the integrity of the sample by maintaining samples as close to *in-situ* conditions as possible.

Among other uses, pore water data have been employed to estimate exposures from contaminated groundwater in discharge zones (Fish 2011), to evaluate and assess impacts of contaminated sediments (Besser et al. 2009a; Ruhl et al. 2012), and as a line of evidence for benthic organism exposure in ecological risk assessments (Besser et al. 2009b; Chapman et al. 2002). Chemical concentration profiles in sediment pore water are used in studies of biogeochemical processes and investigations into contaminant exposure, fate and transport (Di Toro, et al. 1991). Understanding the interrelationships among pore water, groundwater, sediment, and overlying surface waters is important in order to accurately:

- Characterize benthic aquatic environments toxicologically.
- Identify contaminant sources and sinks.
- Evaluate contaminant fluxes across the sediment/surface water interface.

3 Pore Water Collection Considerations and Methods

Pore water data are informative only to the extent that they have been properly collected, analyzed and validated. The US EPA (2001) cited major issues of concern for sample integrity and the ability of the sampling device to maintain natural physiochemical conditions of the pore water and investigators subsequently maintaining samples in an anoxic environment. More recent EPA guidance for pore water sampling recognizes atmospheric oxidation as a potential sampling artifact: "Because pore water is typically collected from an anaerobic environment, it is preferable…to maintain the integrity of the sample by minimizing exposure to air" (US EPA 2013).

The importance of preventing atmospheric exposure during sample collection and processing to minimize the effects of geochemical changes is clear.

Several trace elements (notably iron and manganese) are solubilized in sedimentary reducing environments (e.g., through bacterial reduction; Lovley 1991). Introduction of oxygen can result in redox reactions that may affect the toxicity and bioavailability of trace elements, precipitate less soluble oxidized species, and increase adsorption of dissolved trace elements onto amorphous iron and manganese oxide/hydroxide precipitates. Conversely, exposure of sediments to atmospheric oxygen also can result in dissolution of several trace elements (e.g., arsenic, cadmium, copper, lead, nickel and zinc) that are present as relatively insoluble sulfide species in their native anaerobic sediments (Allen 1995; DeLaune and Smith 1985).

The presence of acid volatile sulfide (AVS) in sediments has the potential to bind certain metals and minimize or negate their toxic effects (USEPA 2005; Simpson et al. 2011). The binding potential of AVS is related to concentrations of simultaneously-extracted metal (SEM), which is discussed in greater detail later in this paper. The binding capacity of this contaminant sink is also influenced by redox conditions, and exposure to dissolved oxygen at the sediment-water interface (Simpson et al. 2012) or direct atmospheric exposure during pore water sampling changes the redox condition increasing the bioavailability of metals (Peterson et al. 1996; De Lange et al. 2008).

Conversely, oxidation can have similar effects on sediments with little to no available sulfide. Kangjoo et al. (2009) offer a mechanism to account for higher As in coal-ash associated pore water exposed to oxidizing conditions. They found that siderite ($FeCO_3$) formed under reducing conditions co-precipitated As and subsequent oxidizing conditions resulted in dissolution of the As-rich siderite, releasing As into the pore water.

Borune et al. (2005) evaluated the importance of the oxidation process on ammonia concentrations in sediment pore water, comparing processing of sediments in oxygen-free conditions to processing exposed to the atmosphere. Higher levels of ammonia were observed when pore water processing and analysis were performed in an oxygen-free atmosphere compared to processing under atmospheric conditions. They observed greater ranges in ammonia concentrations for sediments collected from environments with higher carbon, nitrogen and sulfur content, and with lower sediment redox potential and carbon-sulfur ratios (more anoxic environments). The complexity of these processes clearly demonstrates the importance of maintaining sediment and pore water integrity in order to generate data representative of ambient conditions.

The inherent sensitivity of pore water to geochemical changes would suggest that very prescriptive guidelines for pore water collection might be available. Unfortunately, this is not the case. Commonly used methods for pore water collection are summarized below. The variability in collection methodology and attention (or lack thereof) to preserving *in-situ* conditions demonstrates the need for a more standardized approach and guidance. Regardless of the collection and extraction methods used, pore water samples should be preserved immediately and analyzed as soon as possible (US EPA, 2001).

3.1 In Situ Pore Water Sampling Methods

Drive Point Sampling

Drive point sampling involves collecting pore water directly from sediment in situ. It relies on diffusion of pore water into a sampler in a manner similar to typical groundwater sampling. A close-point pipe constructed of stainless steel or other relatively non-reactive material with screen-covered ports at predetermined sampling intervals is driven into the sediment and the pore water that flows through the screens into the pipe is collected using low-flow groundwater sampling techniques (e.g., Kangjoo et al 2009), a peristaltic pump (Fish 2011), or a syringe (US EPA 2013). This technique is applicable for short-term or long-term monitoring of pore water at specific locations. However, it may allow some exposure to the atmosphere as the pore water sample is discharged into sample containers. In addition, pore water contaminants may adhere to the sampling device during typical sampling periods of a week or more.

Suction Filtration

There are several varieties of suction filtration samplers that involve inserting a device directly into sediment for in situ pore water collection. These include some very simple designs (e.g., a volumetric pipette modified by sealing the tip and covering holes cut into the cylinder with screen mesh) to more complex designs for pore water collection (e.g., devices constructed from glass or other inert material) that utilize vacuum pumps or spring-driven pistons to extract pore water in situ (Bufflap and Allen 1995). These types of samplers typically are limited to use in shallow waters where they can be easily deployed and accessed.

Peepers

Peepers are small porous-walled chambers filled with either distilled or clean water of appropriate hardness or salinity prior to deployment. Depending on study objectives, they may be modified to include sampling ports, larger sample compartments, or different materials with specific pore sizes. If the pore water being sampled is anoxic, the sampler and fluid are deoxygenated prior to deployment. Peepers are buried in sediment for extended periods of time, allowing chemical equilibration to occur between pore water and the fluid in the chamber. Equilibration for two weeks is often recommended but depending on the mobility of the contaminants in question, deployment periods may range from several hours to a month. Peeper sampling enables collection of pore water in an anoxic environment, minimizes opportunities for changes in oxidation states, and provides a more realistic assessment of potential toxicity. However, the results may underestimate equilibrium concentrations (Adams et al. 2000).

Diffusive Gradient Thin-Film Samplers

An alternative to actual pore water collection is use of thin-film diffusive gradient samplers (DGT). DGT is an *in-situ* passive sampling technique designed to accumulate labile metals from the environment (Davision and Zhang 1994; Davison et al. 2000), including metals present in pore water and weakly-bound to sediment solid phases. DGT devices use an adsorbent material specific to the target analyte. These samplers are deployed for pre-determined periods ranging from days to months. The concentration of the analyte is determined by measuring the mass of the analyte per thickness of gel. This allows the opportunity to examine different depth strata and develop a vertical profile of the contamination (Komarova et al. 2012). A recent study indicates that DGT could be used to predict toxicity to the amphipod *Meliata plumulosa* (Amato et al. 2014), highlighting the potential of the DGT technique for assessing contaminant bioavailability.

3.2 Ex Situ Pore Water Sampling

Ex situ pore water sampling involves collecting sediment and pore water together in bulk for subsequent offsite pore water extraction. These techniques are necessary when large pore water volumes are needed or when in situ pore water sampling is not possible (US EPA 2001). Sediment may be retrieved using dredges, box core samplers, or various types of sediment corers. Pore water can be extracted from collected sediments by vacuum filtration (De Coen et al. 1998; Ruhl et al 2010), centrifugation (HydroQual 2003; Oral et al. 2012), or sediment presses (Bender et al. 1987).

Unless filtration or centrifugation is performed under an inert atmosphere, sample integrity is likely to be compromised by atmospheric oxidation (ITRC 2011). Atmospheric exposure during either sediment collection or homogenization introduces opportunity for geochemical changes to pore water samples (NFESC 2003; Bay et al. 2010). Preserving the integrity of sediment throughout the collection and pore water extraction process presents challenges to researchers that can lead to innovative sampling techniques that broaden the range of sampling techniques even more, again illustrating a need for better sampling/processing guidance.

For example, TVA collected sediments with a box core sampler and inserted short sections of tubular core liner into the box corer to collect sub-samples of the sediment. These liners were immediately capped to prevent atmospheric exposure and pore water was extracted by centrifugation under an inert atmosphere in the laboratory (TVA 2012). In other studies, sediment core samples were collected, the overlying water was carefully drained off, the core tubes were capped just above the sediments, then cores were segmented and pore water was extracted in laboratories (e.g., Bufflap and Allen 1995; Cusack and Mihelcic 1999; Lourino-Cabana et al. 2012). Others have conducted the entire process of sediment handling and pore water extraction under an inert atmosphere (Nordstrom et al. 1999).

Sediment compression (Bufflap and Allen 1995) and whole-core squeezer methods (Bender et al. 1987) also have been used to extract pore water with minimal atmospheric exposure. For large-diameter cores, core squeezing allows pore water to be collected from specific depths in the sediment core. By considering the core diameter and sediment porosity and collecting predetermined volumes of pore water (e.g., 3–10 mL aliquots) it is possible to develop pore water depth profiles with millimeter-scale depth resolution. These types of sampling and processing methods help minimize oxidation of reduced chemical species in pore water and the solid sediment phase, inhibit other possible geochemical transformations during sample handling (Allen 1995), and inhibit solubilization of otherwise insoluble compounds (Bufflap and Allen 1995).

4 Exposure and Toxicity Benchmarks

Absent any established regulatory criteria specific to contaminants in pore water, effects of COPECs in pore water on the aquatic system are often gauged by comparing concentrations to surface water criteria (National Recommended Water Quality Criteria [NRWQC]) or drinking water criteria (maximum contaminant levels [MCL]). Site-specific Groundwater Cleanup Levels established with the goal of returning usable groundwater to its beneficial uses condition, when practicable, also are sometimes used for comparison. Although these criteria are established and available, they are not relevant for pore water.

Several different sequential extraction procedures (SEPs) have been developed to evaluate how metals are distributed among various binding phases (e.g., Tessier et al. 1979; Ure et al. 1993; Filgueiras et al. 2002). These extractions provide information on the relative abundances of metals in fractions of varying bioavailability. These typically include (from most- to least-bioavailable) water-soluble, ion-exchangeable, carbonate-bound, iron and manganese oxide-bound, organic matter-bound, and residual fractions. The water-soluble and ion-exchangeable fractions are considered to be readily available for biological uptake; those associated with the carbonate-bound and iron-manganese oxide fraction are considered only partly bioavailable; and the organic-bound fraction even less so. The residual metals fraction is considered to be completely non-bioavailable (Markwiese et al. 2014). Bioavailability is a critical factor in exposure of receptors to contaminants in pore water, thus information on SEPs can be useful for evaluating site-specific potential ecological impacts of contaminated sediments.

Benchmarks based on total dry weight metal concentrations in sediments also have been used for protecting benthic communities (US EPA 2005). These consider total, rather than only bioavailable sediment metals concentrations. Toxicity depends on both physical and chemical characteristics of the sediment (Di Toro et al. 1990). Sulfide reacts with several cationic metal ions to form insoluble compounds that are not readily bioavailable, thus reducing toxicity (Ankley et al. 1996). These insoluble

metal sulfides are important in mediating metal toxicity, but some anaerobic or facultative-anaerobic organisms can derive energy from oxidation of metal sulfides, thus solubilizing sulfide-bound metals (Gadd 2004).

Consequently, AVS in conjunction with the SEM procedure often is considered a more realistic estimate of metals bioavailability than total metals analyses (Paller and Knox 2013). AVS/SEM measures the molar amount of sulfide liberated from sediments by a cold acid leach and the molar amounts of metals simultaneously released during that procedure (Hammerschmidt and Burton 2010). Potential metal toxicity is evaluated indirectly by estimating bioavailability based on the molar concentration ratios of AVS/SEM, focusing on cadmium, copper, nickel, lead, and zinc (Ankley et al. 1996). If the molar concentration of AVS exceeds the combined molar concentrations of SEM (i.e., AVS/SEM > 1), the system is considered to have an excess of sulfides available to react with cationic metals, thereby reducing the probability of metal toxicity.

Several laboratory studies have investigated the influence of AVS on metals solid-to-aqueous phase partitioning using a variety of freshwater and marine organisms, measuring both acute toxicity and chronic sublethal toxicity (e.g., Ankley et al. 1991; Hare et al. 1994; Ingersoll et al. 1994; Ankley et al. 1996; Berry et al. 1996; De Witt et al. 1999; Lee et al. 2000). A preliminary screening level of an AVS/SEM > 1.0 is widely accepted as indicating probable nontoxic sediment. However, Mahony et al. (1996) found a significant number of sediments (26 % of those evaluated) with AVS/SEM < 1.0 also were nontoxic. This indicates that other binding phases such as organic carbon may help reduce the bioavailability of metals in sediments and pore waters. Normalizing the AVS/SEM ratio to the fraction of organic carbon present significantly improves the prediction of sediment toxicity (Di Toro et al. 2001). The US EPA has established screening levels based on this carbon-normalized ratio to estimate risk of adverse ecological impacts to aquatic life (US EPA 2005; Ohio Environmental Protection Agency 2010).

Interstitial Water Toxic Units (IWTU; Berry et al. 1996) represent yet another approach to assess bioavailability of contaminants in pore water. The IWTU pore water compares the dissolved metal concentration in pore water with the concentration of that metal shown to cause 50 % mortality in water-only toxicity testing (US EPA 2005). If the IWTU is ≤0.5 no direct toxicity to benthic organisms is expected from that metal (US EPA 2005). The IWTU approach assumes 100 % bioavailability of dissolved metals, thus may over-estimate actual toxicity.

A variation on the IWTU approach uses the sum of concentrations of contaminants in the pore water divided by their respective water quality criteria final chronic value to predict the influence of a particular contaminant on pore water toxicity. This also is referred to as IWTU by Ankley et al. (1996) or Interstitial Water Benchmark Units (IWBU) by the US EPA (US EPA 2005). If the IWBU is ≤1.0, no direct toxicity to benthic organisms is expected (US EPA 2005). The IWBU also assumes all dissolved metals are bioavailable.

An Equilibrium Partitioning approach that considers both AVS/SEM ratios and IWTU/IWBU has been show to outperform either individual assay in predicting mortality as observed in controlled toxicity tests (Ankley et al. 1996). It is important to note that this method is more useful for predicting a lack of, rather than a presence of toxicity (US EPA 2005).

Considering many of these factors, two groups of researchers have developed recommended sediment quality criteria for freshwater and marine sediments (Ingersoll et al. 1996; MacDonald et al. 2000). These benchmarks are best used as initial screening tools, with follow-up site-specific assessments (Buchman 2008), but are available as supplemental information for consideration in pore water evaluations.

Di Toro et al. (2001) developed a Biotic Ligand Model (BLM) that calculates an estimated toxicity based on predicted chemical speciation of dissolved metals, the protective effects of competing cations, and the biotic ligand associated with the organism investigated. The biotic ligand is a discrete receptor site for each organism where binding of sufficient amounts of metals leads to acute toxicity. For fish, the biotic ligand is the gill surface. For other organisms it is believed that a biotic ligand exists and that the model can apply (Di Toro et al. 2001).

The BLM predicts the amount of metal accumulation at the receptor site for observed water quality parameters and metal concentrations (i.e., the metals concentrations and speciation among inorganic, organic, and biotic forms). It predicts the estimated toxicity based on the estimated biotic accumulation and has been used to predict the effects of water chemistry on the toxicities of Cu, Ag, Cd and Zn (Santore et al. 2002) to aquatic organisms and may be applied to other divalent metals (EPA 2003). The model requires data for temperature, pH, alkalinity, and concentrations of dissolved organic carbon, major cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), and major anions (SO_4^{2-}, Cl^{-}, S^{2-}). In most cases it would be difficult to obtain sufficient pore water to provide data for all these parameters, thus limiting its application to pore water investigations.

Toxicity testing is designed to measure an adverse effect of a substance on a test organism under controlled laboratory conditions at a specified series of dilutions. Direct toxicity testing of pore water is difficult to perform. Adequate volumes of pore water can be difficult to extract, appropriate dilution water is difficult to identify or prepare, and maintaining redox conditions similar to *in-situ* conditions is challenging at best. Additionally, there is no consensus on the type of organism(s) to use in such tests, nor is there consensus on the role of pore water to benthic community toxicity. Pore water has been documented as contributing to potential risk to the benthic community, but is not the only route of exposure (Warren et al. 1998; Hare et al. 2001). Some investigators suggest a general correlation between pore water concentrations and toxicity to benthic organisms (IRTC 2011). Even with appropriate test organisms, interpreting the ecological significance of results of pore water toxicity tests would be challenging.

5 Pore Water Benchmarks: Pros and Cons

Several investigators have directly compared pore water metals concentrations to NRWQC for aquatic life to evaluate potential toxicity (Anon 2000; Fish 2011; Winger et al. 2002; Zimmerman et al. 2005). In most cases NRWQC were developed using toxicity data plotted against total recoverable metals in surface water. Surface water assessments use data obtained for total recoverable metals in surface

waters that are compared to NRWCQs. The bioavailable fraction in hypoxic pore water will likely be significantly lower than the result from a total recoverable metals assay and can lead to overly conservative assumptions (i.e., higher pore water toxicity). At a minimum, comparison to total dissolved trace element concentrations would be preferred, since comparison with total recoverable trace elements is overly conservative and includes fractions that are not bioavailable (Bufflap and Allen 1995; Markwiese et al. 2014). Additionally metals speciation data can further inform data users regarding metals bioavailability and associated ecological risks.

Comparing pore water concentrations to human health drinking water MCLs is a persistent but unfortunate practice (Rees et al. 1995; Ruhl et al. 2010; 2012; Fish 2011). Comparing concentrations of As found in pore water to the drinking water MCL for As (Ruhl et al. 2010), for example, is of questionable relevance given that the MCLs were established for drinking water and designed to protect human health. The pore water comprises a small volume relative to the overlying water column, and is sequestered from human contact. Exposure models that are used to derive the MCLs (e.g. developing child drinking a fixed volume of water per day for an extended period of time) do not apply to pore water. For contaminants of concern to be released to the upper water column would require broad-based turbation. The contaminants would then be very rapidly and highly diluted before becoming bioavailable to anything besides the microbial and invertebrate organisms in the top few centimeters of the sediment. Ultimately, the comparison of pore water constituents to established toxicological benchmarks is inappropriate because they represent sediment or surface water, not the toxicity associated with the medium of concern; i.e., the interstitial water of sediment.

Fish (2011) compared pore water data from samples collected in discharge zones to groundwater cleanup levels to evaluate the effectiveness of a nearby groundwater remediation project. That type of comparison contradicts the stated goal of groundwater cleanup levels since pore water is not "usable" in this context without accounting for the dilution of discharged pore water by groundwater or surface waters.

Pore water toxicity testing would be a more relevant approach to evaluating ecological effects on interstitial aquatic organisms. However, as noted above, that is an easier concept to formulate than to execute. Although various recommended sediment quality criteria are based on exposures of receptors that live in or on sediments, current bioassays use test species that are exposed to both sediment solid phases and to pore water. Organisms on the scale of bacteria are the only ecological receptors solely exposed to pore water. Current bioassays are more directed at the macroinvertebrate scale of ecological receptors which would never be exposed to pore water exclusively (IRTC 2011).

Approaches that consider bioavailability of trace elements are the most ecologically realistic and appropriate methods for evaluating pore water results (Allen 2011). These approaches require considering both the particulate and aqueous components of sediment as a complete system to account for the contaminant binding capacity of the sediment and that of water-borne binding and coordination species. Comparison of AVS to SEM in the sediment, coupled with calculated IWTU/IWBU may be the most useful pore water evaluation approach. That includes consideration of both trace element bioavailability and the potential for observed concentrations to adversely affect living organisms (Ankley et al. 1996).

6 Conclusions and Recommendations

This review of available information has identified three areas where further attention is needed when conducting pore water sampling and evaluation:

1. Although there is a great need for it, no standardized approach or best practice guidance for sediment pore water collection exists. The variety of extant sampling and processing procedures results in pore water concentration data of questionable comparability and quality. No single collection method is suitable or practicable for every situation, but this is true for other media that have recognized best practices or regulatory standards. Others also recognize the need for further development of pore water extraction and chemical analysis that could lead to standard protocols or guidance (e.g., Adams et al. 2000).
2. Protecting sediments from atmospheric exposure during collection and subsequent extraction of pore waters is of utmost importance in order that samples collected appropriately represent *in-situ* conditions. Despite the documented detrimental effects of atmospheric exposure, investigators continue to jeopardize the results of pore water analysis by using procedures that expose sediments and pore waters to the ambient atmosphere.
3. Comparing pore water to existing benchmarks such as drinking water MCLs or ambient water quality criteria for aquatic life is problematic, at best. A better, more relevant approach to evaluating pore water is needed.

The best approach to evaluating pore water considers bioavailability of trace elements in sediments through partitioning of contaminants between the aqueous and solid phases and partitioning within the aqueous phase, in conjunction with potential pore water toxicity as indicated by IWTUs/IWBUs. Sediment/pore water samples should be collected and processed in an oxygen-free atmosphere to minimize the effects of geochemical changes. Collecting additional data (e.g., AVS/SEM, sediment redox potential, temperature, hardness, pH, dissolved organic carbon and anions in the pore water, and observations of benthic community diversity and health) also is important and useful in evaluating ecological significance of pore water contaminant concentrations. This supporting data can provide useful information for an integrated evaluation of pore water as a line of evidence in comprehensive ecological risk assessment. Consideration should also be given to standardizing methods for anaerobic sequentially extracted trace element analysis. Using the US Army Corps of Engineers sequential batch leachate procedure for leach-testing freshwater sediments (Brannon, Myers, and Tardy 1994; USACE 2003) may be a good starting point for developing a standard method.

Sediment researchers and regulatory agencies need to collaborate in developing a consensus-based systematic framework and guidelines for sediment/pore water collection and evaluation. The fundamental understanding and available techniques are available to develop a more standardized approach. Without such guidelines, the number of different pore water collection and extraction techniques will continue to expand, and investigators will continue to evaluate potentially questionable data by comparison to inappropriate criteria.

7 Summary

Investigating the ecological impacts of contaminants released into the environment requires integration of multiple lines of evidence. Collection and analysis of interstitial water is an often-used line of evidence for developing benthic exposure estimates in aquatic ecosystems.

It is a well-established principle that chemical and toxicity data on interstitial water samples should represent *in-situ* conditions; i.e., sample integrity must be maintained throughout the sample collection process to avoid alteration of the *in-situ* geochemical conditions. Unfortunately, collection and processing of pore water is not standardized to address possible geochemical transformations introduced by atmospheric exposure. Furthermore, there are no suitable benchmarks (ecological or human health) against which to evaluate adverse effects from chemicals in pore water; i.e., empirical data is lacking on the toxicity of inorganic contaminants in sediment interstitial water. It is clear that pore water data is best evaluated by considering the bioavailability of trace elements and the partitioning of contaminants between the aqueous and solid phases. It is also evident that there is a need for sediment researchers and regulatory agencies to collaborate in developing a standardized approach for sediment/pore water collection and data evaluation. Without such guidelines, the number of different pore water collection and extraction techniques will continue to expand, and investigators will continue to evaluate potentially questionable data by comparison to inappropriate criteria.

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Environmental Fate and Toxicology of Dimethoate

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Contents

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1 Introduction

Dimethoate ([O,O-Dimethyl S-(N-methylcarbamoylmethyl) phosphorodithioate]) is an organophosphorous insecticide that is used worldwide in agriculture and urban areas due to its high efficacy and rapid environmental degradation. It was registered in 1962 and has been used to control a wide range of insects including mites, flies, aphids, and plant hoppers (Mirajkar and Pope 2005). In 2000, all non-agricultural uses of dimethoate, including residential uses, were cancelled in the United States (US EPA 2008). However, dimethoate can still be applied to many crops such as, fruit, vegetables, grain and ornamentals, in addition to non-agricultural applications for landscape maintenance and structural pest control. Roughly 816,466 kg of active ingredient is applied annually on agricultural sites with the highest applications being on alfalfa, wheat, cotton, and corn (US EPA 2008).

Dimethoate is highly water soluble and has low soil persistence (PPDB 2014; NPIC 2014). Due to these two factors, the potential to runoff into surface waters and/or leaching into groundwater is high. However, a thorough understanding on the environmental fate of dimethoate is needed in order to mitigate the negative impacts it has on the environment. This paper reviews the relevant literature and addresses the environmental fate, chemistry and toxicology of dimethoate.

2 Chemistry

Dimethoate (Fig. 1) is an organophosphorous insecticide that is highly water soluble. When pure, it is a white crystalline solid with a mercaptan odor. At room temperature, it is stable in aqueous solutions of pH 2–7 and is unstable under alkaline conditions. It has a low affinity for soils and a moderate affinity for organic matter. It is susceptible to hydrolysis under acidic conditions, is moderately stable to microbial degradation and is non-volatile as reflected by its low vapor pressure (US EPA 2008). The physiochemical properties of dimethoate are presented in Table 1.

3 Chemodynamics

3.1 Soil

Due to its strong hydrophilic nature, surface and groundwater contamination must be considered. Although adsorption to soils is weak, studies have found organic matter (OM) content to impact its retention in soils.

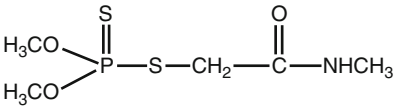

Fig. 1 Structure of dimethoate

Table 1 Physiochemical properties of dimethoate

CAS number[a]	60-51-5
Molecular formula[a]	$C_5H_{12}NO_3PS_2$
Molecular weight (g/mol)[a]	229.3
Density (g/mL)[a]	1.31
Henry's law constant at 25 °C ($Pa \times m^3\ mol^{-1}$)[a]	1.42×10^{-6}
Vapor pressure at 25 °C (mPa)[a]	0.247
Octanol-water partition coefficient at pH 7, 20 °C (log Kow)	0.704
Soil adsorption coefficient (K_{oc})[b]	20
Water solubility at 21 °C (mg/L)[a]	39,800
Half-lives in aqueous solutions (days)[c]	
pH 2–7	Stable
pH 9	12

[a]PPDB (2014)
[b]NPIC (2014)
[c]WHO (2004)

The adsorption and desorption processes of dimethoate was investigated by Vagi et al. (2010) using three Greek soils from the Mytilene Island region, each with different pH, clay and organic matter content. Adsorption isotherms followed a L-shape indicating that the soil surface possessed a high affinity for dimethoate adsorption. Desorbed amounts of the pesticide were only available when the soil was washed with water due to its hydrophilic nature. Furthermore, results indicate hysteresis and was observed in soils with higher OM content. Overall, dimethoate weakly sorbed to all three soils. Al Kuisi (2002) reported similar results. Dimethoate adsorption was measured on eight soil types (pH from 8.0 to 8.5; OM from 0.73 to 2.9 %; clay content from 5.9 to 15 %) in which resulting isotherms also followed a L-shape. Adsorption coefficients (K_{ads}), determined via the Freundlich equation, ranged from 1.0 to 10 suggesting dimethoate is weakly adsorbed and is influenced by a change in organic matter content.

Loam soil half-lives, determined from two field trials held in 1989 and 1990, were approximately 5.1–7.1 days, respectively (Wu and Fan 1997). After each field trial, measured soil residues declined over time. Measurable residues were still present up to 31 days post application. Bohn (1964) studied the accumulation of dimethoate in a sandy loam soil. The insecticide was measured in the top 3 in. of the soil and its half-lives were determined to be 4 days under drought conditions and 2.5 days following a moderate rainfall, respectively. Kolbe et al. (1991) observed rapid degradation 1–2 days post application in a clay loam soil, presumably due to biodegradation. Half-lives were determined for the three soils. For both the humus rich sandy soil and heavy clay soil, at 10 and 20 °C, the half-lives were 15 and 9 days, respectively. However, the half-life in clay loam soil was determined to be 10 and 5 days at 10 and 20 °C, respectively. An increase in organic matter resulted in a shorter half-life.

The mobility of dimethoate from amended and un-amended soil was studied by Antonious et al. (2007). Broccoli plants were grown under three soil management practices: native un-amended soil, native soil amended with sewage sludge, and

native soil amended with yard waste compost. Dimethoate 4E was applied to the broccoli foliage and the amount of pesticide that reached the soil was a result of spray drift or runoff from rain or irrigation. The pesticide residue was higher in the un-amended soil (135 ng/g soil) compared to the amendments with sewage sludge (31 ng/g soil) and yard waste compost (46 ng/g soil); runoff concentrations followed the same trend. Thus, an increased amount of OM in the soil decreased the amount of dimethoate in collected runoff. Overall, studies have indicated that organic matter content in the soil increases dimethoate's sorptive ability and consequently decreases its chances of being transported in runoff water or percolating down into the groundwater.

3.2 Water

Transport through soil via leaching, adsorption, or volatilization are affected by factors such as water solubility, volatility, and stability. Due to dimethoate's high water solubility (39,800 mg/L) and low soil adsorption coefficient ($K_{oc} = 20$), its retention in soil will be low and its dispersion and transport in soils will be affected by soil type and soil moisture content. Under simulated field conditions (19–21 °C; 500 g of soil), El Beit et al. (1977b) found that soil type was an important factor in leaching. They observed an increase in leaching in the following soil order: clay < clay loam < loam < sandy clam loam < sand. The retention within these soils is thought to be impacted by physical forces and hydrogen bonding (El Beit et al. 1977b).

Losses of dimethoate due to soil water can lead to high amounts of chemical leaching into groundwater or transported off soil surfaces into nearby water bodies. El Beit's (1977a) study showed that an increase in the initial soil moisture resulted in an increase in dimethoate's ability to leach. Furthermore, reduction in organic matter content not only reduces the potential for biodegradation, but also accelerates pesticide loss through processes such as evaporation and leaching.

Pesticide leaching through a haplic acrisol soil (rich in clay; classified by the FAO), found in northern Thailand, was assessed by Ciglasch et al. (2005). Dimethoate was applied (2860 g/ha) to plots on a 10-year-old lychee orchard and leachate was monitored for 8 weeks using borosilicate suction lysimeters. The fields received rainfall following application and pesticide residues were found to translocate to a depth of 55 cm in a single flush. Ciglasch et al. (2005) assume that the relative translocation is independent of soil sorption coefficients and that preferential flow dominated the pesticide's displacement. Of all the pesticides applied to these fields, dimethoate was detectable in the leachate for up to 1 month (Ciglasch et al. 2005). However, due to a rainstorm that occurred during this study, it is suggested that further studies be conducted to identify a range of dissipation rates. Another study monitored dimethoate concentrations in the Mae Sa watershed in northern Thailand due to its frequent use (Sangchan et al. 2014). A total of 370 water samples were collected and analyzed from three gauging stations along the watershed; a maximum concentration of 0.4 µg/L was measured. Sangchan et al. (2014)

Table 2 Dimethoate concentrations measured throughout California[a,b,c]

Media	Number of samples	Number of detections	Percent detection (%)	Minimum concentration, µg/L	Maximum concentration, µg/L
Surface water[a]	5945	531	9	0.007	11.5
Ground water[b]	5542	3	0.05	0.38	24
Air[c]	156	0	0	ND	ND

[a]CDPR (2014)
[b]CDPR (2003)
[c]CDPR (2013)

compared the measured contamination level to environmental quality standards set forth by the Canadian Council of Ministers of the Environment. Overall, none of the samples containing dimethoate exceeded the Canadian limit of 0.6 µg/L thus, the measured concentrations are thought to be of little concern for this watershed.

This insecticide has been detected in surface waters throughout California. Ensminger et al. (2009) collected water samples from streams throughout the agriculturally dominated Central Valley of California. Dimethoate was detected in 2 of 21 samples during the irrigation season at 0.074 and 0.19 µg/L, respectively. In California (CDPR 2014), measurable dimethoate concentrations were found in many monitored waterways with the highest residue detected at 11.5 µg/L (Table 2). The maximum measured residue level was above the chronic aquatic life benchmark value for invertebrates (0.5 µg/L) set forth by the US EPA, thus suggesting a higher exposure risk than to fish (chronic aquatic benchmark of 430 µg/L).

Groundwater contamination often occurs due to pesticides leaching yet, it is unknown if measured concentrations within groundwater are of environmental concern. To investigate this, Loewy et al. (2003) sampled 30 groundwater wells over 3 years. Among the detected pesticides, dimethoate was found at concentrations up to 11 µg/L with a mean concentration of 0.22 µg/L. Dimethoate's groundwater ubiquity score (GUS) index value of 3.5 indicates that it has a high potential to leach. A monitoring study conducted in 15 regions of Saudi Arabia, on the persistence of pesticides in ground water, found high concentrations of dimethoate in 13 of the sampled regions (El-Saeid et al. 2011), whereas in China detections were positive in 37 % of collected water samples (Gao et al. 2009). In California (CDPR 2003), measurable dimethoate concentrations were found in 3 of 5542 groundwater samples with the highest residue detected at 24 µg/L (Table 2).

3.3 Air

The volatilization rate of dimethoate from both wet and dry surfaces is low as suggested by its low vapor pressure (0.247 mPa) and Henry's law constant (1.42×10^{-6} Pa m^3/mol). In California (CDPR 2013), a study conducted in 2012 did not measure dimethoate in any of the 156 collected air samples (Table 2).

El Beit (1977b) determined the loss of dimethoate from soil, via evaporation, was impacted by soil type, but independent of soil depth. Furthermore, they note that evaporation was greatest in experiments using sand and less volatilization occurred in experiments using loam. Volatilization is considered a minor route of dissipation. However, if other dissipation routes are found to be minor, volatilization may play a larger role in removing the pesticide from soil over time.

4 Environmental Degradation

4.1 Abiotic Processes

4.1.1 Hydrolysis

The rate of dimethoate hydrolysis is dependent on pH, soil type, temperature and other weather conditions. To demonstrate pH dependency, Ruzicka et al. (1967) carried out hydrolysis studies (21 °C; 20 % ethanol present) using river waters of varying pH and hardness. They found the hydrolytic half-lives to decrease as pH lowered from 8.0 to 7.5 (Thames River water $t_{1/2}=22$ h and Irthing River water $t_{1/2}=18$ h, respectively). Further investigation using an ethanol buffer solution, at pH 6, resulted in a half-life of 12 h. Ruzicka et al. (1967) suggest that the ionic strength of the buffer or a catalytic effect due to ions in the buffer caused the rate of hydrolysis, at pH 6, to be more rapid than at pH 7.5-8.0.

El Beit et al. (1978) looked at the degradation of dimethoate in soil leachates (of distilled water) varying in pH. At the lowest pH (4.2) the pesticide was stable for 19 days; however, as pH increased to 11, degradation occurred within 20 h. Further studies looked at the impact of solutions incubated with either urea or lime ($Ca(OH)_2$). In the presence of either, the pesticide was observed to degrade. Degradation was greater in the lime solution possibly due to becoming an alkaline solution.

Temperature dependency on the rate of hydrolysis was demonstrated by Lartiges and Garrigues (1995). Using four different water types, hydrolysis studies were conducted at 6 and 22 °C, and at three pHs (6.1, 7.3 and 8.1). In ultrapure water, at the lowest pH and temperature, dimethoate was stable to hydrolysis ($t_{1/2}=423$ days) however, when the temperature increased, hydrolysis was observed ($t_{1/2}=193$ days). Hydrolysis in seawater was determined to increase as both the pH and temperature were increased; a half-life of 36 days was measured at 22 °C and pH 8.1. These results indicate hydrolysis occurs more rapidly under alkaline conditions. Druzina and Stegu (2007) reported similar findings. The dissipation of dimethoate in river and groundwater at varying pH and temperature resulted in different half-lives. In groundwater, at pH 6, the half-life was 95 days, whereas at pH 8.5 the half-life was 66 days. When compared to river waters (pH 8), hydrolysis was more rapid as temperature increased from 4 to 25 °C (i.e., $t_{1/2}=169$ days to $t_{1/2}=75$ days, respectively).

Table 3 Suggested photocatalytic transformation by-products for dimethoate using TiO_2 as a catalyst (adapted from Evgenidou et al. 2006)

Dimethoate by-products
O,*O*-Dimethyl phosphonic ester
O,*O*,*O*-Trimethyl phosphoric ester
N-Methyl-2-sulfanylacetamide
O,*O*,*S*-Trimethylphosphorothiate
2-*S*-Methyl-(*N*-methyl) acetamide
O,*O*,*S*-Trimethyl thiophosphorothioate
1-Methyl-2-(acetyl-*N*-methyl-) methane disulfide
Omethoate
1,2-Bis(acetyl-*N*-methyl-) methane disulfide

In northern Vietnam, dimethoate was applied to a combined rice paddy-fish pond farming system to determine dimethoate's environmental fate (Anyusheva et al. 2012). When applied to paddy water (approximately 22 g total; water pH 8.1; sandy loam soil) during the spring crop season, it disappeared rapidly within 14 days and a DT_{50} of 0.3 days was determined. When compared to the summer-autumn crop season, it also disappeared rapidly with a DT_{50} of 0.8 days.

4.1.2 Photolysis

The photocatalytic oxidation of dimethoate was observed by Evgenidou et al. (2006). Using a high-pressure mercury lamp (125 W) and a low amount of titanium dioxide as a catalyst (TiO_2; 100 mg/L), dimethoate (20 mg/L in distilled water) degraded into nine by-products (Table 3). Based on the intermediates formed, it is likely that this pesticide degrades via oxidation and dealkylation reactions that proceed simultaneously. Formation of the secondary intermediates, *O*,*O*-dimethyl phosphoric ester, *O*,*O*,*O*-trimethyl phosphoric ester and *O*,*O*,*S-trimethylphosphorothiate* occur during dimethoate decay. Microtox toxicity tests, using the irradiated solutions, revealed that the transient intermediates (oxon derivative, disulfide, and *O*,*O*,*S*-trimethyl thiophosphorothioate) were more toxic than dimethoate itself (Evgenidou et al. 2006).

Photocatalysts are often utilized in order to advance oxidative processes. Under simulated solar irradiation (300 W xenon lamp), the use of the catalyst 2,4,6-triphenylthiapyrilium cation ($TPTP^+$; 10 mg/L) reduced dimethoate concentrations by 20 % following 60 min of irradiation. This reduction suggests an electron transfer mechanism (Gomis et al. 2012). Chen et al. (2007) identified an increase in the efficiency of dimethoate degradation as TiO_2 catalyst concentrations increased to approximately 0.6 g/L. In particular, irradiating dimethoate (500 W UV-lamp; 120 min) with UV radiation solely resulted in a 3.2 % degradation efficiency, whereas in the presence of the catalyst (0.6 g/L) degradation was 80 % efficient. This is attributed to an increase in the total surface area available for the pesticide to adsorb to. However, higher concentrations of the catalyst may reduce overall efficiency due to a light scattering effect (Chen et al. 2007).

Using a photoreactor containing a Hg lamp (12 W; UV at 254.7 nm), the pesticide, in an aqueous solution, was irradiated in the presence of an oxidizing agent, hydrogen peroxide (H_2O_2) and a catalyst, iron (III) chloride hexahydrate (Nikolaki et al. 2005). Oxidation reactions resulted in the by-products dimethyl phosphite, N-methyl-acetamide, and formic acid. The products were detectable up to 45 min following test initiation and were further oxidized into carbon dioxide, sulfate, phosphate, and ammonium ions. Furthermore, the hydrolysis intermediate omethoate was detected prior to irradiation of the pesticide solution.

4.2 Biotic Processes

Microbial degradation of pesticides plays a significant role in reducing pesticide residues within the environment. To determine the potential for dimethoate to be degraded by microbes, two bacterial strains, *Pseudomonas aeruginosa* W171 (isolated from water) and *Bacillus licheniformis* F102 (isolated from *Labeo rohita* intestine) were used. DebMandal et al. (2008) found both strains to degrade the pesticide. In addition, four metabolites resulted from the degradation by the *P. aeruginosa* strain, whereas *B. licheniformis* degraded the pesticide within 3 days. Liang et al. (2009) observed the bacterium *Raoultella* sp. X1 to degrade dimethoate, however environmental and nutritional conditions were found to be important. Using dimethoate as the sole carbon source resulted in poor degradation yet, 75 % of the initial dimethoate concentration was removed via co-metabolism (Liang et al. 2009).

Microbes found in sewage sludge or wastewater often have the ability to use pesticides as carbon sources for survival, thus leading to decreases in pesticide concentrations. Multiple bacterial strains were isolated from the wastewater treatment pool of a factory that manufactured dimethoate. Out of all of the strains, strain Lgjj-3, having similar lineage to the *Paracoccus* sp., had the highest degrading capabilities. Ultimately, it reduced dimethoate (100 mg/L) to below detection levels within 6 h (Li et al. 2010). Li et al. (2010) also identified seven degradation products and proposed the mechanism shown in Fig 2. It is suggested that this strain degrades dimethoate via hydrolysis, decarboxylation, oxidation and an additional hydroxylation reaction. Isolated from sewage and soil from cotton fields, *Aspergillus niger* ZHY256 has been found to degrade the pesticide via cleavage of the phosphorus-sulfur (P-S) linkage (Liu et al. 2001).

Using an expanded granular sludge bed reactor, Monsalvo et al. (2014) investigated the biodegradability of dimethoate. Under anaerobic conditions, the pesticide was added to the reactor at concentrations up to 500 mg/L and incubated for 21 days. Within the incubation period, dimethoate did not degrade. It was noted that an acclimation period of 50 days was sufficient to observe a complete removal of the pesticide.

Deshpande et al. (2001) tested the ability of 25 bacterial strains to degrade dimethoate. After an 8-day incubation, only two strains, *Pseudomonas aeruginosa* MCMB-427 and *Bacillus megaterium* MCMB-428 were efficient enough to degrade

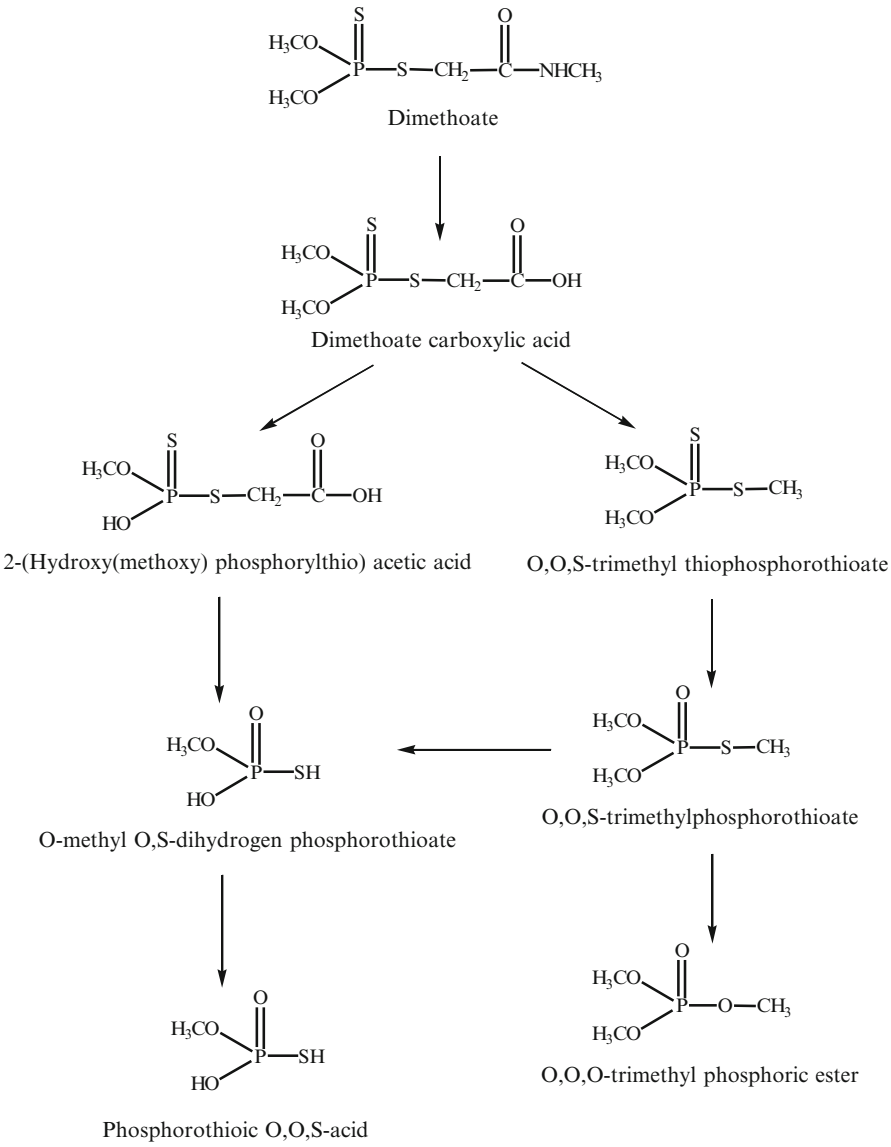

Fig. 2 Suggested microbial transformation pathway for dimethoate by *Paracoccus* sp. Lgjj-3 (adapted from Li et al. 2010)

the pesticide by 95 %. Furthermore, they identified the degradation by *Pseudomonas aeruginosa* MCMB-427 to be plasmid-mediated and thus transferable amongst other strains. This group concluded that in order to understand the genetic basis of this degradation, additional studies are warranted.

5 Ecotoxicology

5.1 Mode of Action

Dimethoate inhibits acetylcholinesterase (AChE) which is present in mammals, fish, birds and insects. AChE is a class of enzymes that initiate the hydrolysis of acetylcholine (ACh), a neurotransmitter, into inactive choline and acetic acid (Fukuto 1990). The inhibition creates a buildup of acetylcholine at the nerve synapses disabling the enzyme cholinesterase that is vital for a functioning central nervous system (Lundebye et al. 1997). The concentration of ACh in the synapses results in continuous stimulation of the muscles eventually leading to seizures, exhaustion and possibly death.

5.2 Insects

Systemic insecticides enter plant tissues and can be translocated into a plant's nectar. For instance, alfalfa treated with the insecticide (at 304 mg/L a.i.) contained 16 mg/L of dimethoate in the nectar 1 day post-application and 1 mg/L within 2 weeks (Barker et al. 1980). Barker et al. (1980) investigated the toxicity of the measured dimethoate concentrations within nectar to honeybees. Worker bees (*Apis mellifera* L.) were fed contaminated and uncontaminated nectar for 7 days; mortality and cholinesterase inhibition resulted. Observations showed that dimethoate is not considered a repellent and will be consumed by bees. Studies conducted by Waller and Barker (1979) observed the impact on bee survival, colony development and comb building. Sucrose solutions, with (up to 5 mg/L) and without dimethoate were provided to bees for 3 weeks. Bees were highly impacted by the highest dose resulting in death within the first week, no new comb and little sugar honey stored. Those dosed with 0.2 mg/L did not show signs of toxicity until the third week of the study where both comb and egg production were reduced (Waller and Barker 1979); colonies were impacted at each tested concentration.

Jepson et al. (1995) investigated the toxicity of topically applied dimethoate to adult coccinellids (*Coccinella septempuncta*) and carabids (*Bembidion obtusum*, *Nebria brevicollis*, *Trechus quadristriatus* and *Demetrias atricapillus*). LD_{50} (48-h) values ranged from 17.7 to 98.8 ng/insect and as body size increased, insect susceptibility decreased.

Midge fourth instar larvae were exposed to a wide range of concentrations up to 4.5 mg/L for *Chironomus riparius* and 7.1 mg/L for *Kiefferulus calligaster*. Both species exhibited significant cholinesterase inhibition. Glutathione S-transferase (GST) activity was not significantly impacted in *K. calligaster* as compared to inhibition in *C. riparius. C. riparius* were more sensitive with a 48-h LC_{50} of 0.48 mg/L, compared to that of *K. calligaster* (1.7 mg/L). In addition, third instar larvae were exposed (up to 0.46 mg/L) to assess the effects on growth and emergence. At the highest concentration, a cholinesterase inhibition of 66 % was observed, whereas each concentration delayed emergence time (Domingues et al. 2007).

Over time, insecticide resistance may occur. Vontas et al. (2001) compared the dimethoate-resistant strain of the olive fruit fly (*Bactrocera oleae*) with a colonized parental strain and field-collected population. Topical applications of the insecticide were placed on the abdominal sternum of the insect; after 24 h, bioassays were conducted. Results identified that oxidative metabolism was not the major factor in resistance, but an altered acetylcholinesterase with poor catalytic efficiency was the major component.

5.3 Aquatic Organisms

Due to dimethoate's hydrophilic nature, its potential to bioaccumulate is insignificant as suggested by its high water solubility (39,800 mg/L) and low log K_{ow} (0.704), however, it is still possible that adverse effects may result. Toxicity values are presented in Table 4.

Beusen and Neven (1989) investigated the toxicity of a high purity (99 % a.i.) dimethoate and an emulsifiable concentrate (10 % a.i.) to freshwater fish and *Daphnia magna*. Zebrafish, guppy and *Daphnia magna* exposed to both were found to be more susceptible to the emulsifiable concentrate with 48-h LC_{50} values of 7.5, 16 and 0.83 mg/L, respectively. This may have been a direct result to the solvent within the concentrate. Exposure to the high purity dimethoate (99 %) did not result in mortality of either the zebrafish or guppy within 96-h. However, a measured 48-h LC_{50} (1.7 mg/L) for *D. magna* was determined. Exposure studies (concentrations from 2.5 to 4.0 mg/L) using catfish (*Heteropneustes fossilis*) observed altered swimming behavior, increased gulping for air and increased mucus secretion over the body. In addition, fish were highly sensitive to low concentrations having a 96-h LC_{50} of 2.9 mg/L (Pandey et al. 2009).

Further studies investigated the biochemical responses resulting from dimethoate exposure. Dogan and Can (2011) exposed adult male rainbow trout (*Oncorhynchus mykiss*) to concentrations of dimethoate under semi-static condition for either 5, 15, or 30 days. Blood and liver samples were taken. Tests revealed that dimethoate did not significantly impact testosterone levels. However, 17β-estradiol levels increased in the 5- and 15-day tests, thus dimethoate may possibly mimic estrogen. In addition, liver tissues showed impaired membrane permeability (Dogan and Can 2011).

Freshwater rotifers, *Brachionus calyciflorus* and *Asplanchna brightwelli*, were exposed to four dimethoate concentrations (0.4, 0.8, 1.2 and 1.6 mg/L) and their

Table 4 Toxicity of organophosphates to aquatic organisms[a]

Aquatic organism	Scientific name	Test	Concentration (mg/L)
Rainbow trout	*Oncorhynchus mykiss*	96-h LC_{50}	6.2
Stonefly	*Pteronarcys californica*	48-h LC_{50}	0.043
Water flea	*Daphnia magna*	96-h LC_{50}	3.32
Mysid shrimp	*Mysidopsis bahia*	96-h LC_{50}	15

[a]US EPA (2008)

swimming responses were recorded. Chen et al. (2014) found dimethoate to significantly inhibit the rotifer's swimming angular and linear speed and this response was dependent on pesticide concentration. Similar results were reported by Guo et al. (2012) with *Brachionus calyciflorus*, exposed to dimethoate concentrations ranging from 0.18 to 1.6 mg/L. In addition to speed inhibition, swimming behavior was negatively impacted suggesting inhibition of AChE.

The acute toxicity of the insecticide to Australian freshwater shrimp, *Paratya australiensis*, was determined by Kumar et al. (2010). Shrimp, collected from a pristine site of the Finniss River area, were exposed to seven nominal concentrations ranging from 0.05 to 20 mg/L; the 96-h LC_{50} was determined to be 800 μg/L. In addition, the authors predicted a 21-day chronic lethality value for shrimp based on a log-log model to be 89 μg/L. Mysid shrimp (*Neomysis integer*) were exposed to concentrations of dimethoate up to 5000 μg/L. Mortality was recorded and a 96-h LC_{50} of 540 μg/L was calculated (Roast et al. 1999).

The freshwater prawn, *Macrobrachium rosenbergii*, at the post-larval stage was used to study the effects of pesticide exposure and its impact of feeding rates. Five concentrations (78–1250 μg/L) were used for lethality tests and surviving prawns were placed into freshwater to assess feeding behavior. Satapornvanit et al. (2009) determined both a 24 and 48-h LC_{50} for dimethoate to be 142 and 103 μg/L, respectively. Post-exposure feeding tests, measuring sublethal effects, resulted in a 24-h EC_{50} of 269 μg/L. Due to the sublethal effects concentration being greater than that of the lethal test, the authors conclude that post-exposure feeding tests cannot be used to detect this pesticide's toxicity.

5.4 Plants

Dimethoate residues on foliar surfaces following application and its residual toxicity were investigated by Chowdhury et al. (2005). The effect leaf wax has on the bioavailablity of dimethoate to arthropods was investigated using leaves from barley, orange, cabbage, sugarcane, tomato, pear, wheat, rape, maize and dwarf bean. They found that as a plant's surface wax increased, the insecticidal efficacy was not impacted. Thus, dimethoate will likely be found in low concentrations in plant waxes due to its hydrophilic nature and its potential affinity for the plant cuticle.

The degradation of the insecticide within yerba mate (*Ilex paraguariensis*) plants was studied using randomly collected field samples. Dimethoate residues on dry leaves were found to decrease from samples collected 1–31 days post application; half-lives ranged from 9.8 to 12 days, respectively (Schmalko et al. 2002).

Wheat plants at 6 days of germination were treated with dimethoate at 50, 100 or 200 mg/L (Pandey and Gopal 2011). Plant leaves were analyzed 10 days post-application. At the lowest dose, plants exhibited an increase in shoot and root length, whereas higher doses decreased growth. Furthermore, an increase in chlorophyll and carotenoids resulted from the 50 mg/L dose. A decrease in the photosynthetic activity and inhibition in growth indicates dimethoate may be hazardous to wheat

plants at high concentrations (Pandey and Gopal 2011). Similar results were observed by Mishra et al. (2008). Dimethoate at 50 mg/L stimulated growth and photosynthesis in cowpea (*Vigna unguiculata*) yet, higher concentrations lead to a reduction in photosynthetic electron transport activity and damage to pigments. Observed inhibition of growth and biomass accumulation may be the result of inhibition of photosystem II, carbon-fixation and photorespiration (Mishra et al. 2008).

5.5 Mammals

Although dimethoate targets insects, studies have shown mammalian impacts as well. Dose–response studies were conducted by Long at al. (2006) using laboratory mice. Single (10 or 30 mg/kg) or short-term repeated doses (three hourly doses of 10 mg/kg) were administered intraperitoneally to mice and brain and serum AChE activity were measured. Single doses of dimethoate did not cause a significant inhibition in AChE activity; however, the repeated doses did decline overall activity. Besides a response in AChE activity, cytochrome P450 (CYP2B) activity was found to be inhibited as well.

Adult Wistar rats were exposed for 30 days to dimethoate in water, feed or feed co-administered with selenium or vitamin E to assess lung damage (Amara et al. 2012). Changes in animal behavior, in dimethoate only tests, were observed and included depression, dyspnea and diarrhea among others. Extracted lung tissue revealed lipid peroxidation. However, in the presence of selenium and/or vitamin E, malondialdehyde concentrations were restored to levels similar to those in the controls. Further observations included histopathological changes such as hemorrhages, increases in glutathione peroxidase and superoxide dismutase and a decrease in acetylcholinesterase (Amara et al. 2012). In the presence of antioxidants, such as those used in this study, there is potential to alleviate damage from dimethoate exposure.

Developmental toxicity was investigated by Farag et al. (2006). Pregnant Fischer-344 rats were dosed via oral gavage with concentrations of 0, 7, 15 and 28 mg/kg/day dimethoate at gestation days 6–15. At the higher doses, clinical signs of toxicity, such as tremors and weakness, occurred. Reduced cholinesterase activity in both maternal and fetal brains was also observed. The number of living fetuses and mean fetal weight was reduced indicating fetotoxicity occurs from exposure to the highest dose in this study (Farag et al. 2006).

Human exposures are possible due to dimethoate's high use. Six workers were exposed dermally and through inhalation when spraying the pesticide onto tomato crops enclosed in plastic houses (Al-Jaghbir et al. 1992). Each sprayman applied dimethoate as a 40 % emulsifiable concentrate in two applications which were 15 days apart. Gauze sponges were placed on the workers to assess dermal exposure and blood samples were collected to identify cholinesterase inhibition. Overall, a reduction in plasma cholinesterase was observed and dermal exposure to the forearms, hands and upper legs was greatest resulting in a mean exposure dose of 914 mg/day (Al-Jaghbir et al. 1992).

5.6 Birds

Field studies assessing bird exposure to spray drift was conducted by Cordi et al. (1997). To do so, four hedgerows which bordered fields sprayed with the pesticide were chosen and nest boxes containing both nestlings and adult great tits (*Parus major*) were placed into the hedges.

Application of dimethoate (1 L/ha) occurred 59 ft from two of the four hedges, on both sides, by using a boom sprayer. Wind direction was measured to be approximately at right angles to the hedges at speeds of 2.6 m/s from the west and 3.6 m/s from the east-south-east, respectively. Responses of exposure by adults included inhibition in serum butyrylcholinesterase (BChE), whereas nestlings experienced significant decreases in BChE and carboxylesterase (CbE) activity; nestling growth rates were also negatively impacted (Cordi et al. 1997). Westlake et al. (1981) dosed Japanese quail (*Coturnix coturnix japonica*) with the pesticide at 8.3, 25 and 75 mg/kg and observed an inhibition in both AChE and cholinesterase (ChE) activity; brain AChE activity was reduced by 85 % at the highest dose.

Martin et al. (1996) fed pesticide treated grasshopper carcasses to 3-day-old ring-necked pheasant chicks (*Phasianus colchicus*) in order to assess the effects of birds consuming treated insects. They determined a dimethoate LD_{50} of 29 mg/kg body weight which was approximately 0.2 LD_{50} doses per day given the body weight of the birds (approximately 30 g). Lower AChE activity was measured in birds consuming treated feed compared to those fed untreated feed.

6 Summary

The insecticide dimethoate, an organophosphate, was first introduced in 1962 for broad spectrum control of a wide range of insects including mites, flies, aphids, and plant hoppers. It inhibits AChE activity, resulting in nerve damage, which may lead to death. It is considered highly toxic to insects although dimethoate resistance has been observed.

Dimethoate has both a low vapor pressure (0.247 mPa) and Henry's law constant (1.42×10^{-6} Pa m^3/mol), thus volatilization is not a major route of dissipation from either water or moist soils. Photolysis is considered a minor dissipation pathway. However, studies have shown that in the presence of a catalyst, the rate of photolysis does increase. The insecticide has high water solubility (39,800 mg/L) and under alkaline conditions, hydrolysis predominates representing a major degradation pathway. It has a low soil sorption capacity ($K_{oc} = 20$) which varies by soil type and organic matter content. Dimethoate is degraded by microbes under anaerobic conditions and bacterial species have been identified that are capable of using dimethoate as a carbon source. Although many intermediate by-products have been identified by abiotic and biotic processes, the major degradation product is omethoate.

Dimethoate has been found to adversely impact many organisms. In plants, photosynthesis and growth are highly impacted, whereas birds exhibit inhibition in brain enzyme activity, thus sublethal effects are apparent. Furthermore, aquatic organisms are expected to be highly impacted via direct exposure, often displaying changes in swimming behavior. Toxicity results include inhibition in growth and more importantly, inhibition of acetylcholinesterase activity.

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Exposure to Crystal Violet, Its Toxic, Genotoxic and Carcinogenic Effects on Environment and Its Degradation and Detoxification for Environmental Safety

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1 Introduction

Rapid industrialization and urbanization around the world has lead to the recognition and understanding of relationship between the environmental pollutants and public health (Upadhyay 2002). Among the different types of environmental pollution, water pollution is regarded as the most serious problem where effluents from different dye-based industries serve as the major source of contamination. Industries generating dye-containing wastewater include pigment manufacture, textile, printing, dyeing, leather, food and cosmetic industries (Ahmad 2009; Amini et al. 2008). It is estimated that upto 15 % of the total textile dyes used in dyeing process remains unreacted and are directly lost in the effluents (O'Neill et al. 1999; Jadhave and Govindwar 2006).

Synthetic textile dyes are one of the major water and soil pollutants released into the environment by different sources (Nelson and Hites 1980). These pollutants are resistant to the ordinary treatment processes and therefore persist in environment for a long period. These dyes severely affect the all living organisms in ecosystem by causing severe health hazards such as skin irritation, digestive tract irritation, nausea, vomiting, liver and kidney damage, etc in humans and animals, reduced seed germination, root and shoot length in plants, and also inhibit the activity of microorganisms contributing soil fertility (Mittal et al. 2010; Senthilkumaar et al. 2006). With the increasing human population and their needs for such dyes, the variety and quantity of these synthetic dyes released into the environment, are continuously increasing. Huge quantities of different groups of synthetic dyes such as azo disperse, acidic, basic triphenylmethane etc. are used in textile dyeing processes and significant amounts of these dyes enter into the environment as wastewater (Raffi et al. 1997; Ahmad 2009). Among different classes of synthetic dyes used in textile, dyeing, paper, leather, cosmetic, and food industries, triphenylmethane dyes are the largest and most versatile group of dyes that plays a major role in various industrial applications (Azmi et al. 1998; Ahmad 2009).

Crystal Violet (CV), (N, N, N^1, N^1, N^{11}, N^{11}-hexa-methyl-para-rosaniline), which is also a triphenylmethane dye has been extensively used in human and veterinary medicine as a biological stain and also as a textile dye in textile processing industries (Au et al. 1978; Azmi et al. 1998). Crystal violet (CV) also known as gentian violet (impure form) is a cationic dye and has one dimethylamino group on each phenyl ring (Fig. 1). It is widely used as a purple dye for textiles such as cotton and

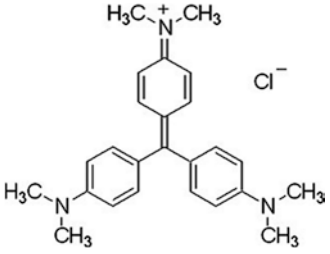

Fig. 1 Chemical structure of crystal violet (Sharma et al. 2011)

silk and provides a deep violet color to paints and printing ink. CV is also used for dyeing nylon, polyacrylonitrile-modified nylon and wool as well as for coloring of plastics, gasoline, varnish, fat, oil and waxes (Daneshvar et al. 2007; Gregory 1993; Parshetti et al. 2006). CV is also used as a mutagenic and bacteriostatic agent in medical solutions and antimicrobial agent to prevent the fungal growth in poultry feed (Littlefield et al. 1985; Mittal et al. 2010). Besides, the medical community also applies CV as a biological stain and is an important ingredient of Gram's stain. The dye is also used as an external skin disinfectant in humans and since proteins are made of different combinations of amino acids, which get easily stained by CV dye and thus, it is used as an enhancer for bloody fingerprints (Chakraborty et al. 2011).

CV is reported as a recalcitrant dye molecule that persists in the environment for a long period and has toxic effects on aquatic as well as terrestrial life (Au et al. 1978; Azmi et al. 1998). *In vitro* investigations have revealed that CV acts as a mitotic poison, potent carcinogen, potent clastogene and promotes tumor growth in some species of fish (Cho et al. 2003; Au et al. 1978; Fan et al. 2009). Hence, CV is regarded as a biohazard substance. The dye is also found to cause moderate eye irritation, painful sensitization to light, permanent injury to cornea and conjunctiva, since the product contains a cationic dye, which is highly toxic to mammalian cells. Nevertheless, in extreme cases it may lead to respiratory and kidney failures also (Ahmad 2009; Amini and Younesi 2009; Azargohar and Dalai 2005). Triphenylmethane dyes are one of the most widely used dermatological agents. Earlier, CV was widely used for the treatment of pinworms through oral route and in topical applications in humans and domestic animals. It has been shown to be effective in controlling the fungal growth under varying conditions and therefore was added to poultry feed to control the fungal growth exposing the human population directly or indirectly to CV through its extensive medicinal and commercial use (Willian et al. 1978; Kumar and Ahmad 2011).

The dark colored wastewater from different industries containing CV significantly affect the photosynthetic activity of aquatic plants because of reduced sunlight penetration and may also be toxic to some other aquatic life due to the presence of aromatics, metals, and chlorides etc. (Gill et al. 2002; Liu et al. 2004). The thin layer of discharged dyes formed over the surface of a receiving water body ultimately reduces the photosynthetic activities and dissolved oxygen content in water whereas in agricultural soil, it inhibits the seed germination and growth of crop plants (Kalyani et al. 2008). In recent years, interest in environmental control of dyes has increased, due to their toxic and genotoxic effects on living organisms as these consists of known carcinogens, such as benzidine and other aromatic compounds.

Due to its adverse effects on human health, CV has been listed as hazardous chemical or material and its use has been prohibited in aquaculture and food industry. However, it is still used in some areas due to its relatively low cost, ready availability and efficacy (Schnick 1988). Dye industries discharge about 10–20 % of the dyes in free state with wastewater during dyeing process, but this value may increase upto 50 % if azo dyes are used. The discharge of this large quantity of dye with wastewater into aquatic resources may affect severely the aesthetic merits, gas solubility as well as water transparency (Moturi and Singara 2009; Shah et al. 2013).

Hence, the removal of CV from wastewater of different industries is essential to not only protect the human health, but also for the protection of soil and water ecosystems. Various methods have been used to remove CV from textile wastewater including chemical oxidation and reduction, physical precipitation and flocculation, photolysis, adsorption, electrochemical treatment, advanced oxidation, reverse osmosis and biodegradation (Azmi et al. 1998). Out of physico-chemical treatment methods, the biological treatment is regarded as cost-effective, simple structural set-up, easy to operate, diverse metabolic pathways, versatility of microorganisms; wider application and environment friendly compared to physical and chemical treatment methods, and produce less amount of sludge (Banat et al. 1996; Mendez-Paz et al. 2005; Pandey et al. 2007).

Several microorganisms like bacterial strains such as *Pseudomonas putida*, *Agrobacterium radiobacter*, *Bacillus* spp., *Sphingomonas paucimobilis* and *Acromonas hydrophila* etc, fungi such as *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium marinum* and *Mycobacterium chelonae*, yeast and actinomycetes has been reported for the effective decolorization of CV dye and thus, can be applied as a bioremediation tools. However, the biological degradation of wastewater containing CV largely depends on pH, temperature and concentration as well as nutrient components. Besides the advantages, biological methods also have certain limitations in their application and suffer due to toxicity of dyestuffs (Daneshvar et al. 2006). Hence, this review article summarizes the structure, chemistry, toxic and genotoxic effects and methods used for the decolorization of CV. The enzymatic degradation of CV by bacteria, fungi, yeast and actinomycetes at various environmental conditions has also been duly emphasized. In addition, focuses on such microorganisms, which are beyond the limitations of all biological methods.

2 Chemical Structure and Properties of Crystal Violet

2.1 Chemical Structure

CV is a typical cationic dye, which has been extensively used as a biological stain, dermatological agent, temporary hair colorant, dyeing cottons, wools and in various other commercial textile operations (Shengfang 2010; Senthilkumaar et al. 2006). It belongs to a class of intensely colored organic compounds that are collectively called triphenylmethane dyes. CV is also known as hexamethyl pararosaniline chloride that is a basic dye with molecular formula $C_{25}H_{30}N_3Cl$ (Sharma et al. 2011). The IUPAC name of crystal violet is Tris (4-(dimethylamino) phenyl) methylium chloride, which is blue-violet color in appearance. The melting point and freezing point of CV is 205 and 40 °C respectively. It is highly soluble in ethanol (13.78 %) and less soluble in water (1.68 %). CV is found to be stable and incompatible with strong oxidizing agents and strong acids. It is light sensitive and combustible. However, the structure and color of crystal violet largely depends on the pH and temperature of medium, which makes it a valuable acid–base indicator as well as an excellent dye (Shah et al. 2013).

The major structural form of CV is the monovalent cation (CV^+), which is the predominant form of CV in solid state as well as in aqueous solution across a broad range of pH values ranging from 1 to 13 (Shah et al. 2013). Moreover, the positive charge on the central carbon atom of CV is delocalized by the mechanism of resonance of the three nitrogen atoms (Sharma et al. 2011). The delocalization of charge across the double bonds in the benzene rings stabilizes the carbonation and is responsible for the vibrant purple color of the CV. In strongly basic solutions, the purple monovalent CV^+ cation slowly combines with hydroxide ions and forms a neutral colorless product (CVOH) as shown in reaction 1.

$$\underset{\textit{Purple}}{CV^+} + OH^- \rightarrow \underset{\textit{Colourless}}{CVOH} \qquad \text{(Reaction 1)}$$

The rate of this reaction is slower than the typical acid–base proton transfer reactions and largely depends on the initial concentration of both crystal violet and hydroxide ions.

2.2 Production

There are number of possible ways to prepare the crystal violet dye in laboratory conditions. However, the original procedure was developed by Caro and Kern (1883), which involves the chemical reaction of dimethylaniline with phosgene to give 4, 4^1-bis (dimethylamino) benzophenone as an intermediate (Reinhardt and Travis 2000). This intermediate form was then reacted with an additional dimethylaniline in presence of phosphorus oxychloride and hydrochloric acid.

The dye can also be synthesized by the condensation of formaldehyde and dimethylaniline to give a leuco dye that is a reduced form of CV as shown in reaction 2 (Gessner and Mayer 2002; Thetner 2000).

$$CH_2O + 3C_6H_5N\left(CH_3\right)_2 \rightarrow CH\left(C_6H_4N\left(CH_3\right)_2\right)_3 + H_2O \qquad \text{(Reaction 2)}$$

Color of Dye

CV has a blue-violet color when dissolved in water with an absorbance of 590 nm at maximum and an extinction coefficient of 87,000 M^{-1} cm^{-1} (Adams and Rosenstein 1914; Cheriaa et al. 2012). The color of dye largely depends on the acidity of medium as at a pH of 1.0, the dye is green with absorption maxima at 420 and 620 nm, while in a strongly acidic solution (pH of −1), and the dye is yellow with an absorption maximum at 420 nm.

The different colors of dye are result of the different charge states of dye molecule. In yellow form, all three nitrogen atoms carry a positive charge, of which two are protonated, while the green color corresponds to a form of dye with two of the nitrogen atoms positively charged, but at neutral pH both the extra protons are lost to the solution leaving only one of the nitrogen atoms positively charged.

3 Sources of CV Contamination in Environment

Industries such as textile, leather, paint, acrylic, cosmetics, plastics, pharmaceutical manufacturers, etc., use dyes in order to color their products and consume substantial volumes of water in the processing of the products. CV is also used for dyeing nylon, polyacrylonitrile-modified nylon and wool (Daneshvar et al. 2007; Gregory 1993; Parshetti et al. 2006) as well as for coloring of plastics, gasoline, varnish, fat, oil and waxes. As a result, a considerable amount of colored wastewater is generated and discharged into the environment. It is estimated that approximately 12 % of synthetic dyes are lost during the manufacturing and processing operation of products. CV is also used as a mutagenic and bacteriostatic agent in medical solutions, also as a biological stain and as an important ingredient in Gram's stain. CV is used as an antimicrobial agent to prevent the fungal growth in poultry feed. Humans also apply the dye as an external skin disinfectant and since, it is a protein dye, it is also used to enhance the bloody fingerprints. These are the major sources of CV contamination/pollution in environment and the wastewater containing high CV concentration when discharged into water and soil environment without adequate treatment, it causes serious environmental and health problems in surrounding areas (Akar et al. 2009; Kiran et al. 2009). Besides these, CV is also one of the major sources of aesthetic pollution and eutrophication in environment (Tsai et al. 2004).

4 Toxic, Genotoxic and Carcinogenic Effects of CV in Environment

The CV dye is reported to cause moderate eye irritation and painful sensitization to light with permanent injury to cornea and conjunctiva. It is highly toxic to mammalian cells and can cause skin irritation and digestive tract irritation, if absorbed in harmful amount through skin. It may also leads to respiratory and kidney failure in extreme conditions (Ahmad 2009; Mittal et al. 2010; Saeed et al. 2010).

CV when released in air at an estimated vapor pressure of 1.0×10^{-13} mm Hg at 25 °C, it exists in atmosphere in the particulate phase. CV when is exposed to skin causes irritation, and if inhaled or is exposed to eyes causes irritation. CV when released in water ecosystems without adequate treatment, it interferes the sunlight penetration power and photosynthetic activity of aquatic plants leading to the reduction in dissolved oxygen content and thus, finally disturbs the normal life process of the aquatic flora and fauna both (Ajao et al. 2011; Cunningham and Siago 2001). In addition, the colloidal and suspended impurities also cause turbidity of the receiving water bodies and the dissolved minerals increase the salinity of water and thus, making it unfit/difficult for recycling process or irrigation process. The wastewaters released from different industries contain high CV content with high BOD and COD values along with various kinds of toxic recalcitrant organic compounds, which decrease dissolved oxygen content significantly in aquatic

environment (Kagalkar et al. 2010; Rajamohan and Karthikeyan 2004). In terrestrial environment, CV adversely affects the seed germination as well as root and shoot length as Parshetti et al. (2011) have studied the effect of CV on these parameters and found that undegraded CV inhibited 50, 70, 100 and 60 % seed germination in *Sorghum bicolor*, *Vigna radiata*, *Lens culinaris* and *Triticum aestivum*, respectively along with significant reduction in root and shoot length. Besides the coloring problem, there is another concern that CV is either toxic or carcinogenic in nature. There is no such universally useful method available to treat the dye wastes because of the complex chemical structures of these dyes. Therefore, CV may be regarded as biohazard substance.

Like malachite green, CV is readily absorbed into fish tissue from water exposure and reduced metabolically by fish to the leucocrystal violet. There are several studies made by the National Toxicology Program, which reported that CV has carcinogenic and mutagenic effects in rodents (Henderson et al. 1997; Littlefield et al. 1985; Michaels and Lewis 2006; Verma and Madamwar 2003). It has also been linked to increased risk of human bladder cancer. The leuco form induces renal, hepatic and lung tumor in mice (US Food and Drug Administration 2009).

4.1 Toxicological Studies on Crystal Violet

According to Hodge et al. (1972), the oral toxicity (LD_{50}) of CV was 1.2 g/kg for mice and 1.0 g/kg for rat. Though the bacterial treatment of textile wastewater is very effective, it is found that microorganisms used in bacterial treatment processes can transform dyes into compounds, which are more toxic than of parent compounds. Hence, there was a need to study the toxic effects of the products formed after the biological treatment processes. To know this, Parshetti et al. (2011) have performed some toxicological studies such as microbial toxicity and phytotoxicity by using bacteria contributing soil fertility and seed germination test on four kinds of plant species respectively to determine the toxicity of degradation products of CV and they found that the degradation products of CV were less toxic to bacteria as well as all four kinds of plant species as compared to undegraded CV.

A number of dye compounds have been tested for mutagenicity using Ames's bioassay test. Several of them have been found to be carcinogenic and mutagenic (Mathur et al. 2005). Parshetti et al. (2011) have performed microbial toxicity tests on various microbes such as *A. radiobacter*, phosphate-solubilising bacterium *Pseudomonas aeruginosa* and a nitrogen-fixing bacterium *Azotobacter vinelandii*. These microbes have shown the zone of inhibition with CV whereas its metabolites showed comparatively less zone of inhibition. Zone of inhibition is the area, which is free from microbial growth in a bacterial lawn resulted due to the toxic effects of compounds that have diffused into the medium from its applied source. These results have revealed that the degradation products were less toxic than CV for the microorganisms studied.

Parshetti et al. (2011) and Kalyani et al. (2008) have also carried out phytotoxicity tests to assess the toxicity of CV and its metabolites on vegetation and to explore the possible reuse of treated wastewater for the irrigation of agricultural fields. They conducted tests on four different plant species, which are fast growing, most sensitive and commonly used in Indian agriculture such as *Sorghum bicolor*, *Vigna radiata*, *Lens culinaris* and *Triticum aestivum* and found that the germination percentage of seeds treated with CV was significantly less in all four plants as compared to the seeds treated with its metabolites obtained after its degradation and distilled water. Therefore, these phytotoxicity studies have revealed that CV is more toxic than its metabolites. In addition, Kalyani et al. (2008) have reported that the seeds of *Sorghum vulgare* and *Phaseolus mungo* have more sensitivity towards the dye molecule and less sensitivity for its metabolic products.

4.2 Genotoxic Effects of CV in Environment

Cytogenetic Toxicity

The cytogenetic toxicity of CV in Chinese Hamster ova cells was studied by Azmi et al. (1998) and results revealed that cultures treated or with a higher dosage (10 g/ml) for a longest period of time (8 h) showed a significant accumulation of abnormal metaphases. Thus, CV might be acting as a mitotic poison. These results have also demonstrated that CV causes severe cytogenetic effects in cultured cell lines (mitotic poison as well as clastogen).

CV was also found to cause reduction in RNA and protein synthesis and decreased oxygen consumption in rabbit granulation tissue. Nelson and Hites (1980) have reported the deposition of crystal violet and malachite green in sediments and water of Buffalo River, New York, USA. These chemicals have been suggested to be responsible for the promotion of tumor growth in several bottom-feeding species of fishes (Cho et al. 2003). Thus, possess a serious threat to aquatic environment as well as human population.

Genetic Toxicity

William et al. (1979) have studied the genetic toxicity of CV by the Ames and the Rosenkranz bacterial assays as well as the cytogenetic assays on Chinese hamster ovary cells *in-vitro* in presence of rat-liver S-9 fractions, the chicken-embryo and mouse-bone-marrow cells *in-vivo*. They found that CV is toxic, but not mutagenic in the Ames assay. However, it is active in the Rosenkranz assay causing reparable DNA damage. The presence of S-9 fraction in the *in-vitro* cytogenetic assay and in bacterial assays showed that the activity of CV can be reduced or eliminated. In the *in-vivo* assays, CV was found to be non-clastogenic and failed to induce sister-chromatid exchanges. Therefore, CV was proved to be highly toxic to growing chick embryos at high concentration and depressed mitotic activities in mouse bone

marrow after prolonged exposure. However, CV was also found to be potentially hazardous to cells e.g. skin epithelium and cell lining of the gastro-intestinal tract that were exposed to dye directly.

Mutagenic Effect

Crystal violet has been used as a fungal growth controlling agent in poultry feed. The mutagenic activity of CV was studied in *Saccharomyces cerevisiae* and *Salmonella typhimurium* by Shahin and Von Borstel in Shahin and Von Borstel 1978 and they found that CV showed a slight increase in the number of revertants in *S. typhimurium* TA1535, but no mutagenic effect was observed in *S. cerevisiae*.

4.3 Chronic Toxicity and Carcinogenicity of CV

Littlefield et al. (1985) to determine the toxicity and carcinogenicity of CV performed a life span dosing study on 720 males and 720 females of B6C3F1, mice (C57BL/6×C3H) at dose concentration of 0, 100, 300, and 600 ppm after 12, 18, and 24 months of continuous dosing. They observed no effect on food consumption or body weight gain; but, a dose effect was noted for mortality rates. They observed that the mortality was less than 15 % at 24 months in controls of both sexes but it was 64 and 23 % higher in female and male respectively given the high dose. In this study, females were appeared to be more susceptible than males. A positive dose response for hepatocellular carcinoma was also observed in males at 24 months and in females at 18 and 24 months.

The statistical tests for dose related trends with respect to (1) mortality due to liver neoplasms, (2) prevalence of liver neoplasms, and (3) time of onset of liver neoplasms showed positive trends in both males and females. Other dose related toxicological responses, particularly in female mice, included erythropoiesis in spleen, atrophy of ovaries, adenoma of the harderian gland, and the presence of type-A reticulum cell sarcomas in urinary bladder, uterus, ovaries, and vagina. The estimation of risk of 10^{-6} over background for malignant liver neoplasms using linear extrapolations showed a lower bound on the virtually safe dose (VSD) to be 2 ppb for female mice and 1 ppb for male mice. For benign and malignant liver tumors together, the lower bound on the VSD was essentially the same as for malignant liver neoplasm alone. Under the experimental conditions described as above, CV appeared to be a carcinogen in mice at several different target organs.

4.4 Some Case Reports of Crystal Violet Human Toxicity

Hashimoto et al. (2013) have reported the case study of chemical cystitis due to the intravesical introduction of CV. Earlier during 1960s; CV was commonly used for the treatment of oral and vaginal candidiasis or for sterilization during operations

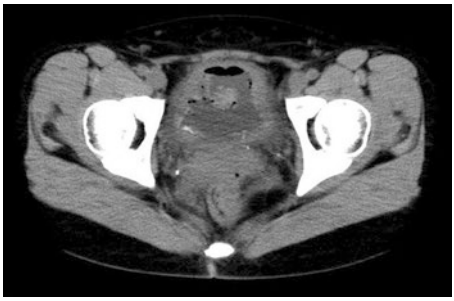

Fig. 2 Computed tomography imaging of whole bladder wall showing the thickening of wall and spotty high-density lesions of the bladder surface with clotting blood (Hashimoto et al. 2013)

(Bonney 1981; Kim et al. 2003; Walsh and Walsh 1986). Since, CV is potentially toxic to mucosal membranes; it has been replaced with other disinfectants. CV was instilled into the bladder of a 47-year-old Japanese woman to confirm the presence of a vesicovaginal fistula. The patient developed symptoms of gross hematuria, frequent urination and lower abdominal pain. Computed tomography had shown the thickening of her whole bladder wall with spotted high-density lesions as shown in Fig. 2. Cystoscopy has demonstrated the desquamated epithelial cells and a hemorrhagic bladder wall. The patient was treated conservatively with non-steroidal anti-inflammatory drugs and glucocorticoids. During follow-up, magnetic resonance images showed that the detrusor muscle of her bladder was normal. Patient's symptoms gradually improved and she completely recovered within 6 months.

Diamante et al. (2009) have reported a case of 32 years old female, who was mistakenly injected 1 % CV (in 2 % alcohol) into her urethra instead of her vagina to treat severe pruritus. Within few seconds, she developed a burning pain in her lower abdomen followed by frequent urination, urgency and dysuria. Two days later, she noticed hematuria, which led to her admission to the hospital. An intravenous pyelogram suggested a mass lesion in the left side of her bladder. Cystoscopy showed gross inflammation and edema of the left side of her bladder with acute ulceration of overlying mucosa and a large mass on the left side of her bladder. Her condition improved with a high intake of fluids, and later cystogram showed a normal outline of bladder with no evidence of mass lesion. Histological examination of a slightly thickened area of her bladder showed extensive ulceration and non-specific inflammatory changes with large number of eosinophils, but no evidences of neoplasia was observed.

Diamante et al. (2009) have also reported another case in which, a 15 days old infant's tongue was appeared white and fuzzy. The mother was advised to use 2 % CV for treatment and she started to use 2 % solution of CV for atleast 10–12 times daily for 4 days. On the fifth day, she noted that the tongue looked unusual and the baby had become increasingly fussy and began to refuse to nurse. She then decreased the applications to twice daily and started formula feeding, but the child remained irritable and was subsequently brought to the emergency room. There, the patient was diagnosed to have severe candidiasis and macroglossia. The undersurface of the

tongue, gingival grooves, and the floor of mouth were covered with large, purplish gray plaques that could be scraped off, leaving a bleeding surface. The lesions appeared similar to a caustic burn of the oral mucosa and tongue. When CV was discontinued, the patient became much less irritable, fed well, and gained weight by the third day of hospitalization.

Diamante et al. (2009) have also reported a third case of 60 years old male patient in which 1 % CV was accidentally instilled into both his eyes by a medical practitioner. The patient complained of irritation, pain, and diminution of vision. On examination, his visual acuity was reduced to counting fingers 1/2 meter OD (right eye) and 6/36 OS (left eye) with development of moderate lid edema and blepharospasm. The conjunctivae were congested and chemosed whereas corneas were hazy and edematous in both eyes. Biomicroscopic examination has revealed the presence of punctate epithelial lesions scattered all over the cornea. The patient was treated with antibiotic, steroid and cycloplegic drops and the eyes were patched. The corneal lesions healed within 4–5 weeks leaving behind fine punctate opacities at the level of Bowman's membrane and at the end of fifth week, the visual acuity had improved to counting fingers at 1 m OD and 6/9 OS. The marginal tear strip was absent and the tear breakup time was 5 s in eyes and after 4 months, the tear secretion had not shown any further improvement.

Thus, all the above studies indicate that CV is a cytogenic, genetic, mutagenic and carcinogenic agent causing severe health hazards in human such as skin irritation, digestive tract irritation, respiratory and kidney failure, etc. In animals the leuco form of CV is reported to induce renal, hepatic and lung tumor. The CV is also reported to cause various toxic effects in aquatic as well as terrestrial environment such as tumor in fishes and inhibition of seed germination, reduction in root and shoot length of plant species and also reduces the activity of microbes contributing soil fertility, respectively. Hence, it becomes very essential to decolorize and detoxify the various types of industrial wastewaters containing CV before its final discharge into the environment.

5 Treatment Methods Used for the Degradation and Detoxification of CV

The removal of color from wastewaters is often more important than the removal of the soluble colorless organic substances, which usually contribute the major fraction of the BOD. However, it is difficult to degrade and detoxify dyes because of their synthetic origin and complex aromatic molecular structures. A wide range of treatment methods have been developed for the removal of synthetic dyes from water and wastewaters to reduce their impact on the environment, which are grouped into three major categories: Physical, chemical and biological treatment methods as shown in Table 1.

Table 1 Advantages and disadvantages of physical, chemical and biological treatment methods used for the treatment of dye wastewaters

Processes	Advantages	Disadvantages	References
Coagulation-flocculation	Elimination of insoluble dyes	Production of sludge blocking filter	Aguilar et al. (2005); Golob and Ojstrsek (2005)
Adsorption on activated carbon	Suspended solids and organic substances well reduced	Cost of activated carbon	Slokar and Le Marechal (1998)
Electrochemical processes	Capacity of adaptation to different volumes and pollution loads	Iron hydroxide sludge	Carneiro et al. (2005)
Reverse osmosis	Removal of all mineral salts, hydrolyzes reactive dyes and chemical auxiliaries	High pressure	Sadrghayeni et al. (1998); Treffry Goatley et al. (1983); Tinghui et al. (1983)
Nanofiltration	Separation of organic compounds of low molecular weight and divalent ions from Monovalent salts	–	Chakraborty et al. (2003)
Ultra filtration and microfiltration	Low pressure	Insufficient quality of the treated wastewater	Sadrghayeni et al. (1998); Watters et al. (1991)
Fenton's reagent	Effective decolorization of both soluble and insoluble dyes	Sludge production	Hao et al. (2000); Nesheiwat and Swanson (2000)
Ozonation	Applied in gaseous state: no alteration of volume	Short half-life (20 min) of O_3	Muthukumar et al. (2005); Aplin and Wait (2000)
Photochemical	No sludge production	Formation of by-products	Gogate and Pandit (2004); Forgacs et al. (2004)
NaOCl	Initiates and accelerates azo bond cleavage	Release of aromatic amines	Robinson et al. (2001)
Stabilization pond	High reduction of solids, BOD and pathogens	Sludge requires proper removal and treatment	Sperling and Chenicharo (2005)
Aerated lagoon	High reduction of BOD and pathogens	Sludge /effluent require further treatment and/or appropriate discharge	Crites and Tchobanoglous (1998)
Trickling filter	Efficient nitrification (ammonium oxidation)	Risk of clogging, depending on pre- and primary treatment	US EPA (2000)
Activated sludge	High reduction of BOD and pathogens (up to 99 %)	Sludge/effluent require further treatment and/or appropriate discharge	Crites and Tchobanoglous (1998)
Anaerobic digestion	Digested slurry therefore provides organic fertilization	Heavy metals cannot be destroyed	Cowley and Wase (1981)

Adopted from Bizuneh (2012); Azmi et al. (1998); Reife (1993)

5.1 Physical Treatment Methods

Adsorption Method

Adsorption technique has gained favor recently due to its higher efficiency for the removal of pollutants in comparison to other treatment methods. Adsorption produces a high quality product, and it is also economically feasible. It is the process by which ions or molecules present in one phase (usually gas or liquid) tend to accumulate and concentrate on the surface of another phase (usually solid). Physical adsorption occurs when weak interspecies bonds exist between the adsorbate and adsorbent, while chemical adsorption occurs when strong interspecies bonds are present between the adsorbate and adsorbent due to an exchange of electrons (Bizuneh 2012). Suzuki (1997) had discussed the role of adsorption in water treatment processes and also evaluated the development of new adsorbents such as eggshells (Chowdhury et al. 2013a, b, c), citric acid modified rice straw (Chowdhury et al. 2013a, b, c; Chakraborty et al. 2013), NaOH modified rice husk (Chowdhury et al. 2013a, b, c), sugarcane bagasse (Chakraborty et al. 2012a, b, c, d), H_2SO_4 modified sugarcane bagasse (Chakraborty et al. 2012a, b, c, d), fish scales (Chakraborty et al. 2012a, b, c, d), hen feathers (Chakraborty et al. 2012a, b, c, d), *Artocarpus heterophyllus* leaf powder (Saha et al. 2012), almond peel (Ahmad and Mondal 2009), water nut carbon (Ahmad and Mondal 2010), ginger waste (Kumar and Ahmad 2010), activated carbon/iron oxide nanocomposite (Ahmad and Kumar 2010a, b, c), conducting polyaniline/iron oxide composite (Ahmad and Kumar 2010a, b, c), bale shell carbon (Ahmad and Kumar 2010a, b, c), alumina reinforced polystyrene (Ahmad and Kumar 2011), PAni/TiO_2 nanocomposite (Ahmad and Mondal 2012a, b) etc. to modernize the treatment systems and role modeling of the findings plays in their development. Adsorption techniques for wastewater treatment have become more popular in recent years owing to their removal efficiency towards pollutants that are not easily biodegradable. Decolorization is a result of two mechanisms—adsorption and ion exchange and is influenced by many physico-chemical factors such as nature of dye, sorbent interaction, sorbent surface area, particle size, temperature, pH, and contact time (Kumar et al. 1998; Shah et al. 2013).

Activated Carbon Method

Activated carbon is the most commonly used treatment method for dye removal by adsorption and is very effective for the adsorption of cationic, mordant and acid dyes and to a slightly lesser extent, dispersed, direct, vat, pigment and reactive dyes (Raghavacharya 1997; Rao et al. 1994). Activated carbon adsorption treatment process is one of the best available treatment technologies. Different materials like peat, wood chips, silica gel, fly ash, corn cobs and rice husks etc. are widely used for the production of commercial activated carbons and for the

removal of dye from wastewaters (Bansode et al. 2003). These materials are advantageous mainly due to their widespread availability and low cost (Robinson et al. 2001). Adsorption methods also have some drawbacks, since the processes are not selective. Thus, the other components of the wastewater may compete for the adsorbing sites, reducing the dye binding capacity of sorbent (Bizuneh 2012). Moreover, an adsorption process removes the synthetic dye by concentrating it on the surface and retaining its structure unchanged. When the support is to be regenerated the concentrated dye sludge causes a problem for its subsequent disposal into the environment (Forgacs et al. 2004).

Membrane Filtration Method

The increasing cost of water and its profligate consumption necessitate a treatment process that is integrated with in-plant water circuits rather than a subsequent treatment (Machenbach 1998). From this standpoint, membrane filtration offers a potential application. This technology has emerged as a feasible alternative to conventional treatment processes used for the removal of CV from wastewaters and has proven to save operational cost and water consumption (Koyuncu 2002). Processes using membranes have provided very interesting possibilities for the separation of hydrolyzed dye-stuffs and dyeing auxiliaries that simultaneously reduce the coloration and BOD/COD of wastewaters; usually used to treat the reactive dye bath effluent, because it could potentially reduce the waste volume and simultaneously recover the salt (Sen and Demirer 2003). The method has the ability to clarify, concentrate and most importantly to separate CV continuously from the effluent (Mishra and Tripathy 1993; Xu and Lebrun 1991). The method has some special features compared to other methods like resistance to temperature and adverse chemical effects. The advantages of the membrane filtration techniques are that it is a quick method with low spatial requirement and the saturate can be reused, but has high capital cost, the possibility of clogging, and membrane replacement affects the applicability of this method. Usually this technique is applied as a tertiary or final treatment process after biological treatment (Bizuneh 2012).

Ion Exchange Method

Ion exchange method has not been widely used for the treatment of dye containing effluents, mainly due to the reason that ion exchangers cannot accommodate a wide range of dyes. In this technique, the wastewater is passed over the ion exchanger resin until all the available exchange sites are saturated (Bizuneh 2012). CV can be efficiently removed by this method, but the limitation of this method is the high cost of organic solvents to regenerate the ion-exchanger (Mishra and Tripathy 1993; Robinson et al. 2001; Slokar and Le Marechal 1998).

5.2 Chemical Treatment Methods

Coagulation and Flocculation Method

Coagulation and flocculation methods are generally used in order to remove the organic materials from the wastewaters by partly removing the TDS, BOD, COD and color through many years (Aguilar et al. 2005). This process is mainly based on the principle of addition of a coagulant followed by a general rapid association between the coagulants and pollutants forming coagulate or flock and subsequently precipitate. The precipitate is then removed by flotation, settling, filtration or other physical techniques to generate a sludge, which is further treated to reduce its toxicity (Golob and Ojstrsek 2005; Mishra and Bajpai 2005). Although these processes effectively remove the insoluble dyes (Gaehr et al. 1994), its value is doubtful because of the cost of treating the sludge and the increasing number of restrictions regarding the disposal of sludge into the environment (Bizuneh 2012).

Photochemical Method

Photocatalytic or photochemical degradation processes are gaining importance in the area of wastewater treatment, since these processes result in complete mineralization of dye molecule with operation at mild temperature and pressure. The photo-activated chemical reactions are characterized by the free radical mechanisms initiated by the interaction of photons of proper energy levels with the chemical molecules present in solution/wastewater in presence/absence of catalysts (Gogate and Pandit 2004). This method causes the degradation of CV molecules into CO_2 and H_2O by UV treatment in presence of H_2O_2 (Peralto-Zamora et al. 1999; Yang et al. 1998). The degradation of CV is caused by the production of high concentrations of hydroxyl radicals, which attack on the unsaturated dye molecules resulting in the destruction of chromophore group with no sludge formation and great reduction in foul odors is the additional benefits of this method. However, the rate of dye removal is greatly influenced by the intensity of UV radiation, pH, dye structure as well as the composition of dye bath solution (Slokar and Le Marechal 1998; Forgacs et al. 2004).

Sodium Hypochloride (NaOCl) Method

This method attacks at the amino group of dye molecules by the Cl^+ and accelerates the subsequent azo bond cleavage. The decolorization is largely affected by pH and NaOCl concentration (Slokar and Le Marechal 1998; Robinson et al. 2001). This method is not an efficient method for the decolorization of disperse dyes as it takes longer times are required to decolorize the reactive and metal-complex dyes. The rate of decolorization increases with an increase in the concentration of chloride ions.

However, the use of chloride ions in high concentration for dye removal is not favorable due to its reactive nature as it leads to the formation of organo-chlorinated compounds, which are highly toxic in nature (Slokar and Le Marechal 1997; Banat et al. 1999).

Ozonation Method

Ozone is an efficient oxidizing agent due to its high reactivity. Effective degradation of chlorinated hydrocarbons, phenols, pesticides and aromatic hydrocarbons can be achieved through oxidation by ozone (Lin and Lin 1993; Xu and Lebrun 1991). The dosage of ozone applied to the dye-containing effluent is largely dependent on the color intensity and total residual COD to be removed. The decomposition of ozone requires a high pH value (pH > 10). In alkaline solutions ozone reacts almost indiscriminately with all compounds present in medium converting them into smaller and biodegradable molecules (Aksu 2005; Chu and Ma 2000; Park et al. 1999). The major advantage of this method is the application of ozone in its gaseous state and therefore, it does not increase the volume of wastewater and sludge generated (Bizuneh 2012). However, the major drawback of this method is the very short half-life of ozone as it decomposes in 20 min, thus requiring continuous ozonation supply which makes this method very expensive (Gogate and Pandit 2004; Gosavi and Sharma 2014; Robinson et al. 2001).

Electrochemical Destruction Method

In this process, the destruction of dyes through oxidation and chlorine evolution from NaCl in the solution takes place at the anode, whereas hydrogen evolution and OH^- ion formation occurs at the cathode during electrolysis process. This is a relatively new technique which was developed in the mid 1990s. It has some significant advantages for use as an effective method for dye removal. This method demands very little or low consumption of chemicals, leading no/very less sludge generation. The breakdown metabolites are generally not hazardous leaving it safe for treated wastewaters to be released into the water ecosystem. However, the high cost of electricity is the major limitation for the application of this method at industrial scale (Ogutveren and Kaparal 1994; Pelegrini et al. 1999).

Fenton Reagent Method

The oxidation system based on the Fenton's reagent has been widely used for the treatment of both organic and inorganic pollutants (Beekeepers 2000). The Fenton's reagent can be used for the effective removal of color and absorbable organohalides from the refining wastewater (Mauskan 2007). Besides heavy metals which are

Table 2 The advantages and disadvantages of the Fenton reagent uses

Advantages	Disadvantages
The first investment cost is low	Additional chemical cost
Decrease in poison for biological refining	Removing mud cost
It can be used in different processes	The potential of polymerization reactions
Getting ineffective of toxic and resistant compounds	Continuing of normal chemical reactions
Sudden beginning time	Potential corrosion problems
Low hydraulic waiting period (1–2 h)	Controlling foam
Chemical mud production	Special safety thoughts

Adopted from Beekeepers (2000)

caused by metal-complex dyes can be precipitated on the neutralization step with the iron oxide. Fenton oxidation process can also be used to decolorize a wide range of dyes because as compared to ozonation, it is relatively cheap and result significant reduction in COD values (Bizuneh 2012; Park et al. 1999). Refining with fenton reagent is more advantageous than the other methods such as flocculation, precipitation, air flotation, filtration, etc. in which H_2O_2 is used (Sewekow 1993). Fenton oxidation is limited only to the fact that the textile wastewaters usually have high pH, whereas Fenton process requires low pH because at higher pH, large volumes of waste sludge is generated due to the precipitation of ferric iron salts and the process looses its effectiveness (Table 2).

In this way, the various physico-chemical treatment methods are thus found to be effective, but their application is limited due to the excess usage of chemicals, sludge generation, subsequent disposal problems, high installation as well as operating costs (Bizuneh 2012; Vandevivere et al. 1998). Therefore, as a viable alternative, biological process have gained increasing interest due to their low cost, generation of less amount of sludge, and most importantly is their environment friendly nature (Banat et al. 1996).

5.3 Biological Treatment Methods

Bioremediation is a process where removal of pollutants and xenobiotics is achieved by using biological systems. Over the past decades, many microorganisms (bacteria, fungi, yeast, actinomycetes and algae) have been reported to have the ability of CV degradation dye, but the effectiveness of biological treatment methods largely depends on the adaptation and activity of the selected microorganisms as well as on pH, temperature, aeration, media composition etc (Bumpus and Brock 1988; Kwasniewska 1985; Yatome et al. 1991). As CV is a stable and long lasting colorant, it is usually not easily degraded. Nevertheless, many researchers have also reported either the partial or complete biodegradation of CV either by pure or mixed cultures of bacteria, fungi and algae (Table 3).

Table 3 Microorganisms used by various workers for the decolorization of CV dye wastewater

Microorganisms	Decolorization (%)	References
Agrobacterium radiobacter	100	Parshetti et al. (2011)
Agrobacterium radiobacter, *Bacillus* spp., *Sphingomonas paucimobilis*, *Aeromonas hydrophila*	91	Cheriaa et al. (2012)
B. subtilis ETL-2211	90	Shah et al. (2013)
Mucor mucedo	78	Moturi and Singara (2009)
Trametes versicolor	72	Moturi and Singara (2009)
Polyporus elegans	73	Moturi and Singara (2009)
Lenzites betulina	75	Moturi and Singara (2009)
Nostoc linckia	72	Sharma et al. (2011)
Pleurotus ostreatus	92	Kunjadia et al. (2012)
Bacillus spp.	99	Azmi and Banerjee (2001)
N. corallina	98.3	Azmi et al. (1998)

Degradation Mechanism by Bacteria

The ability of bacteria to metabolize CV dye has been investigated by a number of researchers (Chen et al. 2007; Parshetti et al. 2011; Yatome et al. 1991; Ahmad et al. 2010; Mondal et al. 2010; Ahmad and Mondal 2012a, b). Earlier, it was predicted that CV is relatively resistant to biodegradation in the environment because the most important environmental factor affecting the CV biodegradation was the pH (Michaels and Lewis 1986).

Yatome et al. (1991) have studied the degradation of CV by *Bacillus subtilis* IFO 13719 and found that CV was remarkably decolorized within the 8 h of incubation period with the low cell growth, but it was completely decolorized in 24 h when the cell growth was higher. They also tried some other bacteria like *E. coli*, but the bacterium was not able to decolorize CV, even the cells were growing remarkably. Similar results were also observed with two other cultures namely *Pseudomonas cepacia* and *Pseudomonas cruciviae*. Roth et al. (1992) have isolated 21 hydrophobic oleophilic bacterial strains having the decolorization activity, but *Mycobacterium* were found to be the most active strain for CV decolorization.

Chen et al. (2007) have elucidated the biodegradation pathway of CV by *Pseudomonas putida* by using High Performance Liquid Chromatography fitted with Atlantis dC18 column (Fig. 3). They identified nine metabolic products resulting from the CV degradation process and concluded that the dye is broken down by the means of demethylation yielding mono-, di-, tri-, tetra- penta- and hexa-demethylated end products. This biodegradation pathway differs from that of *Nocardia coralline* and *Bacillus subtilis*. However, the maximum CV biodegradation efficiency of *P. putida* achieved in the study was 78.5 % at the concentration of 60 μM, which was much higher than that of *Phanerochaete chrysosporium* at the concentration of 12.3 μM as reported by Bumpus and Brock (1988). Many bacteria, which were used to degrade CV were found to be toxic to many other microorganisms (Michaels and Lewis 1986). Chen et al. (2007) results, however,

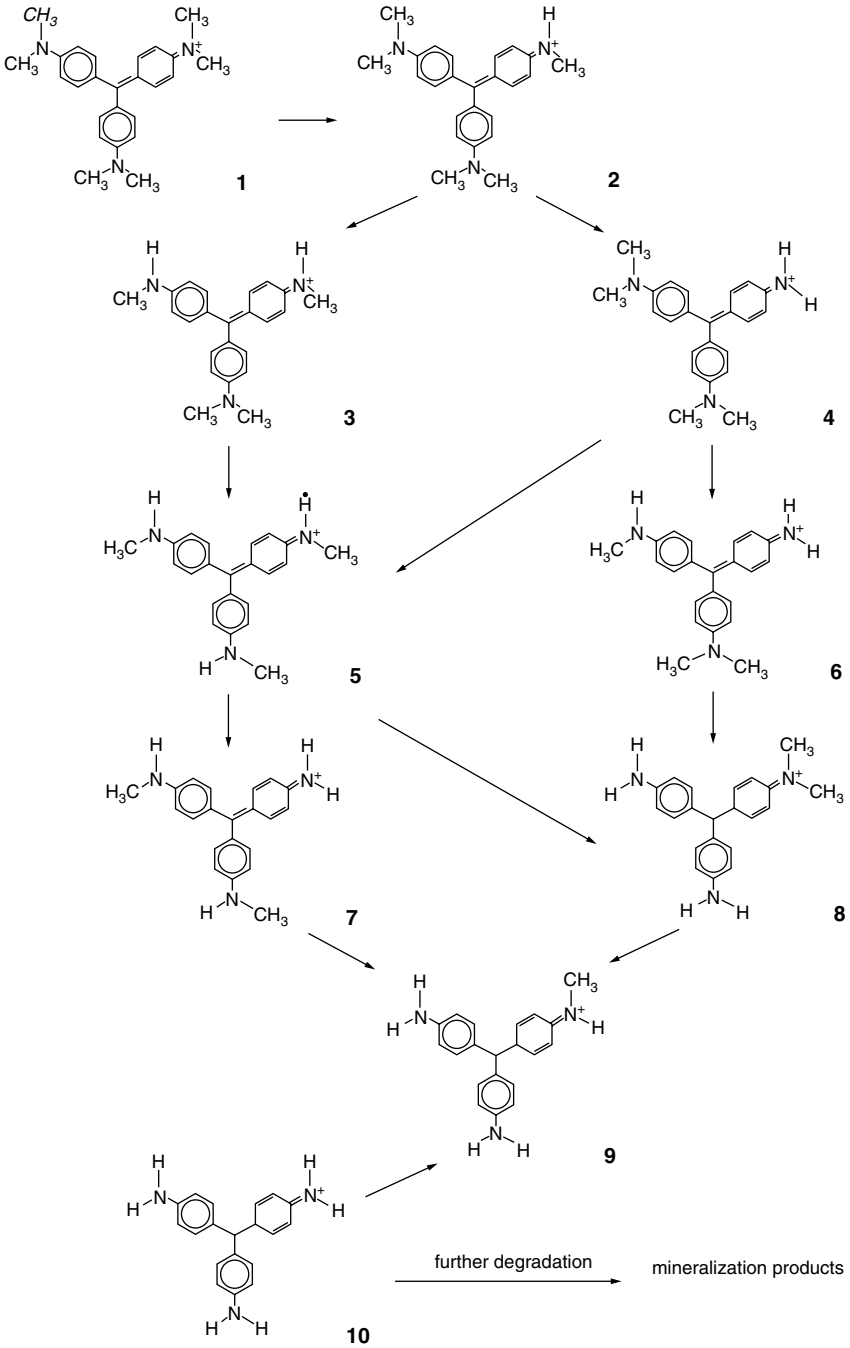

Fig. 3 Proposed pathway of Crystal violet demethylation by *P. putida* (Chen et al. 2007) (**1**: -N, N, N^{1}, N^{1}, N^{11}, N^{11}-hexamethyl pararosaniline; **2**: -N, N-dimethyl-N^{1}, N^{1}-dimethyl-N^{11}-methyl pararosaniline; **3**: -N, N-dimethyl-N^{1}-methyl-N^{11}-methyl pararosaniline; **4**: -N, N-dimethyl-N^{1}, N^{1}-dimethyl pararosaniline; **5**: -N-methyl-N^{1}-methyl-N^{11}-methyl pararosaniline; **6**: -N, N-dimethyl-N1-methyl pararosaniline; **7**: -N-methyl-N^{1}-methyl pararosaniline; **8**: -N, N-dimethyl pararosaniline; **9**: -N-methyl pararosaniline: **10**: -Pararosaniline)

have suggested that CV is non-toxic to *P. putida* and it has the potential to remove CV from the environment without producing toxic byproducts and they proposed the demethylation pathway of CV by *P. putida* depicted in Fig. 3.

They also investigated the effect of pH on the biodegradation of CV and found that the optimum CV degradation takes place at pH 7.5, but at pH higher or lower than 7.5 and 6.0 respectively, the CV degradation capability of *P. putida* was significantly reduced.

Parshetti et al. (2011) have proposed the hypothetical metabolic pathway of CV biodegradation by *A. radiobacter*. The degradation of CV was analyzed by Gas Chromatography and Mass Spectroscopy (GC-MS) and enzyme activities. Five intermediate compounds such as, *N, N, N*^{1}, *N*^{11}-tetramethylpararosaniline, [*N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone, 4-methyl amino phenol, *N*, *N*-dimethylaminobenzaldehyde and phenol were detected during the CV degradation. During the CV degradation, the *N, N*^{1}, *N*^{11}-tetramethyl pararosaniline was first broken down into [*N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone and 4-methyl amino phenol and then in the next step [*N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone was further degraded into *N*, *N* dimethylaminobenzaldehyde and 4-methyl amino phenol and finally phenol was obtained as the final degradation product of CV (Fig. 4).

Degradation Mechanism by Fungi

The role of fungi and their enzymes and their potential use in degradation and detoxification of CV has been well reported and recognized (Ferreira et al. 2000; Mielgo et al. 2001; Claus et al. 2002; Assadi et al. 2003). Concerning the dye degradation, the most widely used fungi are the ligninolytic fungi. Among all the fungi studied, wood degrading white rot fungi are found to be very effective in treatment of colored textile effluents because white rot fungi are reported to produce enzymes such as lignin peroxidase, manganese peroxidase and laccase that can degrade organic pollutants due to their non-specific activities (Toh et al. 2003). Many authors have found these white rot fungi capable to oxidize an array of phenolic, non-phenolic, soluble and in soluble dyes (Libra et al. 2004).

In 1988, Bumpus and Brock have studied the CV biodegradation by using white rot fungus *Phanerochaete chrysosporium* and found that in medium, the CV disappeared jointly with the appearance of three metabolic products: N, N, N^{1}, N^{11}-penta-, N, N, N^{1}, N^{11}-tetra- and N, N^{1}, N^{11}-trimethylpararosaniline. The purified ligninase enzymes were found to catalyze the N-methylation of CV, which proved that the lignin-degrading enzyme system is mainly responsible for the CV degradation.

Vasdev et al. (1995) have reported the decolorization of three triphenylmethane dyes including CV by three birds nest fungi namely *Cyathus bulleri*, *C. stercoreus* and *C. striatus*. They observed that *C. bulleri* have both laccase and ligninase enzyme activity, but the laccase activity was higher than ligninase activity during the dye decolorization. They also reported the decolorization of CV by using the ultra filtered and dialyzed extracellular culture filtrate of *C. bulleri*, which could be due to the presence of active laccase enzyme in ultra filtered and dialyzed extracellular fluid.

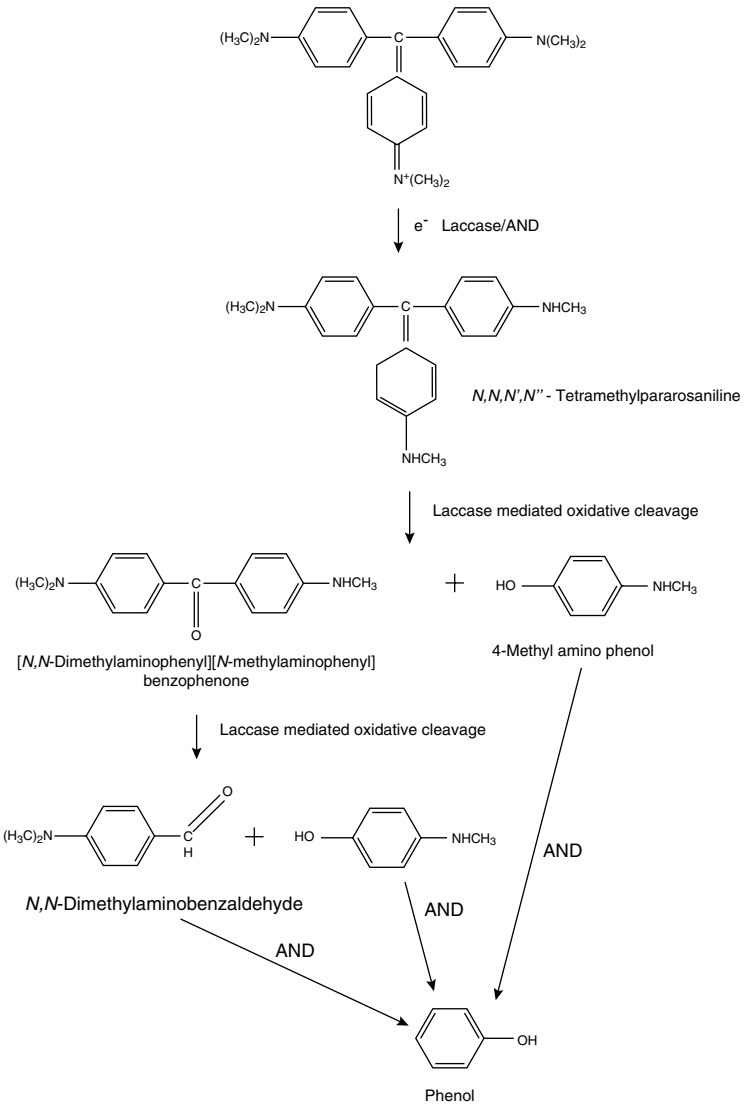

Fig. 4 Mechanism of CV degradation by *Agrobacterium radiobacter* (Parshetti et al. 2011)

However, *C. bulleri* was found capable to decolorize CV dye up to 90 µM, whereas *P. chrysosporium* has been shown to decolorize the dyes to a much lesser extent (12.3 µM) (Bumpus and Brock 1988).

Yesilada (1995) has studied the CV decolorization by three different white-rot fungi such as *Coriolus versicolor*, *Funalia trogii*, and *Phanerochaete chrysosporium* and one brown-rot fungus, *Laetiporus sulphureus* and found that CV undergoes oxidation process only in presence of H_2O_2 suggesting the involvement of an H_2O_2-dependent enzyme produced by the tested fungal strains.

Das et al. (1995) have studied the CV decolorization in a column bioreactor using *P. chrysosporium*. The decolorization process was performed in a glass column bioreactor (31 cm × 5 cm) with an eight tier stainless steel inoculum holder, through which the dye containing medium was recirculated by a peristaltic pump. During the process, CV was passed through column at a concentration of 0.002 % with a recycling rate of 20 ml min^{-1} at 30 °C and results have revealed that almost 92 % CV was decolorized in 82.4 h in recycled medium as compared to 64 % CV decolorization in shake flasks condition in 17 days of incubation period.

Degradation Mechanism by Yeasts

Only few reports are available on the degradation of CV by yeast. Kwasniewska (1985) has studied the biodegradation of CV by some oxidative red yeast and found that these oxidative yeasts such as *Rhodotorula* sp. and *Rhodotorula rubra* were capable of CV degradation in liquid broth, which was measured in terms of decrease in absorbance indicating the continuous decrease in CV concentration and after 4 days of incubation period, the absorbance of culture medium at 600 nm became non-measurable indicating the complete degradation of CV by both the oxidative red yeasts. It was also observed that the fermentative yeast *S. cerevisiae* did not degrade CV in liquid medium even after a prolonged incubation period of 30 days.

Degradation Mechanism by Actinomycetes

Yatome et al. (1991) presented the first report on the CV biodegradation by the actinomycetes. They studied the CV decolorization by two actinomycetes namely *Nocardia coralline* and *N. globerula* and found that the decolorization activity of both the actinomycetes was intracellular and the dye was completely decolorized in 24 h. Further, in 1993, they published a report on CV degradation by *Nocardia coralline* in which they identified Michler's ketone as the main degradation product by Gas chromatography–mass spectrometry (Fig. 5). They also observed that the decolorization rate was dependent upon the initial concentration of *N. coralline* in medium. Besides CV decolorization, *N. coralline* was also found capable to decolorize methyl violet, ethyl violet, basic fuchsin and Victoria blue to a great extent (Table 4).

6 Effects of Nutritional and Environmental Factors on the Microbial Degradation and Detoxification of CV

Environmental factors such as pH, temperature, aeration and nutrients concentration play a vital role in the microbial degradation process of industrial wastes as the activity of enzymes are greatly influenced by these environmental factors. Several studies have been made by various workers to understand the role of various

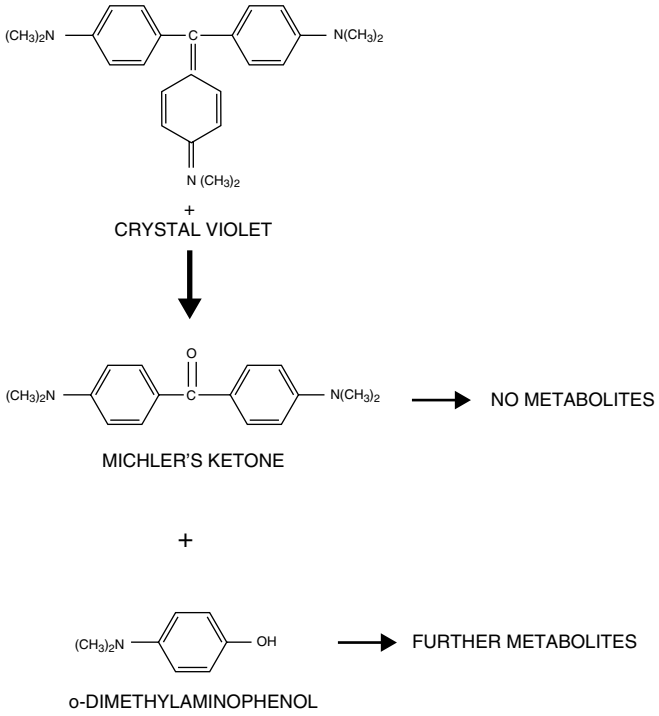

Fig. 5 Degradation pathway of CV by *Nocardia coralline* (Yatome et al. 1993)

Table 4 Decolorization of different triphenylmethane dyes by *N. coralline*

Dyes	λ_{max}	Half life of decolorization (min)	Maximum decolorization (%)
Crystal violet	590	50	98.3
Methyl violet	590	60	71.8
Ethyl violet	600	480	59.8
Basic fuchsin	555	30	70.0
Victoria violet	620	20	33.0

Adopted from Yatome et al. (1991)

environmental factors in microbial degradation and detoxification of CV for environmental safety (Moturi and Singara 2009).

Shah et al. (2013) have studied the effect of different carbon and nitrogen sources on degradation and detoxification of CV by varying 1 % each in medium and they found that dextrose was best carbon source resulting 92 % CV decolorization followed by starch and mannose with 80 and 65 % decolorization, respectively at the end of 24 h of incubation period. However, the decolorization efficiency decreased dramatically with dulcitol, mannitol, lactose, D-xylose and sucrose resulting only 52, 45, 35, 30 and 25 %, decolorization respectively. Further, the maximum decolorization

90 % was achieved when peptone was used as nitrogen source whereas malt extract has resulted only 15 % decolorization of CV.

In addition, Parshetti et al. (2011) have observed 100 % decolorization of CV by using yeast extract and NH_4Cl within the 5 h of incubation period at a concentration of 1 and 0.1 %, respectively. However, in presence of urea, peptone and malt extract, the culture exhibited 87.5, 81.25 and 38.23 % CV decolorization, respectively whereas the presence of sucrose, lactose and glucose at 1 % concentration of each, the culture showed 90, 39.23 and 36.17 % decolorization of CV, respectively.

Shah et al. (2013) while studying the decolorization efficiency of *B. subtilis* ETL-2211 across a wide range of pH have found that CV decolorization was maximum (90 %) at pH 8 and minimum at acidic pH. They also observed that at neutral pH, the strain decolorized 80 % CV, whereas at pH 6 and 9, it was only 45 % and 40 %, respectively. The percentage decolorization of CV decreased markedly at pH 5 (10 %) due to acidic conditions indicating that the maximum growth and decolorization of CV occur at pH 8. Shah et al. (2013) have also studied the effect of temperature on CV decolorization and observed that the dye decolorization activity of the strain decreased with increase in temperature. However, the optimum decolorization of CV (95 %) was achieved at 35 °C and least (25 %) at room temperature (RT) and further increase in temperature has resulted continuous decrease in percent decolorization of CV as at 37, 40, 45 and 50 °C to 85, 70, 55 and 25 %, respectively at the end of 24 h of incubation period. It was also depicted that the percent removal of dye decreases with increase in temperature, which may be due to the weakening of bonds between the dye molecules and the binding sites of the adsorbent (Chowdhury and Saha 2010).

Chen et al. (2007) while studying the biodegradation of CV have also observed that the environmental factors such as pH and temperature play an important role in the microbial degradation and decolorization of CV. They examined the effect of pH only in slightly acidic and slightly alkaline conditions in order to avoid possible chemical degradation of the dye molecule. They found that the optimal pH for CV biodegradation by *P. putida* was 7.5 and resulted 78.5 % CV decolorization. The degradation capacity of *P. putida* decreased significantly at pH higher and lower than 7.5 and 6.0 respectively. They also observed that the optimum temperature for CV degradation was 37 °C by *P. putida*.

Sharma et al. (2011) also studied the effect of pH on the dye removal at different pH (4.0–9.0) at 100 mg/l initial dye concentration and they found that the maximum dye removal was achieved at pH 8.0 and temperature 25 °C. Moturi and Singara (2009) studied the effect of pH on decolorization of CV by using four fungal species such as *Polyporus elegans*, *Trametes versicolor*, *Lenzites betulina* and *Mucor mucedo* and they found that these fungal strains were capable to decolorize the CV upto 73, 72, 75 and 78 % at pH 6.0, 4.5, 4.0 and 2.5, respectively.

Thus, all the above studies showed that the maximum decolorization (95–99 %) of CV can be achieved at pH 7.5, temperature 35 °C and shaking condition (140 rpm) by using 1 % glucose and 1 % peptone as a carbon and nitrogen source respectively.

7 Enzymes Involved in the Degradation and Detoxification of Crystal Violet

In recent years, the use of microbes and plants for the degradation and detoxification of recalcitrant organic pollutants have gained significant recognition as a viable alternative to the existing physical and chemical treatment methods (Movahedin et al. 2006; Franciscon et al. 2009; Kagalkar et al. 2009). Moreover, biological treatment methods are found capable to degrade an array of recalcitrant organic pollutants due to the involvement of different kinds of enzymes. Hence, both intracellular and extracellular enzymes are being explored as biochemical means of wastewater treatment (Nelson and Cox 2004). Thus, the enzymatic treatment may be an innovative approach for the effective treatment of industrial wastewaters containing different types of recalcitrant organic pollutants by removing them either through precipitation or transforming them into other products (Mugdha and Usha 2012). Enzymes offered several advantages such as greater specificity, better standardization, easy handling and storing and no dependence on bacterial growth rates.

The structural complexity of CV and very little knowledge about the enzymatic system involved in its degradation has created many challenges to elucidate the mechanism involved in the enzymatic degradation of CV. However, various workers have studied the enzymes from various microorganisms involved in the CV degradation. Most of the enzymes causing degradation of CV belong to the class of oxidoreductases, which catalyses the electron transfer reactions (Mugdha et al. 2011).

Sometimes, a substrate required may not be oxidized directly by enzyme, if the redox potential of substrate is higher than that of the enzyme. This phenomenon has been observed in the case of laccase enzymes, which require mediators like 2, 2^{1}-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) to act as an intermediate substrate for the enzyme (Kunamneni et al. 2007, 2008). Many processes have been developed based on the laccases due to their potential in degradation of dyes of diverse chemical structures (Rodriguez et al. 2006; Wesenberg et al. 2003). Laccases are copper-containing enzymes that catalyze the oxidation of electron rich substrates. Laccase alone has a limited effect on bioremediation process due to its specificity for phenolic subunits in lignin. Phenolic compounds were shown to be efficient laccase mediators (Camarero et al. 2005). Laccase utilizes molecular oxygen for degradation purposes. Whereas peroxidase enzymes such as lignin peroxidase and manganese peroxidase acts as electron acceptor, which require hydrogen peroxide or alkyl, peroxide that are not specific towards the electron donor in the redox reactions that they catalyze (Moturi and Singara 2009; Kersten et al. 1990).

The role of fungi and their enzymes in degradation and detoxification of organic pollutants has been well reported globally because of their wide versatility and broad range of substrates (Assadi et al. 2003; Claus et al. 2002; Ferreira et al. 2000; Mielgo et al. 2001; Moturi and Singara 2009). Several studies have demonstrated the ability of fungal biomass and purified enzymes to degrade and decolorize a category of different types of organic pollutants (Wesenberg et al. 2003). White rot fungi were able to degrade CV using lignin peroxidase (LiP) and manganese

dependent peroxidase enzymes (MnP) (Muragesan et al. 2007). Other enzymes used for this purpose include H_2O_2 producing enzymes, such as glucose-2-oxidase along with laccase and phenol oxidase enzymes (Husain 2009). The production of enzymes such as lignin peroxidase, manganese peroxidase and laccase by various fungal strains during the decolorization of CV is well reported (Moturi and Singara 2009; Parshetti et al. 2010; Telke et al. 2008). Moturi and Singara (2009) have estimated the percent decolorization of CV by the enzymes lignin peroxidase, manganese peroxidase and laccase produced by four fungal strains such as *Polyporus elegans*, *Trametes versicolor*, *Lenzites betulina* and *Mucor mucedo*. They also observed that the production of lignin peroxidase was high during ten days and reduced in 15 days of incubation period. The highest range of enzyme production was recorded in *Polyporus elegans* whereas the lowest range of enzyme was recorded in *Mucor mucedo. Trametes versicolor* and *Lenzites betulina* showed the maximum production of this enzyme in 5 and 10 days, respectively (Moturi and Singara 2009). In addition, *Trametes versicolor* was also found to produce the maximum quantity of manganese peroxidase enzyme in 5 and 10 days of incubation period whereas *Mucor mucedo* showed the minimum production of manganese peroxidase in 5 days of incubation and remained fail to secrete the enzyme in 10 and 15 days. *Polyporus elegans* and *Lenzites betulina* also showed the maximum production of manganese peroxidase in 10 days of incubation.

In case of laccase enzyme activity, Moturi and Singara (2009) have demonstrated that *Mucor mucedo* remained totally fail to secrete laccase enzyme in all its incubation days. However, the maximum enzyme production by *T. versicolor* was observed in 15 days of incubation period while, the moderate quantity of enzyme was produced by *P. elegans* and lower quantity was produced by *L. betulina* (Moturi and Singara 2009).

The major drawbacks of the enzymatic treatment are the inactivation of enzymes by the conditions normally found in the textile wastewater as well as the high cost of enzyme production has limited its application at industrial scale.

8 Summary

Crystal Violet (CV), a triphenylmethane dye, has been extensively used in human and veterinary medicine as a biological stain, as a textile dye in textile processing industries and also used to provide a deep violet color to paints and printing ink. CV is also used as a mutagenic and bacteriostatic agent in medical solutions and antimicrobial agent to prevent the fungal growth in poultry feed. Inspite of its many uses, CV has been reported as a recalcitrant dye molecule that persists in environment for a long period and pose toxic effects in environment. It acts as a mitotic poison, potent carcinogen and a potent clastogene promoting tumor growth in some species of fish. Thus, CV is regarded as a biohazard substance. Although, there are several physico-chemical methods such as adsorption, coagulation and ion-pair extraction reported for the removal of CV, but these methods are insufficient for the complete

removal of CV from industrial wastewaters and also produce large quantity of sludge containing secondary pollutants. However, biological methods are regarded as cost-effective and eco-friendly for the treatment of industrial wastewaters, but these methods also have certain limitations. Therefore, there is an urgent need to develop such eco-friendly and cost-effective biological treatment methods, which can effectively remove the dye from industrial wastewaters for the safety of environment, as well as human and animal health.

9 Conclusion

For industries using large quantities of water such as textile, leather, paint, acrylic, cosmetic, plastic and pharmaceutical industries, etc. it is essential to treat and reuse their wastewater. The preceding account of CV reveals that this dye has now become one of the most debated and controversial compounds due to its detrimental effects on environment and severe health hazards posed on living organisms. Thus, it is concluded that CV is a recalcitrant pollutant, which possesses toxic effects on aquatic as well as on terrestrial ecosystems. It also acts as a mitotic poisoning, carcinogenic agent and a potent clastogen promoting tumor growth in some species of fishes. Hence, a frequent and effective treatment method should be employed for the degradation and detoxification of wastewater containing CV. In this regard, physical and chemical treatment methods are very costly and also generate large amount of different types of secondary pollutants. Therefore, there is an urgent need to develop an environment friendly and cost effective biological process for the effective degradation and detoxification of CV for environmental safety.

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Metabolic Pathways for Degradation of Aromatic Hydrocarbons by Bacteria

Guillermo Ladino-Orjuela, Eleni Gomes, Roberto da Silva, Christopher Salt, and John R. Parsons

Contents

1 Introduction

The aromatic compounds present in the environment are from natural sources and anthropogenic activities. The chemical characteristic of these compounds is the presence of one benzene ring (monoaromatic hydrocarbon—MAHs) or more than one fused rings (polyaromatic hydrocarbon—PAHs) (Favre and Powell 2013). The ring provides structural and chemical stabilities due to a symmetric π-electron system and therefore recalcitrance of these compounds (Vogt et al. 2011). In accordance with Molecular Orbital Theory, in a molecule of benzene the p electrons on

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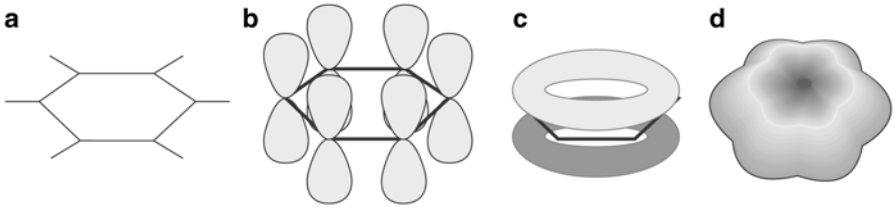

Fig. 1 Electron delocalization of π-electron system in benzene ring. (**a**) Shows the sigma-bonding framework of benzene. (**b**) Shows the *p* orbitals which form the delocalized π-bonding system in benzene. (**c**) Shape of the π-electron clouds above and below the plane of the ring in benzene. (**d**) Electrostatic potential map of benzene (Bruice 2004)

each carbon atom are delocalized and contribute to the development of the so-called π system. An electrostatic potential map of benzene (Fig. 1) shows that the electrons in the π-system are evenly distributed around the ring (Bruice 2004).

Aromatic hydrocarbons are classified as biological and non-biological compounds. Biological aromatic hydrocarbons are produced by plants and by microorganisms. In plants, this is mainly through the shikimic acid pathway (Ghosh et al. 2012) and microorganisms mainly via the malonic acid pathway (Zhan 2009). Capsaicin, estradiol, caffeine, theobromine, gallic acid, aromatic aminoacids (tyrosine, phenylalanine, and tryptophan), salicylic acid and the monolignols (p-coumaryl, coniferyl, and sinapyl) of lignin are the most well-known.

The monolignols are synthesized in plants via the shikimic acid pathway and are the most important natural aromatic compounds since they appear in large quantities in the environment as lignin and humic acids. p-coumaryl is a minor component of grass and forage type lignins, and coniferyl is the predominant lignin monomer found in softwoods (hence the name). Both coniferyl and sinapyl are the building blocks of hardwood lignin (Li and Chapple 2010).

The main sources of non-biological aromatics hydrocarbons are the effluents from fuel, chemical, plastic, explosive, ink, metal, pharmaceutical, and electric industries among others (Table 1). These compounds are called xenobiotics in function of their non-biological origin and their bioaccumulation, toxicity and carcinogenic action are well documented (USEPA 2005).

The physic-chemical properties of aromatic hydrocarbons have environmental significance because they determine fate in soil, water and atmosphere. For instance, adsorption on soils or sediments, due to hydrophobicity, is a major factor in their transportation and eventual degradation (Karickhoff 1981). The soil organic carbon-water partitioning coefficient (K_{OC}), that reflects the ratio between the quantity of the compound absorbed in the soil (normalized to organic carbon content) and the concentration in water has been used in predicting the mobility and bioavailability of organic soil contaminants. Low K_{OC} values correlate to more mobile organic chemicals and higher bioavailability (Wilczyńska-Piliszek et al. 2012) (Table 1).

The United States Environmental Protection Agency (USEPA 2005) published in 1986 guidelines to characterize the human carcinogenic potential of agents according to the Weight of Evidence (WoE). The characterization was done by a six-category

Table 1 Aromatic compounds from industrial activities

Compound	Formula	WoE*	K_{oc}	CASRN
Aniline	$C_6H_5NH_2$	B2	0.96	62-53-3
Phenol	C_6H_6O	D	1.24	108-95-2
Benzene	C_6H_6	A	1.82	71-43-2
2,4,6 Trinitrotoluene	$C_6H_2CH_3(NO_2)_3$	C	2.48	118-96-7
Bisphenol A	$C_{15}H_{16}O_2$	N.A.	2.74	80-05-7
Tetrachlorobenzene 1,2,3,4	$C_6H_2Cl_4$	N.A.	4.60	634-66-2
Benzo-a-pyrene	$C_{20}H_{12}$	B2	5.98	50-32-8

**WoE* weight of evidence approach, *N.A.* not applicable, K_{oc} sorption coefficient (log L/Kg), *CASRN* chemical abstract service registry numbers

alphanumeric classification system (A, B1, B2, C, D and E). Group A includes human carcinogenic agents and Group E is for substances with evidence of non-carcinogenicity. The approach outlined in USEPA's guidelines for carcinogen risk assessment (USEPA 2005) considers all scientific information in determining whether, and under what conditions, an agent may cause cancer in humans and provides a narrative approach to characterize carcinogenicity rather than categories (Table 1).

Among the techniques for the study of metabolic pathways of aromatic hydrocarbon degradation by bacteria, the molecular biology technique Stable Isotope Probing (SIP) is particularly interesting because it allows for detailed metabolic and taxonomic analysis. SIP involves the incorporation of heavy isotopes (^{13}C, ^{15}N or ^{18}O) into newly synthesized nucleic acids allowing the metabolic capacity of cultivated or uncultivated microorganisms to be linked to taxonomic identity (Aanderud and Lennon 2011; Abu Laban et al. 2015; Cupples 2011; Rettedal and Brözel 2015; Taubert et al. 2012; Zhang et al. 2012b). Briefly, in the nucleic acid-based SIP with carbon, light ^{12}C nucleic acid and heavy ^{13}C nucleic acid (DNA or RNA) are separated through ultracentrifugation and then characterized by denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP) and sequencing of 16S rRNA gene. These results are used for genomic and/or metagenomic analysis (Cupples 2011; Kim et al. 2014; Kleinsteuber et al. 2012; Zhang et al. 2012b). The protein-based SIP relies on the detection and quantification of the peptides that incorporate heavy isotope ^{13}C or ^{15}N for proteomic and/or metaproteomic analysis using high-resolution mass spectrometry (Kleinsteuber et al. 2012; Taubert et al. 2012).

2 Biodegradation of Aromatic Compounds

The hydrophobicity and chemical stability of aromatic hydrocarbons, described above, give negligible biological activity to these molecules. Therefore, to break them down, in either aerobic or anaerobic conditions, bacteria need to destabilize the benzene ring through reversible and irreversible chemical modifications (Díaz et al. 2013).

Table 2 TEAs (terminal electron acceptors) used by bacteria for aromatic hydrocarbon degradation

Anaerobic			Aerobic	Environmental condition
Highly reduced	Reduced	Moderately reduced	Oxidized	Redox condition
↓ CO_2 SO_4^{2-}	Fe(III)	Mn(IV) NO_3^-	O_2	Terminal Electron Acceptor (TEA)
CH_4 HS^-	Fe(II)	Mn(II) NO_2	H_2O	Products
Anaerobic	Facultative		Aerobic	Microbial metabolism
−300 −200	−100 0 +100	+200 +300	+400 +500 +600 +700	E° (mV)
←- -				

Downward arrow indicates products of TEAs reduction. Dashed arrow indicates sequential order of TEAs preferences from higher redox potential (E° = +) to lower redox potential (E° = −) (DeLaune and Reddy 2005; Gibson and Harwood C 2002)

In both aerobic and anaerobic pathways of aromatic hydrocarbon biodegradation a Terminal Electron Acceptor (TEA) is required. TEA determines the energy balance and the metabolic reaction used by microorganisms (Table 2) (Philipp and Schink 2012; Schink et al. 2000). However, studies with microcosms and stable isotope probing (SIP) have shown that, in environments dominated by a particular TEA, the dominant bacterial strain was not specialized to degrade the aromatic hydrocarbon being evaluated (Kleinsteuber et al. 2012; Pilloni et al. 2011). These results suggest that aromatic-degrading strains are specialized and the dominant bacterial strains are generalists and are able to use compounds other than aromatic hydrocarbons as carbon and energy sources (Staats et al. 2011).

The aerobic and anaerobic processes of aromatic hydrocarbon biodegradation have been divided (see also below) into upper pathways, which go from the original aromatic compound to so-called central intermediates, and lower pathways, which go from the ring cleavage of intermediates down to molecules for biomass (Cafaro et al. 2004; Carmona et al. 2009).

2.1 Aromatic Hydrocarbon Biodegradation Under Aerobic Conditions

In nature, oxygen is the most common and strongest oxidizing agent found (DeLaune and Reddy 2005). In this sense, bacteria will firstly use oxygen as the TEA to degrade aromatic hydrocarbons.

In aerobic conditions, the first step of upper pathways is an oxidation catalyzed by monooxygenases (hydroxylases) or by dioxygenases (Huijbers et al. 2014; Parales and Resnick 2004).

The monooxygenases catalyze the cleavage of the oxygen-oxygen bond of O_2, inserting one oxygen atom into the aromatic ring while the other is reduced to H_2O (Fig. 2). These enzymes are classified in eight groups according to their structure, sequence, type of reaction catalyzed and type of electron donor. The group A–B

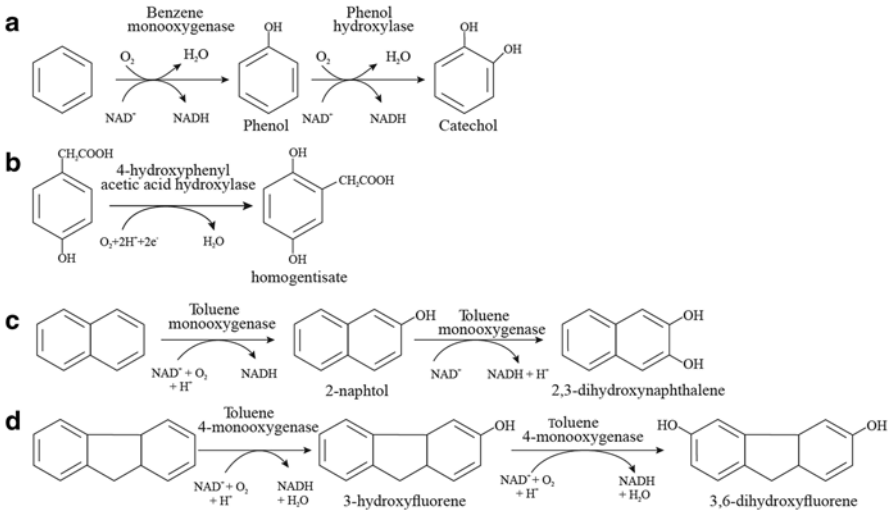

Fig. 2 Process of oxidation of benzene (**a**), 4-hydroxyphenyl acetic acid (**b**), naphthalene (**c**), and fluorene (**d**) catalyzed by monooxygenases

monooxygenases (EC 1.14.13) includes enzymes whose cofactor is the Flavin Adenine Dinucleotide (FAD) and the electron donor is the Nicotinamide Adenine Dinucleotide Phosphate NAD(P)H. They are able to catalyze hydroxylation, sulfoxidation, heteroatom oxygenation, N-hydroxylation and oxidative decarboxylation reactions. Group C-D monooxygenases (EC 1.14.14) require FAD or flavin mononucleotide (FMN) as cofactor and $FMNH_2$ or $FADH_2$ as electron donor and catalyzes hydroxylation, sulfoxidation, oxidation, epoxidation and desulfurization reactions. Group E–G has FAD as cofactor and $FADH_2$ or a substrate as electron donor. This group includes internal flavoprotein monooxygenases that reduce the flavin cofactor through substrate oxidation, and catalyze halogenation, sulfoxidation and oxidative decarboxylation (Huijbers et al. 2014).

Monooxygenases can oxidize both monoaromatic and polyaromatic hydrocarbons. Some examples are phenol hydroxylase and toluene/o-Xylene monooxygenase catalyzing benzene and phenol oxidation as reported by Cafaro et al. (2004) from *Pseudomonas stutzeri* OX1 and 4-hydroxyphenyl acetate 1-monooxygenase catalyzing oxidation of 4-hydroxyphenyl acetate as reported by Hareland et al. (1975) from *P. acidovorans*. In the case of PAHs, there was reported oxidation of naphthalene and fluorene catalyzed by a toluene 4-monooxygenase following a similar route to monoaromatic hydrocarbons (Tao et al. 2005) (Fig. 2).

While monooxygenases sequentially add hydroxyl groups to the aromatic ring, using molecular oxygen as substrate, forming phenols and then catechols, the dioxygenases catalyze, in the upper pathway, the reductive dihydroxylation of the aromatic ring forming cis-dihydrodiols (Fig. 3), then funneled by specific cis-dihydrodiol dehydrogenases to catechols as central intermediates. These dioxygenases are cofactor-requiring multicomponent heteromultimeric proteins (EC 1.14.12) (Parales and Resnick 2004).

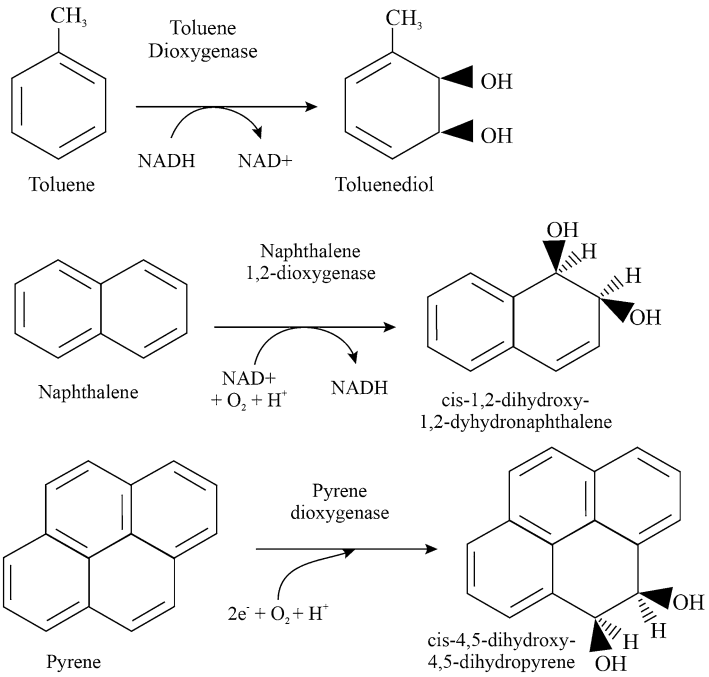

Fig. 3 Formation of cis-dihydrodiols of toluene, naphthalene and pyrene catalyzed by diooxygenases

A group named Rieske non-heme iron-dependent oxygenase systems, also important in aromatic hydrocarbon oxidation, are NADH-dependent and composed of a flavoprotein reductase, an iron-sulfur ferredoxin, and an iron-sulfur ferredoxin as catalytic component (Barry and Challis 2013; Ferraro et al. 2005).

The upper pathways begin with an oxidation and finish with the formation of central intermediates, which can be catechols or non-catecholic compounds (Fig. 4). The former have cis-dihydrodiols groups (Ma et al. 2013; Tao et al. 2005) and latter are hydroxy-substituted aromatic carboxylic acids (Fetzner 2012) resulting from reactions catalyzed by monooxygenases and dioxygenases.

Catechol was found by Loh and Chua (2002) evaluating the ability of *P. putida* ATCC 49451 to degrade sodium benzoate. Catechol was also reported by Karigar et al. (2006) as an intermediate of the phenol metabolic pathway by *Arthrobacter citreus*. (Chloro)hydroxyquinol was reported by Pérez-Pantoja et al. (2008) as a central intermediate of 2,4,6 trichlorophenol degradation by *Cupriavidus necator* JMP134. It was also reported by Khan et al. (2013) in the metabolism of 2-chloro-4-nitroaniline by *Rhodococcus* sp. Strain MB-P1. In the metabolism of para-nitrophenol in *Pseudomonas* sp. strain 1–7, Zhang et al. (2012a, b) found the hydroquinone and hydroxyhydroquinone as central intermediates.

The central intermediates that are non-catechols are hydroxy-substituted aromatic carboxylic acids (Fetzner 2012) (Fig. 4). Among the intermediates reported are the gentisate found by Romero-Silva et al. (2013) studying the peripheral pathways of 3-hydroxybenzoate and 4-hydroxybenzoate degradation by *Burkholderia xenovorans* LB400. Protocatechuate was reported by Kim et al. (2006) as an intermediate in

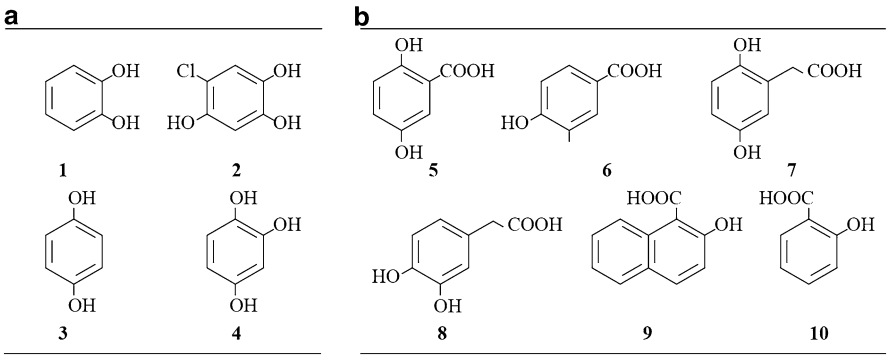

Fig. 4 Central intermediates formed in upper pathway under aerobic conditions. (**a**) Dihydrodiols (*1*) Hydroquinone, (*2*) Catechol, (*3*) (Chloro)hydroxyquinol, (*4*) Hydroxyhydroquinone, (**b**) hydroxy-substituted aromatic carboxylic acids, (*5*) Gentisate, (*6*) Protocatechuate, (*7*) Homogentisate, (*8*) Homoprotocatechuate, (*9*) 2-hydroxy-1-naphtoic acid, (*10*) Salicylic acid

the metabolism of para-hydroxybenzoate and vanilline in *P. putida* KT2440. The homogentisate and homoprotocatechuate were reported by Méndez et al. (2011) in studies with *Burkholderia xenovorans* LB400 degrading 3-hydroxybenzoate.

2-Hydroxy-1-naphtoic acid and salicylic acid were reported by Mallick and Dutta (2008) studying naphthalene degradation by *Staphylococcus* sp. strain PN/Y. The 3-hydroxyanthranilate was reported by Hasegawa et al. (2000) as part of degradative pathway of 2-nitrobenzoate by *P. fluorescens* strain KU-7.

The lower pathways refer to the dearomatization of central intermediates and ring cleavage to tricarboxylic acids (Fig. 5). The first reaction of lower pathways comprises of the de-aromatization of central intermediates, which, thereafter, undergo ortho-, meta- or para-cleavage by dioxygenases (Harwood and Parales 1996). Ortho-cleavage, also known as the β-ketoadipate pathway, is between two hydroxyl groups and catalyzed by intradiol-type dioxygenases using Fe(II) as cofactor (Guzik et al. 2013). Meta-cleavage is on the carbon-carbon bond adjacent to the hydroxyl groups through the formation of an α-keto-lactone intermediate, catalyzed by extradiol-type dioxygenases, using Fe(III) as cofactor (Suenaga et al. 2014). Ortho cleavage of the catechol ring is catalyzed by catechol 1,2-dioxygenase to cis,cis-muconate; catechol meta-cleavage is catalyzed by catechol 2,3-dioxygenase to 2-hydroxy-muconic semialdehyde. Both cleavage pathways were reported by Loh and Chua (2002) in benzoate degradation by *P. putida*. The para-cleavage pathway is followed in hydroxy-substituted aromatic carboxylic acids between the carboxyl-substituted and the adjacent hydroxylated carbon atom. This para-cleavage pathway was reported by Dagley (1971) catalyzed by 2,3-gentisate dioxygenase to gentisate. Crawford (1975) also reported para-cleavage to protocatechuate, catalyzed by 2,3-protocatechuate dioxygenase in *Bacillus circulans* metabolizing 4-hydroxyphenyl propionic acid.

The ring cleavage, of hydroxy-substituted aromatic carboxylic acids, is catalyzed by enzymes that belong to the cupin superfamily. Most cupin-type ring cleavage dioxygenases use an Fe(II) center for catalysis and an action mechanism similar to extradiol dioxygenases (Fetzner 2012).

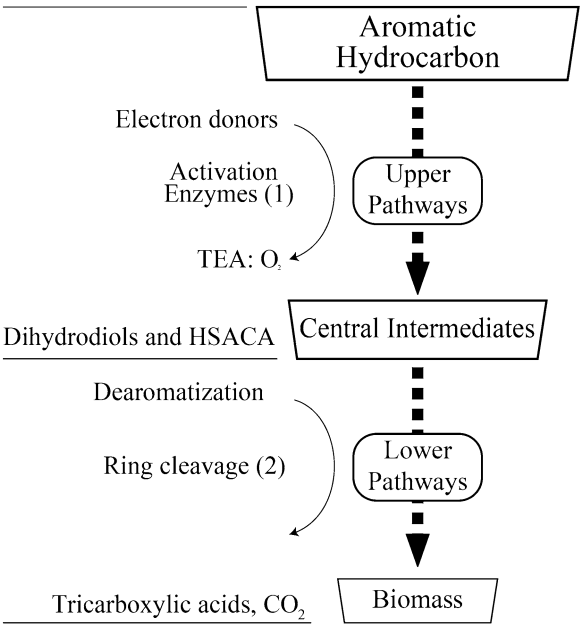

Fig. 5 Schematic representation of metabolic pathways of aromatic hydrocarbons by bacteria under aerobic conditions. Upper pathways are through hydrocarbon molecule to central intermediates. Lower pathways go from destabilization of central intermediates to biomass yield. Electron donors: NAD(P)H, $FMNH_2$ or $FADH_2$, ferredoxin, NADH. *TEA* terminal electron acceptor, Dihydrodiols = catechol, (chloro)catechol, (nitro)catechol, (chloro)hydroxyquinol, hydroquinone, hydroxyhydroquinone, (*1*) Mooxygenases or dioxygenases enzymes. HSACA (hydroxy-substituted aromatic carboxylic acids) = gentisate, protocatechuate, homogentisate, homoprotocatechuate, 2-hydroxy-1-naphtoic acid, salicylic acid, 3-hydroxyanthranilate

2.2 *Aromatic Hydrocarbon Biodegradation Under Anaerobic Conditions*

In the absence of oxygen, oxidized inorganic compounds such as nitrate (NO_3^{-1}), manganese (Mn(IV)), iron (Fe(III)), sulfate (SO_4^{-2})and carbon dioxide (CO_2) act as the terminal electron acceptors (TEAs) (Fuchs et al. 2011; Vogt et al. 2011). The methanogenic reduction also plays an important role in anaerobic biodegradation, particularly at sites that have been contaminated for longer periods of time where other TEAs have been depleted (Lovley 1997).

Most studies reported that, under anaerobic conditions, activation in the upper pathways is by a reduction catalyzed by synthases, dehydrogenases and carboxylases (Chakraborty and Coates 2005; Heider 2007; Meckenstock and Mouttaki 2011). However, Philipp and Schink (1998) reported evidence of two oxidative reactions as the activation reaction in anaerobic degradation of resorcinol.

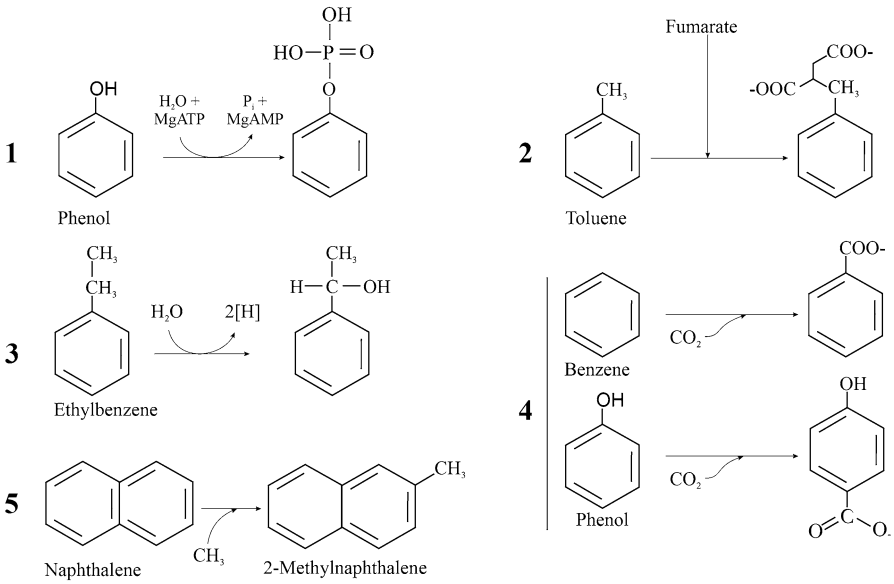

Fig. 6 The current five activation ways studied as the first step of upper pathways under anaerobic conditions. (*1*) Phosphorylation, (*2*) Fumarate insertion, (*3*) O_2-independente hydroxylation, (*4*) Carboxylation, (*5*) Methylation. Phenol can be phosphorylated or carboxylated

Currently, the following five ways of activation of aromatic hydrocarbons activation are being discussed: (1) *Phosphorylation*. Activation occurs by insertion of a phosphate group. In the case of phenol in *Tauera aromatica* catalysis occurs by a phenylphosphate synthase (Narmandakh et al. 2006; Schmeling et al. 2004). (2) *Fumarate insertion*. Alkylated aromatic hydrocarbons such as toluene, cresols and xylenes are activated by radical-based addition of a fumarate to the methyl group and, in the case of ethylbenzene onto the side chain (Heider 2007). (3) *O2-independent hydroxylation*. Vogel and Grbìc-Galìc (1986) using partially ^{18}O-labelled water in an enrichment culture in methanogenic incubations presented toluene and benzene hydroxylation indicating that the hydroxyl group originated from water. Johnson et al. (2001) showed that an ethylbenzene dehydrogenase in *Azoarcus* sp. strain EB1 catalyzes the insertion of one hydroxyl group onto ethylbenzene to produce (S)-(−)-1-phenylethanol (Fig. 6). (4) *Carboxylation*. Also called the "biological Kolbe-Schmitt reaction", the carboxylation reaction has been suggested for monoaromatic hydrocarbons and non-substituted PAHs as follows. The carboxylation of phenol in para-position yielding 4-hydroxybenzoate by strains K172 and S100 was proposed by Tschech and Fuchs (1987). Zhang and Young (1997) working with enrichment cultures with [^{13}C]bicarbonate and sulfate reducing conditions found 2-naphthoate and phenanthrene carboxylic acid indicating that naphthalene and phenanthrene carboxylation. (5) *Methylation*. Also called Friedel-Crafts-type methylation, it was reported by Safinowski and Meckenstock (2006) as the initial reaction in the anaerobic degradation of naphthalene by sulfate-reducing enrichment cultures. Mouttaki et al. (2012) reported the methylation of naphthalene as the first breakdown reaction by enrichment culture N47 in sulphate-reducing conditions.

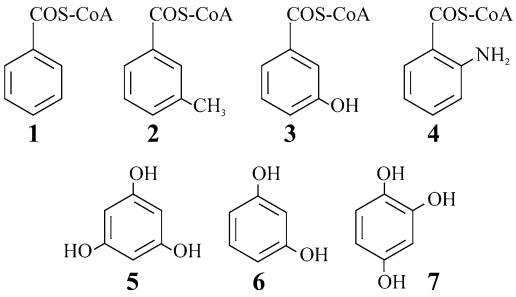

Fig. 7 Central intermediates of anaerobic biodegradation of aromatic hydrocarbons. (*1*) Benzoyl-CoA, (*2*) 3-methyl-benzoyl-CoA, (*3*) 3-hydroxy-benzoyl-CoA, (*4*) 2-amino-benzoyl-CoA, (*5*) Phloroglucinol, (*6*) Resorcinol, (*7*) Hydroxyhydroquinone

The upper pathways are characterized by different metabolic pathways with several central intermediates, here we review seven of them (Fig. 7). The most common is benzoyl-CoA with 2-amino, 3-hydroxy and 3-methyl derivatives (Boll and Fuchs 1995; Koch and Fuchs 1992; Laempe et al. 1999; Merkel et al. 1989; Philipp and Schink 2012). Resorcinol was reported by Boyd et al. (1983) in studies of degradation of several aromatic hydrocarbons. Phloroglucinol was reported by Brune et al. (1992) as the intermediate in the metabolism of pyrogallol by *Pelobacter massiliensis*. Hydroxyhydroquinone (HHQ) was reported in the nitrate-reducing bacterium *Azoarcus anaerobius* strain LuFRes1 from hydroxylation of resorcinol (Philipp and Schink 1998).

The lower pathways begin with dearomatization of the central intermediates and can be by reductive or oxidative reactions throughout as will be described below.

Dearomatization of *benzoyl-CoA* is catalyzed by benzoyl-CoA reductase that seems to follow a Birch-like mechanism to yield cyclohexadiene-carbonyl-CoA (1,5-dienoyl-CoA).

Two classes of benzoyl-CoA reductases (BCR) have been reported. Class I BCR is ATP-consuming and found in facultative microorganisms (Boll and Fuchs 1995; Egland et al. 1997) and class II BCR is found in strictly anaerobic microorganisms being ATP-independent (Holmes et al. 2012). Both classes of BCR produce 1,5-dienoyl-CoA. However, when class I BCR catalyzes, the reaction requires one molecule of the reduced protein ferredoxin as electron donor ($E^{\circ} = -420$ mV), two molecules of ATP and two molecules of water (Boll and Fuchs 1995). The electron donor for class II BCR is unknown (Holmes et al. 2012).

For the de-aromatization of resorcinol (1,3-dihydrobenzene), two different pathways have been reported. The first is a reductive reaction as reported by Tschech and Schink (1985) and by Kluge et al. (1990) in *Clostridium* sp. and *Campylobacter* sp. co-culture named KN245, catalyzed by a resorcinol reductase yielding 1,3 cyclohexanedione. The second one, is an oxidative reaction releasing hydroxyhydroquinone (HHQ) described for the nitrate reducing bacterium *Azoarcus anaerobius* strain LuFRes1 (Philipp and Schink 1998).

De-aromatization of phloroglucinol had been studied in detail with *Eubacterium oxidoreducens* and *Pellobacter acidigallici*. This compound is reduced to dihydro-phloroglucinol by an NADPH-dependent reductase (Armstrong and Patel 1994; Boll 2005; Brune and Schink 1992).

There are three metabolic pathways to de-aromatize Hydroxyhydroquinone (HHQ). The isomerization to phloroglucinol by the fermentative bacterium *Pellobacter massiliensis* (Brune et al. 1992), the reduction to dihydro-HHQ was observed with the sulfate reducing bacterium *Desulfovibrio inopinatus* (Philipp and Schink 2012) and an oxidative degradation to 2-hydroxy-1,4-benzoquinone by the nitrate-reducing bacterium *Azoarcus anaerobius* strain LuFRes1 (Philipp and Schink 1998).

The ring cleavage is a reductive step of the lower pathways. Benzoyl-CoA is transformed to 6-oxo-2-hydroxycyclo-hexane-1-carboxyl-CoA that still maintains the carbon ring. Fission of this ring is catalyzed by the 6-oxo-2-hydroxycyclo-hexane-1-carboxyl-CoA hydrolase using one molecule of water to produce 3-hydroxypimelyl-CoA (UM-BBD 2015).

Philipp and Schink (2012) reported that 1,3-cyclohexadione, a reductive intermediate of resorcinol, is hydrolytically cleaved to 5-oxocaproic acid that is breakdown to an acetate and a butyrate.

Dihydroxyphloroglucinol, originated from phloroglucinol de-aromatization, is cleaved hydrolytically yielding 3-hydroxy-5-oxohexanoic acid, which is then oxidized to triacetic acid (3,5-dioxohexanoic acid) (Brune and Schink 1992).

The first reaction for ring cleavage of HHQ is an oxidation catalyzed by a membrane-bound HHQ-dehydrogenase and then channeled to acetate, malate and succinate (Darley et al. 2007) (Fig. 8). Information about particular compounds undergoing ring cleavage was not found.

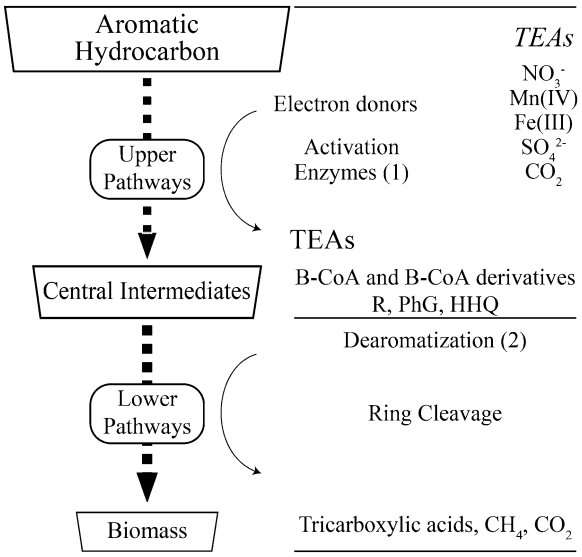

Fig. 8 Schematic representation of upper and lower pathways of aromatic hydrocarbon degradation under anaerobic conditions by bacteria. Electron donors: NAD(P)H, Ferredoxin. Enzymes (*1*): Synthases, Carboxylases, Dehydrogenases. B-Coa: Benzoyl-CoA; B-CoA derivatives are 2-amino benzoyl-CoA, 3-hydroxy benzoyl-CoA, 4-methyl benzoyl-CoA. *R* resorcinol, *PhG* phloroglucinol, *HHQ* hydroxyhydroquinone. dearomatization (*2*) is the initial step of lower pathway

3 Practical Applications of Knowledge About Metabolic Pathways

The knowledge about the metabolism of aromatic hydrocarbons could result in environmentally-friendly practical applications. The cleanup of pollutants by bioaugmentation (introducing bacteria from external sites), and/or biostimulation (providing nutrients or electron acceptors stimulating native populations) in natural or enhanced conditions are the best-known techniques (Prince 2010).

The 1,2- and 1,3-cyclohexanedione, derivatives of the metabolism of resorcinol are cyclic b-triketones able to inactivate toxin A (enterotoxin) and toxin B (cytotoxin) of *C. difficile* with a promising use in medicine (Balfanz and Rautenberg 1989). Their food, antibiotic, antimalarial, antidiabetic and anticancer therapeutical properties have also been reviewed (Blanco et al. 2003).

Muconic acid obtained from the ortho-cleavage of catechol has potential applications in the production of resins, bio-plastics, food additives, agrochemicals and pharmaceuticals (Xie et al. 2014). Lin et al. (2014) devised a novel artificial pathway for the efficient production of muconic acid based on the salicylic acid degradation pathway.

4 Summary

The aim of this review was to build an updated collection of information focused on the mechanisms and elements involved in metabolic pathways of aromatic hydrocarbons by bacteria. Enzymes as an expression of the genetic load and the type of electron acceptor available, as an environmental factor, were highlighted. In general, the review showed that both aerobic routes and anaerobic routes for the degradation of aromatic hydrocarbons are divided into two pathways. The first, named the upper pathways, entails the route from the original compound to central intermediate compounds still containing the aromatic ring but with the benzene nucleus chemically destabilized. The second, named the lower pathway, begins with ring de-aromatization and subsequent cleavage, resulting in metabolites that can be used by bacteria in the production of biomass. Under anaerobic conditions the five mechanisms of activation of the benzene ring described show the diversity of chemical reactions that can take place. Obtaining carbon and energy from an aromatic hydrocarbon molecule is a process that exhibits the high complexity level of the metabolic apparatus of anaerobic microorganisms. The ability of these bacteria to express enzymes that catalyze reactions, known only in non-biological conditions, using final electron acceptors with a low redox potential, is a most interesting topic. The discovery of phylogenetic and functional characteristics of cultivable and non-cultivable hydrocarbon degrading bacteria has been made possible by improvements in molecular research techniques such as SIP (stable isotope probing) tracing the incorporation of ^{13}C, ^{15}N and ^{18}O into nucleic acids and proteins.

Since many metabolic pathways in which enzyme and metabolite participants are still unknown, much new research is required. Therefore, it will surely allow enhancing the known and future applications in practice.

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A Review and Assessment of Spent Lead Ammunition and Its Exposure and Effects to Scavenging Birds in the United States

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1 Introduction

Lead is a naturally occurring and highly toxic element that has no known biological function. Lead can affect all body systems due to its ability to compete with calcium for binding sites, disrupting calcium-mediated functions such as cell to cell communication, cell division and communication, and organization of the cytoskeleton (Goyer and Clarkson 2001). Lead poisoning has been documented in humans for at least 2500 years and in waterfowl from spent lead shot for over 100 years (Grinnell 1894; Hough 1894; Eisler 2000). In that time, the ecotoxicological properties of lead have been extensively reviewed (Eisler 2000). Today lead is primarily used in the manufacture of storage batteries, alloys, pigments and chemicals, and in ammunition.

Wildlife can be exposed to lead from numerous sources, including mining and smelter emissions, lead-based paint, lead fishing sinkers, and spent ammunition. Incidental mortality from waterfowl hunting reached population-level effects when over two million ducks and geese (~2 % of all waterfowl) were poisoned annually by ingestion of spent lead shot deposited in sediments (Bellrose 1951). Later, effects to bald eagles (*Haliaeetus leucocephalus*) preying upon lead exposed waterfowl were documented (Griffin et al. 1980; Pattee and Hennes 1983). To alleviate this problem, between 1986 and 1991 the United States Fish and Wildlife Service (USFWS) phased-in a nationwide restriction on the use of lead shot for hunting waterfowl and American coots (*Fulica Americana*; USFWS 1986, 1995). While this ban resulted in a reduction of exposure in waterfowl (Anderson et al. 2000), lead shot and rifle bullets are still widely used for hunting upland birds and large and small game animals. Therefore, lead poisoning from the ingestion of ammunition and fragments persists in some groups of avian species. In addition to waterfowl, lead exposure and poisoning has been reported in a variety of avian species in the United States, including those protected under the Migratory Bird Treaty Act, the

Endangered Species Act, and the Bald and Golden Eagle Protection Act (Bellrose 1959; Pain et al. 2009). This review focuses specifically on scavenging avian species exposed to spent lead via foraging habits.

2 Objectives and Scope

2.1 Objectives

There are three objectives of this review:

1. To ascertain the contribution of ammunition as a source of lead exposure and its contribution to effects, including mortality, to scavenging birds.
2. To examine whether there are viable pathways of exposure for scavenging birds to other environmental sources of lead.
3. To assess the toxicity of other metals that can be used in ammunition to birds.

For this review, the specific focus is a comprehensive evaluation of the scientific evidence regarding lead exposure and poisoning of scavenging birds from ammunition and other environmental sources. Throughout this review, except where otherwise noted, "poisoning" and "toxicosis" are defined as manifestations of adverse effects that can be observed or measured, up to and including death. The regulation of lead ammunition is discussed to provide the evidence that ultimately resulted in the current ban for waterfowl hunting in the United States and to describe the allowable uses of lead ammunition that remain. Several recent reviews have also examined the effect of environmental lead to birds, but incorporate additional topics such as fishing tackle, upland game birds and other classes of vertebrates, human health, research directions, suggested regulatory pathways, and exposure to wildlife in other countries (Fisher et al. 2006; Rattner et al. 2008; Johnson et al. 2013; Haig et al. 2014).

2.2 Geographic Scope

Exposure to lead ammunition is not unique to scavenging bird species found within the United States; the issue has been documented worldwide (e.g., *Germany*: Nadjafzadeh et al. 2013; *Poland*: Komosa and Kitowski 2008; *South Korea*: Nam and Lee 2009; *Spain*: Mateo et al. 2001; Fernandez et al. 2011; *Sweden*: Helander et al. 2009; *United Kingdom*: Knott et al. 2010). However, the geographic scope of this review is limited to the United States. Whereas much of the information reviewed regarding toxicological information can be applied broadly, narrowing the scope to the United States allows for a more detailed review of the availability of ammunition and other sources of lead in the environment. Some of the species

considered herein migrate across borders, such as the bald eagle into Canada. The regulation of lead shot in Canada differs in that it was banned for hunting most migratory bird game species in 1999 and is currently prohibited for all hunting in National Wildlife Areas (reviewed in Scheuhammer and Thomas 2011). Numerous studies have examined lead exposure of scavenging birds in Canada (e.g., Langelier et al. 1991; Elliott et al. 1992; Miller et al. 1998, 2000, 2001a, b; Wayland et al. 1999, 2003). Where examples from outside the United States can be used to enhance a discussion, they have been included, but are not meant to be comprehensive.

2.3 Taxonomic Scope

For this report, scavenging birds are defined as those which feed on carrion exclusively or almost exclusively (obligate scavengers), or in combination with live prey or other food items. The California condor (*Gymnogyps californianus*), black vulture (*Coragyps atratus*), and turkey vulture (*Cathartes aura*) comprise the obligate scavengers found in the United States. Birds that regularly scavenge on carrion in addition to other dietary items include the bald and golden (*Aquila chrysaetos*) eagle, crows and ravens (*Corvus* spp.), and the Audubon's crested caracara (*Polyborus plancus audubonii*) which has a limited range in the United States. Many other avian species, including *Accipiter* and *Buteo* hawks, may scavenge carrion opportunistically. Obligate and regular scavengers are the focus of this review due to the tendency of these birds to be exposed to and affected by lead, as inferred from the greater abundance of documented poisoning cases. California condors and bald eagles are disproportionately represented in some sections as they are particularly well studied relative to other scavengers and may be more apt to be collected due to their large size, conspicuous plumage, and special protection status. Where literature concerning other species is available, including those which feed only opportunistically on carrion, it is described and incorporated into the analysis.

3 Toxicological Effects of Lead in Birds

Lead has no known beneficial role in biological systems and its adverse effects have been detected in birds for over a century. In North America, mortality due to lead poisoning from the ingestion of lead shot was first reported in waterfowl in 1894 in Texas and North Carolina, and by the 1950s an estimated 2–3 % (1.6–2.4 million) of waterfowl across all North American flyways were dying annually of lead shot poisoning (Grinnell 1894; Hough 1894; Bellrose 1959; Anderson et al. 2000). The early accounts by Grinnell (1894) and Hough (1894) include the first descriptions of gross toxicological effects of lead poisoning in wild birds in the United States. Wetmore (1919) reviewed clinical signs and lesions of lead poisoning in waterfowl and reported the results of an experimental study of lead shot poisoning in ducks.

The study showed that mortality varied in mallards (*Anas platyrhynchos*) dosed with one to three #6 lead shot, but six #6 shot were always fatal. Similar findings were noted in northern pintails (*Anas acuta*) and redheads (*Aythya americana*) (Wetmore 1919). Shillinger and Cottam (1937) reported that the frequency of lead shot detection in several thousand gizzards from various species of ducks ranged from 1 to 39 %, depending on the species, and suggested that lead poisoning may be an important factor in the decline of waterfowl populations. In an early study of a variety of waterbird species, lead poisoning was the third largest cause of mortality noted in 3000 carcasses, and the authors frequently observed poikilocytosis (abnormal shape), anisocytosis (unequal size), and reduced hemoglobin content of red blood cells (Quortrup and Shillinger 1941). These early studies laid the groundwork for a more comprehensive investigation into the toxicological effects of lead in birds.

3.1 Physiological Effects

When metallic lead is ingested by birds, the stomach's acid and grinding action in species with a muscular gizzard begin to dissolve it, resulting in the formation of toxic lead salts. As these salts are absorbed in the intestinal tract, lead enters the bloodstream and measurable increases in blood lead concentrations occur within hours (Roscoe et al. 1979). The first measurable physiological effect of lead exposure is the inhibition of delta-aminolevulinic acid dehydratase (ALAD), an enzyme necessary for hemoglobin synthesis and a very sensitive indicator of lead exposure in birds (Finley et al. 1976). Birds can tolerate considerable reductions in ALAD activity without adverse hematological effects, but ALAD depression by high levels of lead results in anemia characterized by lowered hemoglobin and hematocrit (Franson et al. 1983; Pain and Rattner 1988). Lead also inhibits ferrochelatase (heme synthetase), an enzyme responsible for combining ferrous iron and protoporphyrin IX (PPIX) to form heme. Lead shot dosing studies with mallards and canvasbacks (*Aythya valisineria*) have shown that blood lead and PPIX concentrations may remain elevated, and ALAD activity may remain depressed, for several weeks to as long as 3 months (Finley and Dieter 1978; Roscoe et al. 1979; Franson et al. 1986). Inhibition of ferrochelatase results in the accumulation of PPIX in the erythrocytes, and its quantification in blood samples has been used as an indicator of lead exposure in birds (Roscoe et al. 1979; Franson et al. 1996).

Lead competes with calcium for binding sites within the body, and can sometimes bind with greater affinity than calcium. This disruption in calcium metabolism can result in neurologic and neuromuscular effects via induction or inhibition of neurotransmitter release, alteration of channels or pumps, and interference with protein kinases (Peraza et al. 1998). Learning and behavioral deficits have been linked to these changes in intra- and extra-cellular signaling (Bressler and Goldstein 1991). Neurotoxic effects, including those on learning and memory, have also been observed in birds. In a series of laboratory experiments with young common terns

(*Sterna hirundo*) and herring gulls (*Larus argentatus*), lead was found to exact behavioral changes on a number of parameters relevant to a chick's survival in the wild (e.g., locomotion, begging behavior, individual recognition, balance, depth perception, behavioral thermoregulation; Burger and Gochfeld 2000). When these tests were repeated in the field, lead-injected chicks showed similar behavioral deficits and had a higher susceptibility to predation (Burger and Gochfeld 2000). High doses of lead can also disrupt the blood–brain barrier in immature animals allowing the entrance of molecules, water, and ions otherwise excluded, leading to cephalic edema, a condition observed in lead poisoned geese (Locke and Thomas 1996).

Other endpoints that may be affected by lead exposure include growth, body and organ mass, feeding activity, reproduction, and immune function. For example, common terns and herring gulls exposed to lead showed a variety of abnormalities, including decreased growth, fledging size, locomotion and balance ability, and decreased feeding activity (Burger et al. 1994). Reduced brain weight has been associated with lead exposure in young mallards, American kestrels (*Falco sparverius*), and European starlings (*Sturnus vulgaris*; Hoffman et al. 1985; Grue et al. 1986; Douglas-Stroebel et al. 2004). Mixed results have been reported in studies of lead effects on immune response. Antibody-mediated immunity was suppressed in Japanese quail (*Coturnix coturnix*) and mallards exposed to lead at levels resulting in other clinical signs (Trust et al. 1990; Rocke and Samuel 1991; Grasman and Scanlon 1995). However, lead ingestion at levels that impaired growth and hematology in Japanese quail did not affect humoral immune response (Morgan et al. 1975). No evidence of immunotoxicity was reported with low-level exposure to lead in Japanese quail and red-tailed hawks (*Buteo jamaicensis*) (Redig et al. 1991; Nain and Smits 2011). Effects of lead exposure on reproduction include .lower fertilization rate in ring-necked pheasants (*Phasianus colchicus*), lower egg production in Japanese quail, reduced hatchability in ring-necked pheasants and mourning doves, and smaller clutches and increased nestling mortality in pied flycatcher (*Ficedula hypoleuca*) (Edens et al. 1976; Buerger et al. 1986; Berglund et al. 2010; Gasparik et al. 2012). Testicular changes have been noted in chickens (*Gallus gallus domesticus*), Japanese quail, and ringed turtle-doves (*Streptopelia risoria*) (Morgan et al. 1975; Veit et al. 1983; Mazliah et al. 1989). .Decreased mineralization in bones with increased concentrations of lead has been reported in Egyptian vultures (*Neophron percnopterus*) and red-legged partridges (*Alectoris rufa*; Gangoso et al. 2009; Álvarez-Lloret et al. 2014). Bellrose (1959) reported that mallards dosed with lead shot and released were 1.5 times more vulnerable to being shot by hunters than controls.

While some sublethal effects alter health directly, others may render birds more susceptible to causes of mortality such as predation, hunting mortality, collisions with objects, and illness or death from disease. However, it is often difficult to establish a definitive relationship between the detection of elevated concentrations of a contaminant and proximate cause of death. Hunt (2012) argues that population impacts to avian scavengers are likely underestimated, in part due to the difficulty in detecting the health manifestations of sublethal lead exposure. In cases suggestive of lead as a contributing factor, elevated concentrations have been associated

with avian mortality from other causes such as collisions with power lines, cables, or other objects (O'Halloran et al. 1989; Kelly and Kelly 2005; Helander et al. 2009). Elevated lead concentrations were also detected through routine or retroactive screening in a portion of eagles admitted to rehabilitation centers for trauma (Kramer and Redig 1997; Neumann 2009; Nam et al. 2011). However, none of the 1733 bald and 491 golden eagles diagnosed as having succumbed from causes other than lead poisoning (e.g., collision, trauma, electrocution, emaciation, infectious disease) by the National Wildlife Health Center from 1975 to 2013 contained concentrations of lead above background (>1 ppm wet weight in liver; Franson and Russell 2014).

3.2 Clinical Signs of Lead Poisoning

In addition to changes in ALAD and PPIX, signs of lead poisoning vary somewhat among species groups, and include submandibular edema, lethargy, wing droop, ataxia, anorexia, green bile staining of the vent, leg paralysis, and convulsions (Fig. 1; Locke and Thomas 1996; Rattner et al. 2008; Franson and Pain 2011). In a study of lead acetate poisoning in six captive avian species, the most consistent clinical signs across all taxa were weight loss, anemia, and increased concentrations of PPIX (Beyer 1988). Bald eagles dosed with lead shot lost weight and had

Fig. 1 Bald eagle (*Haliaeetus leucocephalus*) at a rehabilitation center displaying clinical signs of lead poisoning, including wing droop and lethargy. Photo courtesy of Kay Neumann, Saving Our Avian Resources, 25494 320th Str, Dedham, IA 51440

reduced hematocrit, hemoglobin, and ALAD activity, as well as changes in serum biochemistries (Hoffman et al. 1981; Pattee et al. 1981).

Birds that die within a few days from an acute exposure to a large concentration or dose of lead may be in good flesh. However, lead poisoning is typically a chronic condition resulting in anorexia, loss of fat reserves, muscle wasting, and debilitation (Locke and Thomas 1996). Time to death in experimental studies varies by species and dosage regimen, with waterfowl generally succumbing within 2–4 weeks, although some raptors survived for more than 15 weeks (Barrett and Karstad 1971; Pattee et al. 1981, 2006; Franson et al. 1986; Beyer et al. 1998). Other gross lesions include impaction of the esophagus, proventriculus, and ventriculus with food (particularly in waterfowl), bile staining of the ventriculus and intestinal contents, distension of the gall bladder with dark green viscous bile, necrosis evidenced by light streaks on the surface of the heart or the cut surface of the gizzard muscle, pale and atrophied internal organs, and flabby heart (Locke and Thomas 1996; Rattner et al. 2008; Franson and Pain 2011; Franson and Russell 2014). In a study of 421 lead-poisoned waterfowl of various species, the most reliable gross indications of lead poisoning were reported to be impactions of the alimentary tract, submandibular edema, necrosis of heart muscle, and bile staining of the liver (Beyer et al. 1998).

Locke et al. (1966) were the first to report inclusion bodies in histologic sections of kidney tissue of lead poisoned birds, in that case mallards. These structures occur within the nuclei of cells in the proximal convoluted tubules of the kidney, and when stained with the Ziehl-Neelson acid-fast technique appear scarlet in color. Other gross and microscopic lesions of lead poisoning are nonspecific and may be observed in association with other conditions, but only lead exposure is known to produce acid-fast intranuclear inclusion bodies in the kidneys of birds. Renal inclusions have been reported in several other species of birds poisoned by lead, including mourning doves (*Zenaida macroura*), rock doves (*Columba livia*), mute swans (*Cygnus olor*), whooper swans (*Cygnus cygnus*), Andean condors (*Vultur gryphus*), turkey vultures, bald eagles, golden eagles, and white-tailed eagle (*Haliaeetus albicilla*; Locke and Bagley 1967; Simpson et al. 1979; DeMent et al. 1987; Ochiai et al. 1992; Kenntner et al. 2001; Carpenter et al. 2003; Pattee et al. 2006; Franson and Russell 2014). However, even though the inclusion bodies are indicative of lead poisoning, they are not present in all cases. Renal inclusions occurred in 64 % of lead poisoned red-winged blackbirds (*Agelaius phoeniceus*), 69 % of brown-headed cowbirds (*Molothrus ater*), 75 % of mallards, 86 % of northern bobwhites (*Colinus virginianus*), and 100 % of common grackles (*Quiscalus quiscula*) and eastern screech-owls (*Otus asio*) that died from lead acetate poisoning (Beyer 1988) and have been infrequently reported in poisoned Canada geese (*Branta Canadensis;* Bagley et al. 1967; Locke et al. 1967; Barrett and Karstad 1971; Sileo et al. 2001). No inclusion bodies were found in bald eagles experimentally dosed with lead, nor observed in 17 lead poisoned eagles (13 bald and 4 golden) submitted to the National Wildlife Health Center (Pattee et al. 1981; Franson and Russell 2014). Additional histopathologic lesions noted in lead poisoned birds include hepatic hemosiderosis, renal tubular cell degeneration, myocardial and gizzard muscle necrosis, fibrinoid necrosis of arterioles, erythroid hyperplasia, encephalopathy, and peripheral neuropathy (Locke and Thomas 1996; Wobeser 1997; Franson and Pain 2011).

3.3 Tissue Distribution and Thresholds for Toxicosis

Lead is distributed throughout the body, including growing feathers, via the circulatory system and a dynamic equilibrium controls deposition and removal in various tissues. Franson and Pain (2011) reviewed the distribution of lead in avian tissues and factors influencing the concentrations of lead in tissues. In general, the highest concentrations are found in bone, liver, and kidney, with intermediate concentrations in brain and blood, and low concentrations in muscle. In birds that survive lead exposure, concentrations in soft tissues will decline over time. Because lead is released from bone far more slowly than from soft tissues, bone functions as a long term repository. Bone lead concentrations may also differ between males and females and among females, depending on season. Finley and Dieter (1978) reported that lead concentrations in femurs of laying mallards were four times higher than in nonlaying females. As calcium is utilized for eggshell formation, intestinal absorption of calcium increases, as well as lead (Krementz and Ankney 1995; Scheuhammer 1996).

Concentrations of lead in blood of live birds and in liver and kidney of dead birds are the tissues commonly used to assess exposure. Concentrations in bone are not a good reflection of recent lead exposure and remain difficult to interpret because of continual accumulation and slow release. Lead concentrations in birds with no history of lead exposure are typically <0.2 ppm wet weight in blood (all blood lead reported herein is wet weight), <2 ppm wet weight in liver and kidney, and <10 ppm dry weight in bone (Table 1; Franson and Pain 2011). Some bird species appear to be more resistant to lead intoxication than others, as indicated by higher lead concentrations in tissues reported in association with lead poisoning. However, suggested guidelines are that lead concentrations >0.2 ppm in blood or >2 ppm wet weight in liver and kidney are evidence of subclinical poisoning, and >0.5 ppm in blood or >6 ppm wet weight in liver and kidney are evidence of clinical poisoning (Table 1; Franson and Pain 2011). Birds with subclinical poisoning are expected to experience physiological effects that are unlikely to severely impair normal biological function and would be likely to recover if lead exposure ceased. Clinical poisoning would likely be accompanied by signs such as anemia, weight loss, and muscular incoordination, and could result in death if lead exposure continued. When monitoring

Table 1 Thresholds for lead toxicosis

Tissue	Background	Subclinical	Clinical poisoning
Blood (ppm, wet weight)	<0.2	0.2–0.5	>0.5
Liver (ppm, wet weight)	<2	2–6	>6
Kidney (ppm, wet weight)	<2	2–6	>6

Note that all tissue concentrations herein have been converted from their original unit to parts per million (ppm) for ease of comparison across studies and against diagnostic thresholds. For measurements taken for blood, the margin of error can be up to 4 % as a result of converting a volumetric measurement to standard SI units. By the nature of the tissue, all blood lead reported is wet weight. Thresholds from Franson and Pain (2011)

reveals elevated blood lead concentrations in live birds, individuals may be given chelation therapy, a treatment used to remove heavy metals. The chelating agent EDTA (ethylenediaminetetraacetic acid) binds lead from soft tissue and bone for excretion (Goyer and Clarkson 2001). Tissues of birds that have undergone recent chelation therapy are likely to have lower concentrations of lead than prior to treatment (e.g. California condor; Rideout et al. 2012), and therefore may not be suitable for diagnostic purposes.

The use of feathers to monitor lead levels can provide a simple, non-invasive method to determine exposure in birds. Although many investigators have measured concentrations of lead in this manner, there have been a limited number of controlled exposure studies to validate this approach and aid in the interpretation of such field data (Kendall and Scanlon 1981; Burger and Gochfeld 1990; Dauwe et al. 2002; Golden et al. 2003). These investigations have shown that under certain conditions feathers can be a reliable indicator of dietary exposure to lead that is associated with lead accumulation in internal organs and biochemical measures of effect. Golden et al. (2003) used ratios of lead concentrations found in these studies as well as those in juvenile birds collected from the field to calculate feather to tissue ratios for nestling or juvenile birds (1:2 for liver, 1:5 for kidney, 1:10 for bone). These ratios, however, may vary based on the specific type of tissue analyzed (e.g., femur versus tibia, primary versus body feather) and in situations of very low or high lead exposure. Golden et al. (2003) also cautioned that adult feathers appear to be a less reliable indicator of endogenous lead exposure, primarily due to complications from external deposition of lead. Feather parts openly exposed to the environment have been found to have higher concentrations of lead than those covered by other feathers and a lack of correlation with concentrations of lead in internal organs (Goede and de Voogt 1985; Goede and de Bruin 1986; Hahn et al. 1993). Rinsing of feathers does not necessarily remove external contamination (Weyers et al. 1988). Dauwe et al. (2002) found evidence that feathers may be also subject to exogenous contamination of lead by excretion from the uropygial gland.

The measurement of lead concentrations in egg content is not known to be particularly useful for monitoring lead exposure in birds. A laboratory experiment with Japanese quail showed that although some lead is transferred to eggs, concentrations were much lower than in the diet (Leonzio and Massi 1989). Field studies with common eiders (*Somateria mollissima*) failed to detect correlations between lead concentrations in eggs and concentrations in feathers or blood of laying females (Grand et al. 2002; Burger et al. 2008). Lead concentrations in eggshells have been measured in several species (Burger 1994; Mora 2003; Dauwe et al. 2005) and may be a suitable indicator of lead contamination (Dauwe et al. 1999).

Wildlife suspected to have died of lead poisoning should be subjected to a complete necropsy to the extent feasible for the condition of the carcass. Although interpretive guidelines are available, lead poisoning as a cause of death should not be distinguished from simple lead exposure based solely on tissue residues. Ideally, a determination of lead poisoning as cause of mortality should be based on an evaluation of field circumstances, observed clinical signs, gross lesions and pathological findings, tissue residues, and when possible, laboratory testing to rule out other

contaminants and infectious or parasitic diseases. However, since birds are generally collected opportunistically and often after death has occurred, observations of clinical signs and comprehensive necropsy findings may not be available due to carcass condition. In these cases, a more conservative diagnostic approach may be warranted. In a study of lead poisoned waterfowl, Beyer et al. (1998) reported that 95 % of fatalities had liver lead concentrations of at least 38 ppm dry weight (10 ppm wet weight), but fewer than 1 % of birds that died of other causes had a concentration that high. The authors concluded that 38 ppm dry weight lead in liver is a defensible criterion for identifying lead poisoning in waterfowl in the absence of pathologic observations.

Key Points: Toxicological Effects of Lead on Birds

- Lead toxicity in birds has been studied in a variety of species and is relatively well understood.
- The progression of lead poisoning can ultimately result in mortality.
- Sublethal effects of lead may render a bird more susceptible to mortality from other causes.
- Lead exposure results in numerous physiological responses that are measurable and specific for diagnostic purposes, but may vary among individuals and species.
- General thresholds have been established in birds to help diagnose lead exposure and poisoning. However, tissue residues can vary and should be interpreted in conjunction with other diagnostic signs, when available.

4 Spent Lead Ammunition in the Environment

4.1 Regulation of Lead Ammunition

Studies conducted in the 1960s further confirmed the toxicity of lead to waterfowl and determined clinical signs such as impairments to normal biological functions, severe weight loss, and mortality (Bellrose 1965; Irby et al. 1967; Locke et al. 1967). Irby et al. (1967) and Locke et al. (1967) examined the effects of lead on mallards dosed with three types of commonly used shot: lead, plastic-coated lead, and lead-magnesium. The study found mortality to 96 % of the mallards dosed with lead, 93 % dosed with plastic-coated shot, and 58 % dosed with lead-magnesium (Irby et al. 1967). The lead-dosed mallards developed anemia, atrophy of adipose and liver tissue, and enlarged gall bladders distended with bile, as well as cellular effects such as hemosiderosis and destruction of the kidney tubule cells, acid-fast intranuclear inclusion bodies, and enlarged nuclei in the cells of the proximal convoluted tubules (Locke et al. 1967). These early studies provided the initial data on the susceptibility of waterfowl to lead poisoning that led to a progression of further studies.

In the early 1980s, an association between bald eagle deaths and lead shot used in waterfowl hunting became apparent. At that time, the bald eagle was listed under the Endangered Species Act. A number of cases determined that bald eagles were exposed to lead by feeding on crippled or non-retrieved hunter-shot waterfowl containing ingested or embedded shot (Griffin et al. 1980; Pattee and Hennes 1983). Between 1967 and 1982, an estimated 7 % of the bald eagle population in the United States was lead poisoned (Pattee and Hennes 1983). In 1986, the USFWS required lead shot in hunting waterfowl and American coots to be phased-out over a 5-year timeframe initiated during the 1987–1988 hunting season (USFWS 1986, 1995). The regulation became a nationwide restriction in 1991 and the USFWS started developing an approval process for nontoxic shot. The USFWS defined that word "non-toxic" as shot that does not cause sickness and death when ingested by migratory birds, and developed testing guidelines to develop and approve new types of shot (USFWS 1997, 2013a, b). More information regarding these testing protocols and the outcome of their use is provided in Sect. 9 of this review.

4.2 Sources of Spent Lead Ammunition Remaining After the Ban

The effectiveness of the 1991 regulations that banned lead for waterfowl hunting in the United States has been evaluated by comparing lead shot ingestion rates before and after the ban. Five years after the ban, a study of 16,651 mallards along the Mississippi Flyway estimated that mortality from lead shot exposure declined 64 %, equating to 1.4 million of 90 million ducks in the 1997 fall flight spared from lead poisoning (Anderson et al. 2000). Although the study indicated a similar overall rate of shot ingestion for these birds (8.9 % post-ban versus 8 % pre-ban; Bellrose 1959; Sanderson and Bellrose 1986), 2/3 of shot ingested were nontoxic. Samuel et al. (1992) and Samuel and Bowers (2000) investigated the effectiveness by studying blood lead concentrations in American black ducks (*Anas rubripes*) in Tennessee before (1986–1988) and after (1997–1999) the ban. Using blood lead concentrations of ≥0.2 ppm as an indication of lead exposure, exposure rates were 11.7 % pre-ban and 6.5 % post-ban in adult black ducks. No difference in the exposure rate was detected for juveniles, with differences in food habits or habitat use theorized as a cause. In mottled ducks (*Anas fulvigula*) collected in Texas during the 1987–2002 hunting seasons, lead shot ingestion rates were 7 % along the upper coast and 18 % in those from the central coast (Merendino et al. 2013). This contrasts with an ingestion rate of about 32 % in mottled ducks collected along the gulf coast of Texas from 1973 to 1975, before non-toxic regulations were implemented in the early 1980s (Moulton et al. 1988). A study in Canada reported that bone lead concentrations in ducks declined by about 50 % after the Canadian ban on lead shot for waterfowl hunting (Stevenson et al. 2005).

These studies demonstrate that the lead shot ban was effective at reducing, but not eliminating, lead exposure in waterfowl and birds that prey upon waterfowl.

Lead shot discharged prior to the ban remains on the landscape and is available to be ingested by waterfowl and wading birds unless removed from the environment. Strom et al. (2009) documented lead exposure in trumpeter swans (*Cygnus buccinator*) in the upper Midwest after the lead shot ban. Twenty-five percent of swan mortality from 1991 to 2007 was attributed to lead toxicosis and 39 % of the lead poisoned swans had lead artifacts in their ventriculus at the time of necropsy. The authors suggest that the foraging habitats of swans (digging up sediments to find food) may predispose these species to lead exposure. Bald eagles submitted to the National Wildlife Health Center and diagnosed with lead poisoning significantly increased in all four migratory bird flyways in the United States (Atlantic, Pacific, Central, and Mississippi) after the autumn 1991 ban on the use of lead shot for waterfowl hunting (Russell and Franson 2014). The authors concluded that lead ammunition for use on game other than waterfowl versus the impacts of lead on wildlife populations needs further evaluation.

In addition to the lead remaining from waterfowl hunting, lead ammunition continues to enter the environment from other types of hunting and shooting activities in the United States. Deer hunting is the most popular type of hunting in the United States, with 10.1 million participants nationwide in 2006 (USFWS 2006). At least 3.8 million deer were harvested by resident hunters and 0.7 million deer harvested by non-resident hunters (USFWS 2006). It is common practice to field dress game and discard the internal organs and tissues on the landscape, leaving a lighter carcass to transport out of the field. As a result, each harvested deer may result in an offal pile that may be accessed by scavenging birds and other wildlife. In addition, studies have revealed that a significant number of deer are shot but not retrieved by hunters. Though exact wounding rates are difficult to enumerate and are likely to vary across regions, attempts at estimations have produced similar results: 21–24 % in Illinois, 24 % in Montana, and 17–32 % in Indiana (Stormer et al. 1979; Dusek et al. 1989; Nixon et al. 2001). Other types of hunting also generate substantial participation. Small game such as rabbit, squirrel, pheasant, and quail comprised the next largest group after deer with about 7.5 million hunters followed by turkey (2.6 million) and dove (1.2 million) hunters. Waterfowl hunting accounts for 1.8 million hunters, though no longer contributes to new lead in the environment. Hunting for varmint (coyote, raccoon, fox) and other game (e.g., upland birds and mammals) also takes place and the extent varies by region (USFWS 2006). Poaching of deer and other game species may add to these estimates of wounded prey, carcasses, offal piles, and lead ammunition in the environment, though the magnitude of illegal hunting activity is difficult to evaluate.

4.3 Fragmentation of Ammunition

Modern firearms used for hunting in the United States discharge projectiles of various size and shapes, such as clusters of shot, rifle bullets, and shotgun slugs (Thomas and Guitart 2013). Bullets for hunting are designed to transfer energy from

Fig. 2 The spent remains of the copper jacket and fragmented lead core of a lead-based bullet (*left*) compared with a copper (lead-free) expanding bullet that remained intact. Fragmentation of spent lead ammunition, copper jacket with lead core and pure copper. Photo courtesy of Institute for Wildlife Studies, P.O. Box 1137 Tres Pinos, California 95075

the projectile to the target to quickly maximize power and humanely kill game. This is accomplished by the projectile expanding in diameter when it strikes the animal. For bullets made of frangible metals, like lead, the availability from carcasses and offal left by hunters is greatly enhanced by the tendency to break into small pieces and disperse within a carcass. Several studies have documented that lead-containing bullets fragment and radiate a considerable distance in target animals upon impact. This property makes bullet fragments easily ingested, difficult to avoid when consuming contaminated tissue, and potentially available to multiple scavengers. Fragmentation can also increase the surface area of the ingested material for digestion by stomach acids. Copper bullets, designed to expand into 4–6 frontal petals (or "mushroom"), exhibit much less frangibility and tend to remain intact (as described below; Fig. 2); thus they are less likely to be incidentally consumed than shot or bullet fragments. There exists at least one case of an intact bullet retrieved from an historical California condor nest-site, though evidence suggests that this bullet was fired into the nest rather than having been incidentally ingested by a foraging bird (Snyder et al. 1986).

Fragmentation in Large Game

Several experimental studies of bullet fragmentation in large game, and examination of carcasses and offal piles showed similar patterns of bullet fragment numbers, size, and radiation from the wound site.

Hunt et al. (2006) collected whole or partial remains of white-tailed and mule deer (*Odocoileus virginianus* and *O. hemionus*) killed by hunters with centerfire, breach-loading rifles in California and Wyoming between 2002 and 2004. Thirty-four were killed using copper-jacketed bullets with lead cores, and four with monolithic copper expanding bullets. Local veterinarians radiographed areas of bullet transition, and fragments were counted manually; the presence of metal was verified by dissection in one sample. No attempt was made to distinguish between. lead and copper fragments. All whole or eviscerated deer killed .with lead-containing bullets (N = 24) contained fragments (38–738), with over 100 fragments counted in 74 % of samples. Fragment samples ranged in size about 0.5 to >5 mm, and clusters radiated as far as 15 cm from the wound channel. Ninety percent of offal piles (N = 20) contained fragments with counts ranging from 2 to 521. For deer shot with copper bullets, only six fragments in total were found in four whole carcasses, and one fragment in four offal piles. Warner et al. (2014) also observed lead fragmentation in hunter-killed white-tailed deer shot with different firearm types (12 and 20 G shotgun, muzzleloader rifle). Of 25 offal piles examined by radiography, 36 % contained lead fragments, ranging from 1 to 107 per pile (Fig. 3).

Knott et al. (2010) radiographed carcasses and abdominal viscera (stomach, spleen and intestines) of ten red deer (*Cervus elaphus*) and two roe deer (*Capreolus capreolus*) killed by a single shot to the thorax with copper-jacketed lead-core

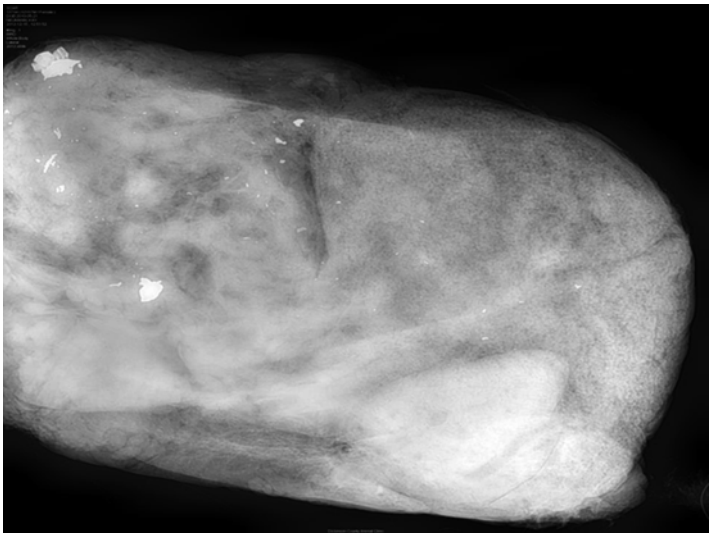

Fig. 3 Radiograph shows lead fragments as white specks in the offal pile from a white-tailed deer (*Odocoileus virginianus*) shot with a .50 caliber muzzleloader in 2012 on the Upper Mississippi River National Wildlife and Fish Refuge. The bullet's intact copper jacket (shown in the *upper left corner*) mushroomed, exposing the lead core which fragmented into 107 pieces that were spread throughout the offal pile. Photo courtesy of USFWS Upper Mississippi River National Wildlife and Fish Refuge, 7071 Riverview Rd. Thomson IL 61285

bullets. Most of the abdominal viscera were removed from each. carcass to mimic .local practice and the remaining internal organs (heart, lungs, liver, and kidneys) were retained in the thoracic cavity of the carcass. Fragments on radiographs were compared with images of known lead fragments obtained from copper-jacketed lead bullets fired into a water jug, and bone fragments and grit collected from the study site. The authors found bone and grit to be obviously less opaque than metal fragments and easy to distinguish. An average of 356 metal fragments were visible on radiographs of the carcass and 180 fragments in viscera, accounting for an estimated 17 % of the weight of the bullet. The authors made no attempt to distinguish between copper and lead fragments, but presumed the majority of smaller fragments to be lead due to the larger size of copper fragments produced from firing into water jugs. Fragment counts of carcass and viscera from the same animal were only weakly correlated, and fragments in viscera tended to be smaller: 91 % were <0.01 g as compared to 34 % in carcass. Though mean fragment counts taken from opposite sides of the thoracic area were similar, there was sometimes a substantial difference between counts (a difference of over 300 fragments in one case), suggesting that a single radiograph may underestimate the total number of fragments in a carcass.

Grund et al. (2010) shot euthanized domestic sheep (*Ovis aries)* broadside from a range of 50 m as surrogates for deer. Six types of lead-containing bullets and one copper bullet were studied. Fragments were detected in all carcasses shot with lead-containing bullets, but varied according to bullet type. While all expansion bullets had similar mass and velocity, one of two bullets marketed as "controlled expansion" (i.e., designed to achieve deeper penetration by deforming or "mushrooming" rather than fragmenting) produced fewer fragments and less radiation from the wound site (2–28 fragments per carcass within 25 cm) than other expansion bullets (21–498 fragments per carcass within 45 cm). Similar results were obtained from eight deer shot with rapid expansion bullets as part of a disease management program (Fig. 4). Slugs and muzzleloaders had greater mass and lower velocity than expansion bullets, though still fragmented, albeit to a lesser extent (slugs produced 3–127 fragments, muzzleloaders 1–105 fragments). Lead fragments from these bullet types tended to radiate less. Rinsing of carcasses caused lead to be detected further from the exit wound for all bullet types. Copper bullets produced between 1 and 4 fragments per carcass. The authors concluded that all lead-containing bullets produced fragments, and that hunters could not rely on advertised claims that certain bullets minimized fragmentation and lead deposition into the carcass. The authors also concluded that all meat from deer harvested with lead bullets has the potential to contain at least some lead (e.g., Fig. 4—radiation of lead shot throughout the body cavity).

Dobrowolska and Melosik (2008) collected muscle tissue from wild boar (*Sus scrofa*) and red deer killed by hunters in Poland. Bullet types were selected by hunters and varied, though no attempt was made to establish a relationship between bullet type and lead contamination in their analysis. Elevated lead (>0.3 ppm wet weight, the greatest value detected in control tissues) was detected at least 15 cm from the bullet pathway in all boar carcasses (N = 10) and 30 cm from the bullet

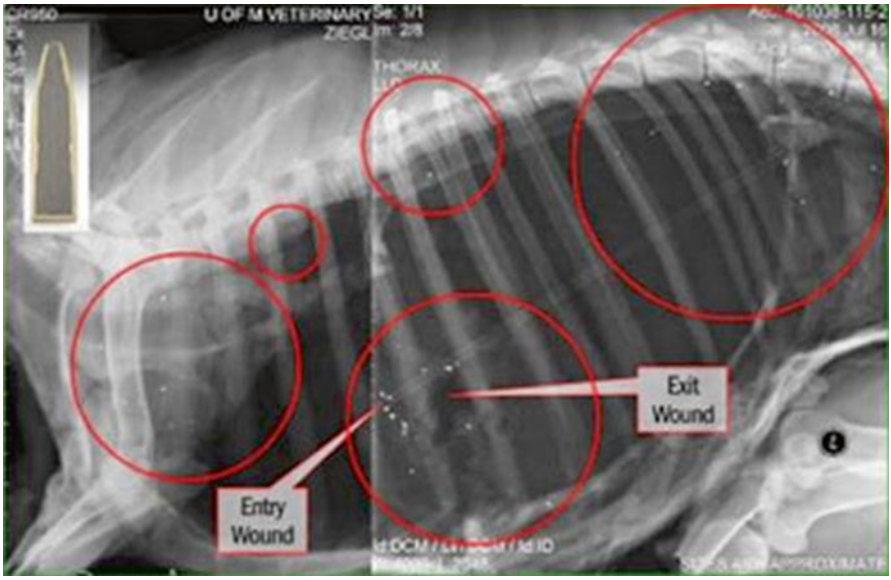

Fig. 4 Radiograph of domestic sheep (*Ovis aries)* shot with lead ammunition (rapid expansion bullet fired from a 0.308 Winchester) behind the scapula. Bullet fragments are within the *red circles* and are throughout the thoracic cavity and into the pelvic cavity. Photo courtesy of Minnesota Department of Natural Resources, Farmland Wildlife Populations and Research Group, 35365 800th Ave, Madelia, MN 56062

pathway in three carcasses. Similarly, elevated lead was detected at least 15 cm from the bullet pathway in all deer carcasses (N = 10) and 30 cm from the bullet pathway in three carcasses.

Fragmentation in Small Game and Varmints

To study the potential hazard of predators and scavengers consuming small game or varmints, 15 Richardson's ground squirrels (*Spermophilus richardsonii*) were shot using a .22 caliber rifle and hollow-point rimfire ammunition (Knopper et al. 2006). Bullet fragments were visible in 14 of 15 carcasses as minute debris rather than larger pieces of bullet. The area containing fragments (as determined by radiography) was removed from each carcass and the entire section analyzed for lead (bone, hair, and tissue combined). Concentrations in these sections ranged from 0.01 to 17.21 mg (median = 3.23 mg). The authors theorized that larger fragments have enough momentum to leave a small carcass, but dust-like lead debris left behind may be an appreciable source of lead for consumers.

In black-tailed prairie dogs (*Cynomys ludovicianus*), fragmentation was studied in individuals shot with non-expanding lead bullets enclosed by a copper jacket and

expanding soft-point lead bullets (Pauli and Buskirk 2007). All prairie dogs were shot at 20–200 m to mimic typical shooting events. Radiographs revealed fragments in 26 of 30 prairie dogs shot with expanding bullets and 2 of 29 shot with non-expanding bullets. Fragments extracted for analysis averaged 317.8 mg (235.7 mg from the lead core, 23.2 mg from the copper jacket) for expanding bullets, and 43.0 mg (19.8 mg lead core, 23.2 mg copper jacket) for the non-expanding bullets. The authors hypothesized that most fragments were too small (<25 mg) to either be avoided during ingestion or egested through regurgitation by a predator or scavenger. Stephens et al. (2008) detected bullet fragments in four of ten black-tailed prairie dog carcasses from shooters at Thunder Basin National Grassland, Wyoming. Lead was the primary metal in fragments of one carcass, and copper the primary metal in the other three carcasses. Lead content averaged 57.3 mg in fragments from three carcasses with measurable amounts of lead.

In three rifle-shot coyotes obtained from a taxidermist, fluoroscopy revealed that lead fragments were distributed in the chest and abdominal cavity (Stauber et al. 2010). Fragments numbered in the hundreds in one carcass to less than ten in another.

Fragments in Meat from Hunter-Killed Game

Lead fragments have also been detected in meat processed from hunter-killed carcasses. Fragments were commonly observed via radiograph in white-tailed deer killed using copper-jacketed lead bullets (all of the same brand, caliber, and bullet weight) during the Wyoming hunting season and then commercially processed, each at a separate facility (Hunt et al. 2009). All 30 carcasses contained bullet fragments (15–409 fragments per carcass), with fragment separation up to 45 cm. In ground meat, fragments were visible in packages from 24 of 30 deer (80 %) and in 74 of 234 packages (32 %). Analysis of fragments excised from ground meat from 13 deer identified lead in 25 of 27 samples (93 %). Nine samples contained copper at greater than background levels. In Wisconsin, lead was detected in 30 of 199 (15 %) commercially processed packages of venison, and 8 of 98 (8 %) samples collected from hunters (Thiboldeaux 2008). Lead concentrations averaged 15.9 ppm wet weight in commercially processed samples that tested positive for lead and 21.8 ppm wet weight in hunter submitted samples that tested positive for lead. Pharmacokinetic modeling using the U.S. EPA *Integrated Exposure Uptake Biokinetic Model* predicted a risk of elevated blood lead concentrations in children (>0.1 ppm) from eating as little as one venison meal per month. In North Dakota, 100 samples of ground venison packages were selected from 15,250 packages donated to food pantries (Cornatzer et al. 2009). High definition computer tomography revealed metal fragments in 59 of 100 packages. Of 15 random samples from all 100 packages, one tested positive for 120 ppm lead dry weight. Lead concentrations in five samples known to contain metal fragments ranged from 4200 to 55,000 ppm dry weight.

In a follow-up investigation to the study by Cornatzer et al. (2009), the Centers for Disease Control and Prevention (CDC) collected blood samples from 736 vol-

unteers in six cities in North Dakota (Iqbal et al. 2009). Participants that consumed wild game had higher blood lead levels than those who did not, with those consuming the greatest serving sizes having the highest concentrations. The average blood lead concentration was 0.0117 ppm, with 1.1 % of samples ≥0.05 ppm. No samples exceeded the CDC recommended case management threshold of 0.1 ppm, though the authors note that there is no clinical threshold of lead in the human body that is considered safe, and that increased risk of factors such as myocardial and stroke mortality has been observed at ≥0.02 ppm. Since the publication of this investigation, the CDC has lowered its threshold to identify children at risk to 0.05 ppm, and removed the phrase "level of concern" from its publications, noting that no safe blood lead level in children has been identified (CDC 2012).

Key Points: Spent Lead Ammunition in the Environment

Regulation of ammunition

- Studies in the 1960s found waterfowl poisoning from lead shot to affect populations.
- Secondary poisoning to scavenging birds, including bald eagles, resulted from consumption of waterfowl containing embedded or ingested lead shot.
- Lead shot for waterfowl hunting became a nationwide restriction in 1991.

Sources of spent lead ammunition remaining after the ban

- The switch to non-toxic shot resulted in lower rates of lead shot ingestion in waterfowl and was effective at reducing, but not eliminating, lead shot exposure in waterfowl and birds that prey upon waterfowl.
- Lead fragments from bullets and slugs, and lead shot remain on the landscape in game animals that are wounded or killed by hunters but not retrieved, and from discarded offal piles.

Fragmentation

- Regardless of the type of game or lead-based bullet, all studies showed that lead bullets fragment, sometimes substantially, when fired into an animal.
- In samples collected from the field, fragments were often confirmed to contain lead by analysis of the fragments themselves or detection of lead in surrounding tissue.
- Metal fragments on radiographs are more opaque than bone or grit and easily distinguished.
- Fragment size and number varied, with some carcasses containing hundreds of fragments.
- Fragments radiated significantly (up to 45 cm) from the wound channel in large game.

(continued)

(continued)

- The creation of small lead fragments increases availability to scavengers due to the tendency to radiate from wound channels, avoid detection or regurgitation by scavengers, and be abundant enough to expose several scavengers feeding on a single carcass.
- In contrast to lead-containing bullets, monolithic copper bullets produced few, if any, fragments, within carcasses.

5 Vulnerability of Avian Scavengers

The vulnerability of an individual to a toxicant is based on its exposure potential and sensitivity (Golden and Rattner 2003). When coupled with demographic parameters, vulnerability of populations may be assessed.

5.1 Exposure Potential

The potential for a bird to be exposed to lead ammunition is a function of multiple factors, including its diet, foraging strategy, and the frequency that lead will be encountered in the environment. Birds that scavenge on carcasses or offal piles are likely to be exposed to spent ammunition in the tissues of hunter-killed game. Scavengers can feed on carcasses and offal, thus removing them from the environment, with efficiency. Removal rates have been documented as high as 98 % after 24 h in controlled studies, but can vary widely, influenced by factors such as season, location, and species (Prosser et al. 2008). Wildlife can also be exposed to spent ammunition via direct ingestion of shot or fragments from the ground; however, this route is unlikely to pose significant risk to scavenging birds.

Influence of Diet on Exposure

Birds can either be obligate scavengers, meaning they feed exclusively (or almost exclusively) on carrion, or combine scavenging with predation on live prey or other food items. Obligate North American avian scavengers include the California condor, black vulture, and turkey vulture, although the diet of the latter may include a high proportion of plant food (Kirk and Mossman 1998). Specific dietary items are dependent on geographic location, but condors and black vultures rely heavily on large mammals, while turkey vultures are more adaptable and forage on smaller and more varied food items (Kirk and Mossman 1998; Buckley 1999; USFWS 2013c). Other avian species such as bald eagles, golden eagles, caracaras, crows, and ravens

consume carrion as part of a broader diet (Boarman and Heinrich 1999; Buehler 2000; Kochert et al. 2002; Verbeek and Caffrey 2002; Morrison and Dwyer 2012). These species tend to be opportunistic, and like obligate scavengers, diets vary with geography, habitat, and availability. For some species, such as eagles, adults may be more apt than immatures to capture live prey due to better developed foraging ability (Buehler 2000). Crows and ravens consume the widest variety of food of birds that scavenge, including plant material, insects, garbage, eggs, small animals, and carcasses (Boarman and Heinrich 1999; Verbeek and Caffrey 2002). Some species, such as the northern harrier (*Circus cyaneus*) and ferruginous hawk (*Buteo regalis*), generally prey on live animals, but may scavenge when resources are available (Peterson et al. 2001; Stephens et al. 2008).

Scavenging birds may vary their diet according to availability. Both species of eagles have been documented to consume a greater percentage of carrion during winter months (Buehler 2000; Kochert et al 2002). Several studies show a large percentage of deer, in particular, in the diet of bald eagles. In 949 feeding observations of bald eagles in New Brunswick, Canada, white-tailed deer and deer offal accounted for 40 and 30 % of the diet, respectively (Stocek 2000). Of 339 feeding observations of bald eagles wintering in the lower Great Lakes basin, 47 % were on carcasses of white-tailed deer (Ewins and Andress 1995). White-tailed deer remains were the most frequently detected dietary item (found in 67–72 % of the regurgitated castings) of bald eagles wintering along the St. Lawrence River (Lang et al. 2001).

Foraging Strategy

While most scavenging species will do so either individually or in groups, several factors related to foraging strategies can result in group feeding behavior at carcasses (Fig. 5). Turkey vultures have well-developed olfactory organs and are often the first to locate carrion (Kirk and Mossman 1998). Species such as black vultures, condors, and bald eagles are more reliant on visual clues, such as other scavengers feeding on a carcass, and thus tend to fly high, keeping other scavengers in view to follow them to carcasses (Buckley 1999; Buehler 2000; USFWS 2013c). Vultures and smaller birds cannot open thicker skin of some carcasses, so must wait for larger scavengers such as eagles or condors to open intact carcasses, which may displace them in the process (Kirk and Mossman 1998; Buckley 1999). However, specialization by location, type of carcass, and even within the carcass can result in no single species dominating a food source. For example, turkey vultures tend to feed on smaller items, less often near humans, and favor muscle and connective tissue, as opposed to viscera (Kirk and Mossman 1998). Species such as black vultures and bald eagles may visit the same carcasses for several days, thus providing a pathway for others in the roost to discover the carcass and increasing the number of individuals exposed to a single carcass (Buckley 1999; Buehler 2000). A single carcass may attract numerous scavengers. In a study of waterfowl carcass scavenging in agricultural fields, group sizes at a single carcass ranged from 1 to 79 individual birds (Peterson et al. 2001). Mean group size was 16.6 individuals, with

Fig. 5 *Top*: Bald eagles (*Haliaeetus leucocephalus)* feeding on the offal pile from a white-tailed deer (*Odocoileus virginianus*) killed in Monroe County, Iowa in January 2013. *Bottom*: Juvenile bald eagle feeding on a white-tailed deer carcass with an adult bald eagle and an American crow at the Lost Mound Unit of the Upper Mississippi River National Wildlife and Fish Refuge. Top photo courtesy of Peter Eyerhalde. Iowa State University; Bottom photo courtesy of USFWS Upper Mississippi River National Wildlife and Fish Refuge, 7071 Riverview Rd. Thomson IL 61285

maximums of 79 northwestern crows (*Corvus caurinus*) and 16 bald eagles recorded at a carcass at one time. In a study of black vultures, group size increased with carcass size, with as many as 98 individuals recorded at a single feral hog (*Sus scrofa)* carcass (Buckley 1999).

The tendency of scavengers to exhibit group feeding behavior may enhance their vulnerability to lead exposure in a number of ways. The probability of vultures finding food has been found to be related to the density of vultures in the habitat (Jackson

et al. 2008). As the number of foragers increases, so does the probability of finding a carcass. Likewise, if vulture populations experience decline and fall below a critical level, the feeding efficiency of each individual falls dramatically. In addition, the propensity for many individuals to feed on a single food source can expose them to a common source of contamination, as exhibited by group foraging in agricultural fields where carcasses may contain pesticide residues (Peterson et al. 2001). Demographic modeling of vultures in the Indian subcontinent affected by poisoning from use of the non-steroidal anti-inflammatory drug diclofenac revealed that observed population declines (22–50 % annually) could be attributed to a very small proportion of carcasses (between 1:130 and 1:760) containing concentrations of diclofenac that were lethal to old world vultures (Green et al. 2004).

5.2 Sensitivity to Lead

Avian sensitivity to lead is influenced by diet, anatomy, and physiology. Species and individuals may also possess inherent sensitivity driven by genetic makeup: three polymorphic genes have been identified that influence the bioaccumulation and toxicokinetics of lead in mammals though this has not yet been investigated in birds (Onalaja and Claudio 2000).

Physiology

Birds have a unique physiology that can enhance their vulnerability to lead toxicosis by facilitating breakdown and absorption into tissue. Species such as waterfowl that feed on coarse objects like grain or plant material have muscular gizzards for grinding that are larger than birds whose diet is largely meat (Farner 1960). This grinding facilitates the erosion of ingested metallic lead, making it more bioavailable for absorption in the gastrointestinal tract and subsequent transport to other organs (Jordan and Bellrose 1951). Although carnivorous birds may have highly reduced gizzards (and omnivorous birds intermediate to the two groups), other digestive characteristics facilitate the absorption of items from the gastrointestinal tract. While specific values are not available for scavenging birds such as eagles or vultures, some closely related raptors have been found to have especially acidic stomach fluids as compared to other bird species (Duke 1997). The average pH of 1.6 measured during gastric digestion in falcons translates to about six times more hydrogen ion per ml in their basal gastric secretion than was measured in owls (average pH 2.35; Duke 1997). Other species studied included turkeys (*Meleagris gallopavo*; pH 3.0) and domestic ducks (*Anas platyrhynchos x Cairina moscata* hybrid; pH 2.1). Raptors have the unique ability to egest indigestible materials, such as bone and fur via casts (Duke et al. 1976; Griffin et al. 1980; Nelson et al. 1989). Casts of raptors exhibit more thorough corrosion of bone as compared to those of owls, likely due to this difference in gastric pH (Duke et al. 1975). In addition, birds

possess a distinctive trait in gastrointestinal mobility that increases residence time of ingested materials in the digestive system, including the highly acidic stomach. Periodic reverse peristalsis moves the contents of the upper ileum and duodenum back into the stomach, an adaptation hypothesized to allow for greater digestion of nutrients without lengthening the gastrointestinal tract, which would be disadvantageous to flying due to added weight (Duke 1997). Raptors feeding on mice had 1–2 refluxes of this nature per hour to aid in digestion (Duke 1997).

Influence of Diet on Sensitivity

Diet can effect lead's toxicity and storage in tissues. Jordan and Bellrose (1951) reported that diet was a more important influence on the toxicity of lead in waterfowl than was the dosage of lead, within a range of one to four #6 shot. Toxicity can be enhanced by nutritional deficiencies in the diet (e.g., calcium, zinc, iron, protein, fat) and decreased by excesses of others (e.g., zinc, protein, fat; Eisler 2000). In particular, because of lead's physical similarity to calcium, these substances compete for absorption in the gut (Quarterman 1986). Studies in waterfowl have found that individuals ingesting foods high in protein and calcium were less susceptible to toxic effects of lead (Sanderson and Bellrose 1986). Dietary influence on lead toxicity has since been studied in other species, and findings indicate that toxic effects are less severe when birds intake nutritionally balanced diets high in protein and calcium. The mitigating effects of such diets may be the result of high calcium and protein levels reducing lead absorption in the gastrointestinal tract and lowering the body burden of lead (Koranda et al. 1979; Sanderson 1992; Scheuhammer 1996). Differences in lead concentrations found in species with similar ingestion rates have been attributed to this variation in diet, with species feeding on increasing amounts of animal matter showing a lower accumulation of lead in tissues (Stendell et al. 1979). Reproductively active female birds have been found to accumulate lead at a greater rate than males, a characteristic presumably linked to increased intestinal calcium absorption during eggshell formation (Scheuhammer 1987). The effects of dietary preferences upon lead toxicity have not been specifically studied in scavengers, but their high intake of animal matter may influence the effects of ingested lead, and could vary among species that specialize on different parts of the carcass.

Results of Toxicity Testing of Scavengers

Laboratory toxicity studies of lead ammunition have been performed on three species of scavenging birds, the Andean condor (Pattee et al. 2006), turkey vulture (Carpenter et al. 2003), and bald eagle (Hoffman et al. 1981; Pattee et al. 1981). For all three species, dosed birds exhibited individual variability in sensitivity, possibly related to factors such as length of shot retention, number of shot retained, amount of lead eroded, and individual susceptibility. Though protocols differed across studies (Table 2), the Andean condor appeared to be more sensitive than other raptor

Table 2 Comparison of dosing protocols in three studies of scavenging species experimentally treated with lead shot

Species	Number shot dosed	Shot number	Shot diameter (mm)	Approx. number shot in 1 oz	Total lead eroded (mg)	Outcome
Andean condor[a]	2 or 6	00	9.14	6.2	126–603	4/4 died or euthanized; clinical signs in 39–49 days
Turkey vulture[b]	3 or 10	BB	4.57	50	111–247	4/6 died or euthanized; clinical signs in 143–183 days; 2/6 survived to study end (211 days)
Bald eagle[c]	10–156	4	3.28	135	19–185	5/5 died in 10–133 days

[a]Pattee et al. (2006)
[b]Carpenter et al. (2003)
[c]Pattee et al. (1981)

species tested, with all four birds dosed with either two or six lead shot (size 00) showing signs of lead poisoning within 50 days, and two of those succumbing. This contrasts with a time to mortality of 10–155 days in five bald eagles dosed with 10–156 lead shot (size 4), and a minimum time to death of 143 days in six turkey vultures dosed with up to three or six lead shot (size BB). Two turkey vultures were euthanized at 211 days showing no overt signs of lead poisoning. The authors concluded that the vultures showed considerable tolerance to lead shot when compared to other experimentally treated raptors (Carpenter et al. 2003).

Shot retention varied among individuals of each species. Redosing of turkey vultures was described as "constant" due to defecation or regurgitation of shot (Carpenter et al 2003). Bald eagles retained shot as little as 12 h and as long as 48 days (Pattee et al 1981). Dosed shot was recovered from all four condors at necropsy 39–49 days after dosing (with one bird having been redosed at day 7 following regurgitation) (Pattee et al. 2006). Shot retention has been reported in eagles that have succumbed to lead poisoning in the wild (e.g., 77 shot recovered in one eagle, Jacobson et al. 1977; Fig. 6). The variability in shot retention seen here is consistent with that seen in other species that ingest lead shot, which may erode or pass through the gut before death occurs (Roscoe et al. 1979; Schulz et al. 2006).

Studies on all three scavenging birds confirmed the solubility of dosed metallic lead shot in the digestive tract either by measuring erosion of lead shot post-dosing or by detection of lead in blood and other internal organs shortly after dosing. Total lead eroded after dosing varied among individuals and ranged from 126 to 603 mg (condors), 111–247 mg (turkey vultures), and 19–185 mg (bald eagles) (Table 2). For all species, an increase in blood lead concentrations and a decrease in ALAD activity followed treatment, with blood lead continuing to rise after dosing. These changes were detected in bald eagles within 24 h of treatment (Hoffman et al. 1981), and in condors and vultures at the first blood collection 7 days after dosing.

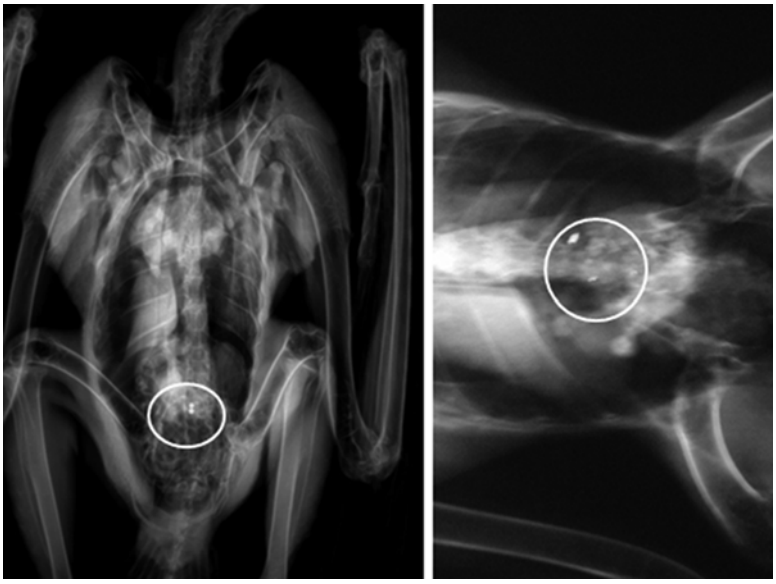

Fig. 6 *Left:* Radiograph showing two #5 lead shot (*circled*) in the digestive tract of a bald eagle (*Haliaeetus leucocephalus*) admitted to a wildlife rehabilitation center in Iowa. *Right*: Radiograph showing ammunition fragments (*circled*) in bald eagle carcass. Photos courtesy of Kay Neumann, Saving Our Avian Resources, 25494 320th Str, Dedham, IA 51440

Table 3 Comparison of hepatic lead concentrations in scavenging birds showing varying levels of intoxication after being experimentally treated with lead shot

	Lead in liver at necropsy (ppm wet weight)			
Species	Control	No signs of intoxication	Intoxication, euthanized	Mortality
Andean condor[a]	Not analyzed	–	49.88, 109.09	45.46, 58.52
Turkey vulture[b]	0.05, 0.10	1.48, 2.22	6.79, 18.71	20.73, 33.78
Bald eagle[c]	0.4	–	3.4	11.5–27.0

[a]Pattee et al. (2006)
[b]Carpenter et al. (2003)
[c]Pattee et al. (1981)

Liver concentrations for these three species (Table 3) were in general agreement with previously described diagnostic thresholds (<2 ppm wet weight in liver for birds with no history of lead exposure and >6 ppm wet weight in liver in for birds with evidence of clinical poisoning; see Table 1). However, individual variability among treated birds exhibits the utility in incorporating diagnostic criteria such as field observations, clinical signs, gross lesions and pathological findings, when available, in making a finding of lead poisoning.

Signs of toxicosis in condors, vultures, and eagles showing clinical poisoning included lack of coordination, loss of appetite, lethargy, weakness, reduced activity,

postural change, drooped wing, and frequent opening of the mouth, each occurring in one or more species (Pattee et al. 1981, 2006; Carpenter et al. 2003). All three species exhibited emaciation, with turkey vultures showing a loss of pectoral muscle, subcutaneous fat, and coelomic fat, and condors showing a loss of subcutaneous, abdominal, and coronary fat. Histological findings included spongiosis in brain tissue of vultures and condors, and nephrosis in some individuals of all species. All four eagles examined for myocardial necrosis exhibited this lesion. It is notable that acid-fast inclusions were present in renal tubular epithelium in turkey vultures and condors, but not in bald eagles. As noted above (see Sect. 3), inclusion bodies are indicative of lead exposure, but are not consistently present in all species or all individuals within a species. In general, despite overall evidence of clinical poisoning, individual gross and histological signs varied among individuals and were not consistently displayed.

5.3 Demographic Vulnerability

Species that are long-lived, have delayed maturation, and low reproductive output can be disproportionately sensitive to the loss of breeding adults as opposed to immature individuals. Scavenging species such as eagles, condors, and vultures possess these life history traits (USFWS 1996; Buckley 1999; Buehler 2000; Kochert et al 2002). For example, bald eagles and condors can live well over 20 years, may not begin breeding until 6–7 years old, and typically have only 1–2 offspring (Gerrard et al. 1992; Schempf 1997; Snyder and Schmitt 2002). One study that examined blood lead concentrations in bald eagles admitted to a rehabilitation center found that a greater proportion of admitted adults had elevated lead concentrations compared to juveniles and hatch-years (Cruz-Martinez et al. 2012). The authors hypothesized that the discrepancy was due to the aggressive behavior of adult eagles at scavenging sites. A comprehensive examination of lead poisoning cases of eagles submitted to the National Wildlife Health Center between 1975 and 2013 found that the odds of lead poisoning were greater in adults versus juveniles (Franson and Russell 2014).

Key Points: Vulnerability of Avian Scavengers

Exposure potential

- Birds that feed on carcasses are at risk of ingesting lead ammunition from hunter-killed game.
- The tendency of scavengers to exhibit group feeding behavior can enhance their vulnerability to lead exposure by increasing their ability to locate contaminated carcasses and subsequently exposing multiple individuals simultaneously.

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Sensitivity

- Despite having a reduced gizzard, carnivorous birds contain physiological adaptations (low pH, reverse peristalsis) that facilitate the dissolution of lead after ingestion.
- Dietary components, including calcium and protein, may influence the susceptibility of birds to lead toxicity.
- Scavenging birds can retain ammunition in the gastrointestinal tract. However, when ammunition is not detected this could indicate either a lack of exposure, exposure followed by regurgitation, or complete absorption (e.g., for smaller fragments).
- Metallic lead is soluble in the gastrointestinal tract and is demonstrated in experimental studies by (1) measuring blood lead concentrations or enzyme activity after treatment with lead shot or (2) weighing shot before and after they have passed through the digestive system.
- In controlled studies with scavenging birds, signs considered diagnostic of lead poisoning were not exhibited uniformly by exposed species or individuals, despite known toxicosis.

Demographic vulnerability

- Because lead can kill breeding adults, species that are long-lived, have delayed maturation, and low reproductive output, such as condors and eagles, may be especially vulnerable to its effects.

6 Occurrence of Lead in Avian Scavengers

The sensitivity of scavenging birds coupled with the availability of lead ammunition in the environment has resulted in numerous documented cases of exposure and mortality. One study found that of 130 species documented to be affected by lead ammunition, 24 % were raptors and scavengers (Tranel and Kimmel 2009). Herein, we discuss examples of lead exposure or poisoning in predatory and scavenging birds that are representative of several regions across the United States. For ease of comparison, and to the extent possible, results are presented using tissue thresholds and diagnostic descriptors as defined above (background: <0.2 ppm blood, <2 ppm wet weight liver; subclinical: 0.2–0.5 ppm blood, 2–6 ppm wet weight liver; clinical poisoning: >0.5 ppm blood, >6 ppm wet weight liver; Table 1). Where study authors provided results using other thresholds or criteria, those are described. We only included examples after 1991 when the United States lead shot ban for waterfowl was in full effect in order to focus on exposures that are less likely to be associated with the consumption of waterfowl. Though waterfowl were not devoid of lead shot

following the ban, documented decreases in exposure, as described above, rendered waterfowl a less likely source than prior to the ban. In addition, only cases from the published literature are included, although a number of additional cases have been documented from wildlife rehabilitators, and state agency, university, and federal government labs. Table 4 lists such examples of lead exposure (>0.2 ppm blood or 2 ppm wet weight liver) in raptors. Some cases include evidence suggestive of exposure to lead ammunition, such as seasonality contemporary with hunting activities or radiography of gastrointestinal contents.

Table 4 Examples of lead exposure indicative of subclinical or clinical poisoning (>0.2 ppm in blood or >2 ppm wet weight in liver) in predatory and scavenging birds in the United States

Species	References	Clinical poisoning[a]	Hunting season[b]	Ammunition detected[c]
Bald eagle (*Haliaeetus leucocephalus*)	Kramer and Redig (1997)	•	•	•
	Strom et al. (2009)	•	•	
	Harris and Sleeman (2007)	•		
	Bedrosian and Craighead (2009)	•	•	
	Neumann et al. (2009)	•	•	•
	Stauber et al. (2010)	•	•	
	Harmata (2011)	•		
	Nam et al. (2011)	•		•
	Bedrosian et al. (2012)	•	•	
	Cruz-Martinez et al. (2012)	•	•	•
	Pagel et al. (2012)	•	•	
	Mierzykowski et al. (2013)	•	•	
	Franson and Russell (2014)	•	•	•
	Warner et al. (2014)	•		
Golden eagle (*Aquila chrysaetos*)	Kramer and Redig (1997)	•	•	•
	Bedrosian and Craighead (2009)	•	•	
	Stephens et al. (2008)			
	Domenech and Langer (2009)	•		
	Stauber et al. (2010)	•	•	
	Kelly et al. (2011)	•		
	Harmata and Restini (2013)	•		
	Franson and Russell (2014)	•	•	•
	Kelly et al. (2014)	•		
	Langner et al. (2015)	•	•	
	Watson and Davies (2015)	•		
California condor (*Gymnogyps californianus*)	Parish et al. (2009)	•	•	
	Finkelstein et al. (2012)	•		
	Rideout et al. (2012)	•		•
	USFWS (2012)	•	•	•
	USFWS (2013c)	•		•

(continued)

Table 4 (continued)

Species	References	Clinical poisoning[a]	Hunting season[b]	Ammunition detected[c]
Turkey vulture (*Cathartes aura*)	Kelly et al. (2011)		·	
	Kelly and Johnson (2011)	·	·	
	Kelly et al. (2014)	·		·
Black vulture (*Coragyps atrauts*)	Behmke et al. (2015)	·		
Cooper's hawk (*Accipiter cooperii*)	McBride et al. (2004)		·	
Red-tailed hawk (*Bueto jamaicensis*)	Stansley and Murphy (2011)	·	·	
Common raven (*Corvus corax*)	Craighead and Bedrosian (2008)	·		
	Craighead and Bedrosian (2009)		·	

Columns indicate if any individuals had tissue concentrations consistent with clinical poisoning (>0.5 ppm blood or >6 ppm wet weight liver)a, if authors indicated that elevated lead levels coincided with the hunting seasonb, or if shot or fragments were detected in the gastrointestinal tract via radiography or necropsyc. All individuals were collected or sampled after the 1991 United States ban on lead shot use for waterfowl hunting

Though the cases described below are somewhat widespread geographically, they are largely based on opportunistic sampling. For any contaminant, collection of dead or moribund birds is likely to represent only a subset of the actual exposure or mortality attributable to that contaminant. In order to document mortality, a carcass must be observed, reported, collected, and chemically analyzed while still relatively fresh (Vyas 1999). The loss rate of dead birds to scavengers may be up to 98 % in the wild, depending on season, location, and species, with losses generally occurring within 24–96 h after placement of a carcass in experimental studies (Peterson et al. 2001; Prosser et al. 2008). Carcass detection studies have found that even when searches are performed on carcasses known to exist (e.g., placed by a researcher for study), a percentage will never be found due to scavenging, location in remote and inaccessible areas, or size or coloration that renders the carcass inconspicuous (Vyas 1999; Elliott et al. 2008). For these reasons, in addition to the exclusion of unpublished data (as described above), the cases that follow are likely representative of a wider breadth of lead exposure and poisoning, including those jurisdictions where it has not been well documented to date.

Moreover, isolated or infrequent monitoring of lead in the blood of live birds is likely to miss peak values or entire exposure events due to lead's short half-life in blood (e.g., estimated at ~14 days for condors; Fry et al. 2009). A single sample provides information on concentrations at the time of collection only. There is no way to ascertain if blood concentrations are rising, falling, or static, or if previously exposed birds no longer contain detectable concentrations in blood (Barbosa et al. 2005).

Furthermore, lead concentrations from blood can occur from either current exposure or mobilization of previously stored lead from internal tissues (Barbosa et al. 2005). Information from blood samples should be interpreted accordingly.

6.1 Nationwide

United States (Bald and Golden eagles): Demographic and pathologic data obtained from case files for lead poisoned bald and golden eagles previously necropsied at the National Wildlife Health Center were analyzed for 484 bald eagles and 68 golden eagles (Franson and Russell 2014). The diagnosis of clinical poisoning was made by pathologists performing necropies, generally based on gross observations including emaciation and characteristics of bile stasis, microscopic lesions such as damage to the kidney and heart, in combination with liver lead concentrations >6 ppm wet weight. Carcasses were collected from 1982 to 2013 for bald eagles and 1975–2013 for golden eagles from 38 states. The mean liver lead concentration was 28.9 ppm wet weight for bald eagles and 19.4 ppm wet weight for golden eagles. Lead ammunition or fragments were detected in 14.2 % of bald eagles and 11.8 % of golden eagles. Lead poisoned carcasses were found in greater frequency in the late autumn and winter than spring and summer months, and the odds of lead poisoning were greater in eagles from the Mississippi and Central flyway versus the Atlantic and Pacific flyway. In addition, the probability of lead poisoning was greater for bald eagles versus golden eagles, females versus males, and adults versus juveniles. Out of 4064 eagles submitted in total, trauma and poisonings were the leading causes of death for bald eagles, with lead accounting for the greatest percentage of poisonings (Russell and Franson 2014).

6.2 Northeast

Maine (Bald eagles): Mierzykowski et al. (2013) found that 19 out of 127 (15 %) bald eagles collected dead in the state of Maine between the years 2001 and 2012 had liver lead concentrations indicative of clinical poisoning (>6 ppm wet weight). The highest lead concentrations were detected in the eagles collected during the winter and early spring months.

New Jersey (Red-tailed hawks, Black vultures, Turkey vultures): Carcasses collected from wildlife rehabilitators in New Jersey from 2008 and 2010 were analyzed for lead exposure in liver (Stansley and Murphy 2011). Of 221 individuals representing 13 raptor species, two red-tailed hawks contained elevated liver concentrations (2.1 ppm wet weight, consistent with subclinical poisoning, and 7.4 ppm wet weight, consistent with clinical poisoning). The red-tailed hawk with the highest lead concentration was submitted during the hunting season for several small game

species in New Jersey. Of 104 red-tailed hawks in total, lead was detected in 52 birds at background concentrations (<2 ppm wet weight). Of scavenging species analyzed, background levels of lead were detected in 1 of 1 black vulture and 5 of 7 turkey vultures. Other species with background levels of lead were barred owl (*Strix varia*), broad-winged hawk (*Buteo platypterus*), Cooper's hawk (*Accipiter cooperii*), great horned owl (*Bubo virginianus)*, red-shouldered hawk (*Buteo lineatus*), peregrine falcon (*Falco peregrinus*), eastern screech-owl, and sharp-shinned hawk (*Accipiter striatus*).

6.3 Southeast

Virginia (Bald eagles): From 1993 to 2003, 4 of 95 bald eagles admitted to the Wildlife Center of Virginia were diagnosed with lead poisoning based on blood concentrations of >0.2 ppm and clinical signs, or, when available, elevated liver concentrations (Harris and Sleeman 2007). Trauma was the most common diagnosis (71 %). Eagles were not routinely screened for lead unless showing clinical signs (Jonathan Sleeman, USGS National Wildlife Health Center, personal communication).

Virginia (Black vultures, Turkey vultures): Lead was measured in black vultures and turkey vultures culled by the U.S. Department of Agriculture, Wildlife Services in Chesterfield County, Virginia from July 2011 to May 2012 (Behmke et al. 2015). Mean lead concentrations in liver were 0.78 ppm wet weight (range: 0.012–6.17 ppm, N = 96) in black vultures and 0.55 ppm (0.23–1.3 ppm, N = 9) in turkey vultures. Of these, concentrations in livers of five black vultures fell within the range of subclinical poisoning (2–6 ppm) and one was over the threshold of clinical poisoning (>6 ppm). Concentrations in livers of turkey vultures did not exceed background levels (>2 ppm). Mean concentrations in femur were 36.99 ppm wet weight (4.5–540 pm, N = 98) in black vultures and 23.02 (6.16–70, N = 10) in turkey vultures, above thresholds that have been associated with background exposure (10 ppm dry weight). The authors indicated that these concentrations were indicative of long-term lead exposure and used isotopic analysis to attribute their origin to multiple potential sources of lead including ammunition, gasoline, coal-fired power plants, and zinc smelting.

6.4 Northwest

Montana (Golden eagles): Of 42 migrant golden eagles sampled in Montana during the fall of 2006 and 2007, 58 % had elevated blood lead levels (Domenech and Langer 2009). For all eagles, blood lead ranged from <0.005 to 4.81 ppm, with 18 exhibiting background levels (0–0.1 ppm), 19 subclinical poisoning (0.1–0.6 ppm), 5 clinical exposure (>0.6 ppm).

Of 74 golden eagles captured in southwestern Montana from 2008 to 2010, 70 (97 %) contained detectable levels of lead in blood (Harmata and Restani 2013). Of these eagles, concentrations were indicative of subclinical poisoning (0.2–0.5 ppm) in 29 %, and clinical poisoning (>0.5 ppm) in 16 %. Blood lead concentrations decreased from winter to spring, which the authors attributed to either a change to a less contaminated food supply locally or population turnover during migration.

Of 178 golden eagles sampled in the Helena National Forest in Montana during fall migration from 2006 to 2012, 118 (66 %) contained blood lead concentrations below background levels (<0.2 ppm), 42 (24 %) within the range of subclinical poisoning (0.2–0.6 ppm), and 18 (10 %) above the threshold for clinical poisoning (>0.6 ppm; Langner et al. 2015). Seven eagles contained blood lead >1.2 ppm and all were captured during the second half of fall migration, when hunter-killed carcasses were presumed to be increasing due to the start of the big-game hunting seasons along the migratory route. Golden eagles trapped using road-killed deer carcasses contained significantly higher concentrations than those trapped using bow-nets with rock pigeons. The authors suggest that these individuals may specialize on carcasses and therefore be more susceptible to increased lead exposure from bullet fragments. Hatch-year birds had lower concentrations than subadults and adults, suggesting that blood lead concentrations may be a function of cumulative lead exposure over time.

Montana (Bald eagles): Bald eagles were sampled from nestling, free-flying, and rehabilitation populations in southwestern Montana from 2006 to 2008 (Harmata 2011). Mean blood lead concentration in nestlings (N = 17) was 0.037 ppm and none exceeded the threshold for clinical poisoning (>0.6 ppm). The mean concentration in free-flying individuals (N = 88) was 0.272 ppm, 70 % of which exceeded background levels (>0.2 ppm), and 9 % exceeded 1.0 ppm. For birds sampled at rehabilitation centers (N = 23), mean concentrations were 0.183 ppm; most were within the range of background concentrations and none exceeded 1 ppm.

Wyoming (Ferruginous hawks, Golden eagles): In 2001, Stephens et al. (2008) investigated blood lead concentrations in nestling ferruginous hawks and golden eagles collected from Thunder Basin National Grasslands, Wyoming, due to local increases of lead poisoned raptors being admitted to wildlife rehabilitation facilities. Lead was detected in blood of all nestlings, but at less than subclinical concentrations (<0.2 ppm). Ferruginous hawks and golden eagles are known to scavenge on black-tailed prairie dogs at this location that have been killed by shooters and not recovered. Concentrations of lead in nestlings were similar to those at a reference site where no hunting took place. As scavenging on prairie dog carcasses is a known exposure pathway (bullet fragments were detected in four of ten carcasses collected), the authors hypothesized that lead levels may have been low due to decreased shooting at the site that season and an increase in alternate food sources for raptors.

Wyoming (Bald eagles, Golden eagles): Bedrosian and Craighead (2009) found that bald eagles and golden eagles accumulated more lead in blood samples during the

hunting season (median 0.56 ppm, just above the threshold for clinical poisoning) in the southern Yellowstone River ecosystem compared to the non-hunting season (median 0.277 ppm, in the range of subclinical poisoning). Nine blood samples out of 63 had concentrations >1.0 ppm.

Wyoming (Bald eagles): Bedrosian et al. (2012) studied free-flying bald eagles near Jackson Hole, Wyoming. The study area included all hunt zones on the National Elk Refuge and Grand Teton National Park where there is an estimated 3000 big game animals harvested annually. There are no other potential sources of lead in the study area as recreational varmint hunting, waterfowl hunting, upland game hunting, and fishing with live bait are not permitted on the Refuge or the National Park Service land. Blood samples were analyzed from 71 free-flying eagles, which included three re-captures and eight nestlings. Samples were taken during and after the big game hunting season for elk and bison in 2005–2010, excluding 2008. The analysis found 93 % (68) of all free-flying eagles tested had concentrations above background levels (>0.1 ppm). Thirty-three percent (14) of these had exposure indicative of clinical poisoning (>0.6 ppm), all of which were sampled during hunting season. For all eagles tested (free-flying and nestlings) 24 % had blood levels indicative of clinical poisoning during the hunting season, and none during the non-hunting season. There was an increase of eagle abundance during the hunting season as compared to after the season. The study tracked ten free-flying eagles with satellite transmitters. These eagles were captured at the site during the hunting season. After the hunting season, the ten eagles migrated south. The following year during the hunting season, 80 % of the eagles with transmitters returned to the site. The authors suggest that offal provides a seasonal attractant for the eagles.

Wyoming (Common ravens): Craighead and Bedrosian (2008) found that of 302 ravens sampled in the southern Yellowstone River ecosystem during 2004 and 2005, 47 % exhibited elevated blood lead levels (≥0.1 ppm) during the hunting season as compared to 2 % during the nonhunting season. Expanding upon this data, additional samples were collected through 2008 (Craighead and Bedrosian 2009). For all samples pooled (N = 539), median blood lead levels were 0.10 ppm for the hunting season and 0.02 ppm during the nonhunting season. A significant relationship was detected between annual median blood lead levels and the combined large-game harvest success from the National Elk Refuge and Grand Teton National Park.

Inland Pacific Northwest (Bald eagles, Golden eagles): Of 67 golden eagles and 63 bald eagles admitted to a raptor rehabilitation program from 1991 to 2008, 46 and 50 were tested for blood lead, respectively (Stauber et al. 2010). Blood lead concentrations above background levels (>0.2 ppm) were detected in 48 % of bald eagles and 62 % of golden eagles, and these eagles were submitted from eastern Washington, northern Idaho, northeast Oregon, and Alaska. Of these, 46 % of bald eagles and 52 % of golden eagles had concentrations associated with clinical poisoning (>0.5 ppm). Admission of eagles to the rehabilitation center with lead concentrations greater than background was strongly seasonal: 91 % of bald eagles were admitted January to March, and 58 and 32 % of golden eagles were admitted January

to March, and October to December, respectively. Elk (*Cervus canadensis*) and deer hunting seasons run from October to December, and the authors indicated that shooting of coyotes intensifies after December and could contribute to lead exposure after that time. All eagles were radiographed and none had evidence of ingested lead particles.

Washington (Golden eagles): From 2005 to 2013, lead was analyzed in blood of resident golden eagles on their nesting territories in the Columbia Basin of eastern Washington between January and June (Watson and Davies 2015). Of 17 eagles sampled, 11 had elevated concentrations (>0.2 ppm), four of which were at levels consistent with clinical poisoning (>0.5 ppm). None of these four exhibited physical impairment upon capture and all survived at least 1 year following capture. Two eagles recovered after colliding with wind turbines had background concentrations of lead (≤0.2 ppm).

6.5 Southwest

New Mexico (Cooper's hawk): Blood samples were collected from Cooper's hawks in the Cibola National Forest of north-central New Mexico during fall migration of 2001 and at the spring migration of 2002 (McBride et al. 2004). Three of 98 samples contained lead concentrations indicative of subclinical or clinical poisoning (0.2–1.5 ppm), all collected during spring migration. In total, 50 individuals contained detectable levels of lead. Cooper's hawks may feed on upland game birds such as dove and quail which may contain embedded lead shot. The authors hypothesized that blood lead levels were higher in spring migrants due to the timing of the hunting season in their winter range.

California (Turkey vultures): Lead was elevated (>0.1 ppm) in the blood of 83 of 172 turkey vultures sampled across California in 2008 and 2009 (Kelly and Johnson 2011). Some vultures contained lead concentrations >1.0 ppm, but showed no overt signs of toxicosis. In an intensely hunted area, average blood lead levels were twice as high during the hunting season as during the off-season, with 76 % having elevated concentrations during the hunting season as compared to 36 % prevalence outside of the big game hunting season. Blood lead was also measured in turkey vultures collected within the area of the California condor rangewide ban on lead ammunition for big game hunting (see Sect. 6.7 for more information on this ban; Kelly et al. 2011). Concentrations were elevated (>0.1 ppm) in significantly more vultures collected in 2008 prior to this ban (23 of 38; 61 %) than those collected in 2009 after this ban (3 of 33; 9 %). Blood lead levels indicated subclinical poisoning (0.2–0.49 ppm) in seven vultures (18 %) prior to the ban and one vulture (3 %) after the ban. None of the vultures contained blood lead concentrations associated with clinical poisoning (>0.5 ppm).

California (Golden eagles): Blood lead was measured in golden eagles that were collected within the range of the lead ammunition ban for big game hunting in southern California (Kelly et al. 2011). Concentrations were elevated (>0.1 ppm) in significantly more eagles collected in 2007–2008 prior to the ban (13 of 17) than those collected in 2008–2009 after the ban (12 of 38).

Of non-migratory eagles, the prevalence of elevated lead dropped from 83 % (5 of 6) to 0 % (0 of 9) after the ban. Blood lead levels indicated subclinical poisoning (0.2–0.49 ppm) in eight eagles (48 %) prior to the ban and four eagles (11 % after the ban). Blood levels exceeded the threshold for clinical poisoning (>0.5 ppm) in one eagle (6 %) prior to the ban and three eagles (8 %) after the ban.

California (Bald eagles): Of seven bald eagles released in the northern Channel Islands between 2002 and 2006, and subsequently found dead, three had concentrations of lead in bone considered to be above background levels (>10 ppm wet weight) (Pagel et al 2012). Eagles that spent the most time on Santa Rosa Island, where a deer and elk hunting program occurred, had the highest concentrations of lead. One bird found alive with a broken wing had a blood lead level of 0.522 ppm, above the threshold for clinical poisoning (>0.5 ppm).

California (Golden eagles, Turkey vultures, Common ravens): Cause of death was determined in 21 golden eagles, 23 turkey vultures, and 4 common ravens collected from wildlife biologists and wildlife rehabilitators in 13 counties throughout California between 2007 and 2009 (Kelly et al. 2014a). Forty-five of these birds were found alive and three (two golden eagles and one turkey vulture) were found dead. Lead poisoning (diagnosed from pathological lesions plus lead concentrations >1 ppm in blood or >5 ppm wet weight in liver) was the primary cause of mortality in 17 % (8/48) of cases. Of these, five were turkey vultures, three were golden eagles, and none were ravens. A lead-based fragment was retrieved from the gastrointestinal tract of one vulture. Lead-related mortalities occurred during the winter and early spring months (i.e., outside of the big game hunting season) and were found in various areas throughout the state. Of 39 birds total analyzed for lead, subclinical or clinical concentrations were detected in 21 % of livers (>2 ppm wet weight) and 48 % of bone samples (>6 ppm dry weight).

6.6 Bald Eagles in the Midwest

The Great Lakes region is an important habitat for bald eagles in the winter (Millsap 1986; Steenhof et al. 2002) and some of the highest mid-winter bald eagle counts have been recorded for the central United States (Steenhof et al. 2008). During the winter season, bald eagles congregate and forage along lakes and tributaries to feed on fish and waterfowl. As winter progresses in the Midwest and ice freezes over many of the open waterways, bald eagles opportunistically search for other food sources that are available on the landscape. One readily available food source in the winter is discarded offal from hunter-killed white-tailed deer, or deer that were shot

and not retrieved (Harper et al. 1988; Ewins and Andress 1995; Lang et al. 2001; Cruz-Martinez et al. 2012; e.g., Fig. 5).

Iowa: Wildlife rehabilitation facilities in Iowa processed 62 moribund bald eagles from 2004 to 2008 with 39 (62.9 %) of the birds having concentrations >0.2 ppm in the blood or >6 ppm wet weight in liver (Neumann 2009). The radiographs of seven of the 59 (12 %) birds admitted in Iowa showed fragments in the digestive tract presumed to be ammunition. Some of the birds admitted for traumatic injuries had elevated lead exposure. Most of the lead poisoned birds were admitted between the months of September and April, overlapping with the deer hunting season.

Minnesota, Iowa, and Wisconsin: At a raptor rehabilitation center in Minnesota 1227 moribund bald eagles were admitted from 1996 to 2009, with 331 (27 %) of the birds having blood lead concentrations above background levels (>0.2 ppm) (Cruz-Martinez et al. 2012). Over 90 % of these eagles were from Minnesota, Iowa, and Wisconsin. The chance of elevated lead concentrations increased based on hunting season, deer hunting zones, and age of bird. Metal objects were visible by radiograph in the stomachs of 34 eagles with lead levels above background (10 with shot, 24 with metallic shrapnel). An investigation conducted by the USFWS found that 35 of livers from the 58 (60 %) dead bald eagles found between 2009 and 2012 in the states of Iowa, Minnesota, and Wisconsin had detectable concentrations of lead and 22 (37.9 %) had concentrations consistent with clinical poisoning (>6 ppm wet weight in liver; Warner et al. 2014).

Michigan and Minnesota: In a retrospective analysis of 46 eagles that had been diagnosed with various causes of death (mainly trauma) between 2002 and 2010, 30 % exceeded levels suggestive of lead poisoning (>26.4 ppm dry weight in liver) (Nam et al. 2011). Fluoroscopic analysis of 26 birds from Michigan revealed lead shot or fragments (1–19 pieces) in the digestive tract of eight birds.

Wisconsin: The State of Wisconsin diagnostic laboratory processed 583 dead bald eagles between 2000 and 2008 with 87 (16 %) diagnosed with lead poisoning (>6 ppm wet weight in liver; Strom et al. 2009). This study associated bald eagle exposure rates with the hunting season in Wisconsin. Of the remaining admitted eagles, 48 % were diagnosed with trauma; however, lead concentrations were not presented.

Minnesota and Wisconsin: Of 654 bald and golden eagles admitted to a rehabilitation center from 1980 to 1995, 138 contained blood lead levels above background (>0.2 ppm) (Kramer and Redig 1997). Of these eagles, approximately 75 % were collected in Minnesota and Wisconsin, with the remaining from Alaska, Iowa, Illinois, Indiana, Michigan Nebraska, North Dakota, South Dakota, and Ohio. All eagles were routinely screened for lead upon admittance. The prevalence of lead-exposed eagles did not change after lead shot restrictions were implemented (17.5 % before 1991 versus 26.8 % after 1991). Of the 66 cases between 1991 and 1995, 36 % had blood lead concentrations indicative of clinical poisoning (>0.6 ppm). Eagles were admitted in all months of the year, with >40 % in November and

December for eagles collected after 1991, coinciding with deer seasons in Minnesota and Wisconsin. Of the 66 lead exposed birds admitted after 1991, 32 % were admitted for trauma, 23 % for projectile injury, and 17 % for lead toxicity. One eagle had radiographic evidence of lead shot in its gastrointestinal tract.

6.7 California Condor

The California condor, once distributed throughout North America, is now considered one of the rarest bird species in the world. The overall population has steadily increased over the past two decades, chiefly from the propagation and release of captive-reared individuals. Condors can currently be found in two distinct populations in the United States based on release sites: Arizona and Utah (referred to herein as the "Arizona" population), and California. Condors have also been released in Baja, Mexico. As of December 2012 there were 235 free-flying birds in the wild and 169 in captivity. However, the condor population's high rate of mortality and its low reproductive success have prevented recovery of the species. The leading causes of mortality in birds from all release sites were determined to be anthropogenic, with lead poisoning deemed the most important of those identified (Rideout et al. 2012). Extensive conservation efforts towards recovery continue, including annual captive rearing and release, bi-yearly trapping and drawing of a blood sample, chelation treatment of condors that exhibit elevated lead concentrations, supplemental feeding, radio tracking, and daily monitoring.

While exposure to other sources of lead cannot be definitively ruled out, and some have been documented in select cases (e.g., lead paint; Finkelstein et al. 2012), the feeding habits of the condor make it particularly vulnerable to lead exposure from hunter-killed carcasses left in the field. Condors in interior habitats rely heavily on mule deer, elk, pronghorn antelope (*Antilocapra americana*), feral hogs, and domestic ungulates, but may also scavenge smaller mammals such as rabbits, ground squirrels, and coyotes (USFWS 2012; 2013c). Condors maintain wide-ranging foraging patterns throughout the year, allowing for opportunistic feeding in accordance with food supplies (Meretsky and Snyder 1992).

California: In California, the total harvest of game and varmints from the counties in the condor's range can be quite high, and in 2010 included approximately 9000 deer, 17,000 cottontail rabbits, 9500 jackrabbits, 8500 wild pigs, 13,000 coyotes, and 7000 tree squirrels (California Department of Fish and Game 2011). In 2007, the State of California passed a law that banned the use of lead ammunition in the range of the condor for big game and varmint hunting. Despite this, declines in blood lead concentrations were not found in the California population in the years following the ban (Finkelstein et al. 2012). Kelly et al. (2014b) suggest that this lack of decline may be attributable to a lesser reliance on intensive management in condors sampled after the ban. Based on their analysis of blood samples collected from

the California population between 1997 and 2011, lead concentrations increased as the time an individual went undetected near provisioning and release sites increased, and as reliance on food provisioning decreased. Time undetected was significantly higher and reliance on food provisioning was significantly lower and post-ban years as compared to pre-ban years (Kelly et al. 2014b). Condors foraging independently of provisioned food may be vulnerable to exposure from lead ammunition remaining on the landscape from poaching, non-compliance with the ban, and shooting activities not addressed under the ban (such as upland game hunting, nuisance animal control, and dispatching domestic livestock).

The USFWS summarized condor mortality up to 2012 for the California population (USFWS 2013c). Condors were first released in California in 1992. By December of 2012, 42 (34 %) out of 123 cases where a cause of death was known were attributed to lead poisoning (USFWS 2013c). Rideout et al. (2012) provided details of condor fatalities occurring between 1993 and 2009. Diagnostic criteria for clinical poisoning in these cases were >0.5 ppm lead in blood (antemortem) or >6 ppm wet weight in liver or kidney (post-mortem). Some cases had additional supporting evidence such as lead fragments in gastrointestinal tracts, or histological findings consistent with lead exposure. In California, three of nine lead poisoning cases were based on liver lead concentrations of 11, 21, and 26.4 ppm wet weight (Rideout et al. 2012). In five cases, condors received chelation therapy but did not recover. Chelation therapy results in lowered lead concentrations in tissues, and as such, these condors had lead concentrations in liver (<3.7 ppm wet weight) below the threshold level for clinical poisoning at the time of death. The remaining case involved a condor with lead toxicosis that did not recover after receiving therapy. Based on feather analysis, this condor was exposed to lead four times since its release 180 days earlier, including one event in which lead concentrations were severely elevated (3 ppm) in blood.

Finkelstein et al. (2012) tested blood samples multiple times throughout the year from up to 150 free-flying condors in the California population between 1997 and 2010. Based on 1154 samples analyzed for lead and lead isotope ratios, an average of 71 % of the birds tested each year were exposed to lead above the selected background concentration of 0.1 ppm (approximately threefold higher than the average background lead level of pre-release condors with no history of lead exposure, 0.0303 ppm). Thirty percent of blood samples collected each year indicated exposure to lead >0.2 ppm, indicative of subclinical health effects (>60 % inhibition of ALAD). About 20 % of the birds tested each year were exposed to lead concentrations >0.45 ppm, the threshold selected for chelation therapy to avert morbidity or mortality. Over the period of 1997–2010, 48 % of the free-flying birds had blood lead concentrations over this threshold. The use of isotopic analysis to identify possible sources of lead exposure revealed that 79 % of the 150 condors sampled contained lead with ratios that overlapped with those measured in ammunition by the researchers (Finkelstein et al. 2012). Other samples contained isotope ratios consistent with leaded paint from a tower where condors were observed roosting. The remaining isotopic ratios were not consistent with known sources of lead.

Arizona: In Arizona, harvest in 2011 within the game management units in the condor's range included over 1000 mule deer, several hundred elk and pronghorn, and potentially thousands of cottontail rabbits, coyotes, and tree squirrels based on statewide totals (Arizona Game and Fish Department 2012). In 2003, the State of Arizona instituted a voluntary program offering incentives for the use of non-toxic ammunition for big game hunting or the removal of offal harvested with lead ammunition. Despite these measures, declines in blood lead concentrations were not found in the Arizona population in the years following the instatement of this program (Parish et al. 2009). Movement from Arizona to the Utah highlands for mule deer and elk hunting season, where no lead reduction program existed until 2012, was associated with a spike of blood lead concentrations during and just after that state's big game hunting season (Parish et al. 2009). Additionally, poaching, noncompliance with lead reduction programs, and shooting activities not addressed by these programs (such as small game and varmint hunting) likely contributes to lead remaining on the landscape throughout the range in Arizona.

The USFWS summarized condor mortality through 2011 for the Arizona population. Condors were first released in Arizona in December of 1996. By the end of 2011, 69 out of 134 released condors had died (USFWS 2012). Of 44 cases where the cause of death was determined, 21 deaths (48 %) have been attributed to lead poisoning (USFWS 2012). Of these birds, eight carcasses contained bullet fragments, one had a whole bullet, and five had shot. Rideout et al. (2012) provided details of condor fatalities occurring between 1993 and 2009. In Arizona, 7 of the 12 lead poisoning cases were based on liver lead concentrations ranging from 17 to 62 ppm wet weight (Rideout et al. 2012). Lead fragments were identified in the gastrointestinal tract of three of these individuals. The remaining five lead poisoning cases were for condors that received chelation therapy but did not recover. As a result of therapy, lead concentrations in liver (<5.2 ppm wet weight) were below the threshold for clinical poisoning at the time of death. Metallic fragments were detected in the gastrointestinal tracts of three of these five treated condors, though were not identified as lead or another metal. Condor mortality in Arizona has predominantly occurred in the fall and winter months and is associated with the big-game hunting seasons (Parish et al. 2009).

Parish et al. (2009) collected blood multiple times throughout the year from up to 64 condors in the Arizona population between 2000 and 2007. Depending on the year, an average of 48–95 % of the birds tested were exposed to lead. Birds were held and monitored when blood lead concentrations were >0.3 ppm. Chelation therapy was administered to condors with concentrations above this threshold whose blood lead continued to increase, or those with concentrations >0.6 ppm, amounting to 4–70 % of the Arizona population treated per year. Since 2002, an increase of condor blood lead concentrations has been found to correspond with deer hunting areas in southern Utah (USFWS 2012).

Key Points: Occurrence of Lead in Avian Scavengers

- Lead exposure and poisoning has been broadly documented in predatory and scavenging birds including eagles, condors, vultures, hawks, and ravens.
- There is broad geographic distribution of lead exposure and poisoning of scavenging birds across the United States.
- Elevated lead concentrations have not only been detected in tissues of dead or moribund birds, but also in random sampling of free-flying birds.
- Elevated lead was detected in tissues of some birds that were diagnosed with mortality from trauma.
- For bald eagles wintering in the Midwest, lead poisoning is a common diagnosis in moribund and dead eagles submitted to rehabilitators and diagnostic labs annually.
- For condors in Arizona and California, nearly half of all fatalities through 2011 and 2012, respectively, have been attributed to lead poisoning.
- Measures to reduce the amount of lead ammunition in the condor's range have likely contributed to a decline in lead availability, but lead ammunition may still be present from upland game hunting, nuisance animal depredation, dispatching domestic livestock, poaching, and non-compliance of lead ban regulations.

7 Association of Lead Exposure with Spent Ammunition

In addition to the diet and foraging strategies discussed above that make scavenging birds susceptible to ingesting ammunition, there are additional factors that support ammunition as the primary source of lead exposure in the cases just described: greater frequency and/or magnitude of lead exposure during or following hunting seasons, observation of ammunition in birds with lead exposure, and isotopic signatures of lead within the range used for the manufacture of ammunition.

7.1 Temporal Association with Hunting Season

Seasonality can play an important role in lead exposure as birds opportunistically forage on readily available food sources such as hunter-killed deer carcasses and offal piles. An increase in the frequency of lead exposure events and mortality during or immediately after game hunting seasons was formerly observed in scavenging birds prior to the lead shot ban for waterfowl in the United States (Bloom et al. 1989; Wiemeyer et al. 1988). This temporal trend continues to be observed, as presented in Table 4 and in the previous section (Kramer and Redig 1997; McBride

et al. 2004; Hunt et al. 2007; Craighead and Bedrosian 2008; Bedrosian and Craighead 2009; Parish et al. 2009; Strom et al. 2009; Stauber et al. 2010; Kelly and Johnson 2011; Bedrosian et al. 2012; Cruz-Martinez et al. 2012; USFWS 2012; Mierzykowski et al. 2013; Russell and Franson 2014; Langner et al. 2015). Instances of poaching that occur outside of legal hunting seasons or exposure to small game or varmints shot with lead in other seasons can weaken these temporal trends.

7.2 Detection of Ammunition

Lead fragments or shot may be recovered at necropsy and removed surgically (Fig. 7), or present on radiographs (Fig. 6). In some cases, lead poisoned birds have also been observed feeding on contaminated carcasses. While many cases lack this direct line of evidence, there are numerous citations of such that complete the pathway, as presented in Table 4 and above (Kramer and Redig 1997; Neumann 2009; Nam et al. 2011; Cruz-Martinez et al. 2012; Rideout et al. 2012; USFWS 2012; Franson and Russell 2014; Kelly et al. 2014a).

Birds may also be exposed to lead ammunition despite no detection or recovery of lead fragments from the gastrointestinal tract. As described previously, the frangibility of lead can produce fragments small enough to be completely dissolved in the gastrointestinal tract. Absorption of fragments is enhanced by the physiology of

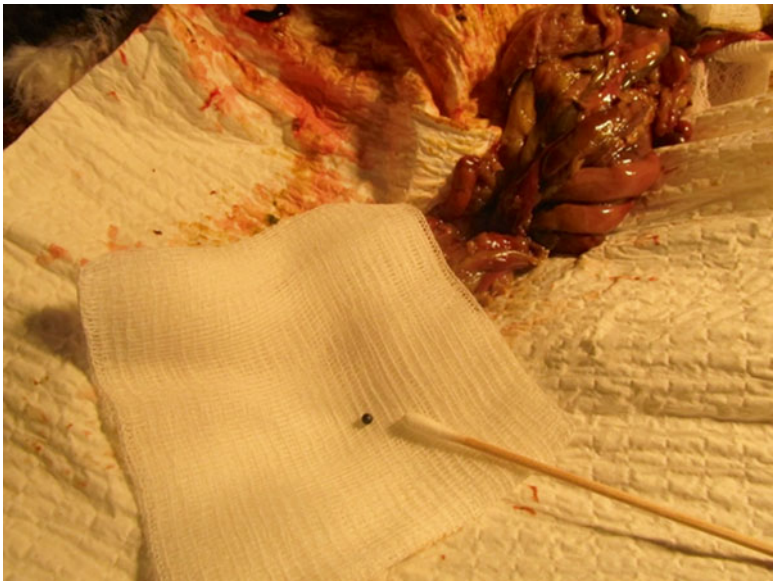

Fig. 7 Retrieval of lead shot from gastrointestinal tract of a bald eagle (*Haliaeetus leucocephalus*). Photo courtesy of Kay Neumann, Saving Our Avian Resources, 25494 320th Str, Dedham, IA 51440

raptors that combines an extremely acidic environment with prolonged digestion. Fragments that are not completely absorbed may also be passed by birds prior to death or capture, leaving no evidence in radiographs or post-mortem examination (Rideout et al. 2012). Fragments that are not completely absorbed may also be egested and lead shot has been found in casts for raptors sampled in the wild (Griffin et al. 1980; Nelson et al. 1989). In one laboratory study, the time between consumption of a meal and cast egestion in captive bald eagles was about 22 h (Duke et al. 1976). Therefore, cast egestion is not a means of completely eliminating ingested lead, as lead can be retained in the stomach for a period of time and absorbed before cast egestion. In laboratory studies of scavenging birds dosed with lead shot, retention of shot varied among individuals of the same species and under similar treatment regimens (Pattee et al. 1981, 2006; Carpenter et al. 2003).

7.3 Isotopic Ratios in Lead-Exposed Birds

Lead has four stable isotopes that occur naturally: ^{204}Pb, ^{206}Pb, ^{207}Pb, and ^{208}Pb. Ores from which lead is mined are made up of various percentages of these four isotopes, and those percentages are maintained as lead is extracted and manufactured into products. The ratio of any two of these isotopes is sufficient to provide a unique identifier for the lead, with the ratio of ^{207}Pb:^{206}Pb most commonly reported (all ratios are reported as such herein). Measuring these ratios in human or wildlife tissue and comparing them to ratios in particular or potential sources of lead (e.g., paint, ammunition, mine tailings) can help to elucidate how biota are exposed to this toxicant.

In an attempt to identify sources of lead exposure for wildlife, several studies have examined isotopic signatures of ammunition for comparison with ratios measured in tissues of exposed individuals. Scheuhammer and Templeton (1998) measured ^{207}Pb:^{206}Pb ratios in 22 brands of lead shotshells, finding low variability within cartridges and boxes, but higher variability between brands. Ratios from tissues of lead-exposed birds (game birds, waterfowl, and bald and golden eagles) showed ranges and patterns similar to those measured for shot (0.787–0.935). Few samples had ratios within the narrow range associated with environmental lead from gasoline (0.8658–0.8811), with the exception of bones from ten lead-exposed herring gulls, a species that rarely ingests lead ammunition. None of the samples analyzed in this study were consistent with ratios associated with mine and smelter waste.

Church et al. (2006) compared ^{207}Pb:^{206}Pb isotopic ratios measured in ammunition, condor dietary items, and condor tissue. Ratios from 13 boxes of purchased ammunition and nine individual rifle bullets donated from hunters ranged from 0.8054 to 0.8145, much narrower than that determined by Scheuhammer and Templeton (1998). Lead isotope ratios in seven samples of dietary items (0.8253–0.8394) were similar to background environmental ratios in California obtained from literature (0.8338–0.8453). Blood was collected from both captive (pre-release) and free-ranging condors. Lead levels and isotopic ratios were strongly inversely

associated; as condors were exposed to greater lead concentrations, isotopic ratios declined. Isotopic ratios from pre-release condors (0.8296–0.8351) were significantly different than free-flying condors with blood lead above 0.0375 ppm (0.8101–0.8307), indicating that higher lead concentrations were associated with exposure to a novel source of lead and perhaps shifting downwards towards ratios associated with ammunition.

Whereas Church et al. (2006) contend that the narrow range of isotopic ratios measured in ammunition purchased locally for their analysis is representative for California condor exposure, subsequent studies expanded this range by testing a more expansive set of ammunition. Finkelstein et al. (2012) collected ammunition from an exchange program, hunters, or from shot carcasses and found an isotopic range of approximately 0.78–0.87 ^{207}Pb:^{206}Pb. Results from condor blood analyses in this study were similar to Church et al. (2006) in that condors exposed to greater lead concentrations showed a decline in isotopic ratios. Isotopic ratios ranged from 0.8296 to 0.8483 in pre-release condors and 0.7602–0.9145 in wild condors. Lead exposure for five birds at the high end of the range (>0.9) was attributed to lead-based paint from a fire lookout tower where condors had been observed roosting (for further discussion, see below: Alternative Sources of Lead: Paint). Lead fragments recovered from six condors that were either lead poisoned or observed feeding on a carcass shot with lead-based ammunition had isotopic ratios that were similar to blood collected from the same bird. The isotopic range of the fragment/blood pairs were approximately 0.81–0.83, overlapping slightly with ratios of pre-release condors. Nine of the 110 condors sampled had isotopic ratios that could not be explained by background, ammunition, or paint.

Ranges of ^{207}Pb:^{206}Pb ratios were relatively wide in femurs of black vultures (0.8055–0.8813, N = 98) and turkey vultures (0.8121–0.8513, N = 10) collected from Virginia between July 2011 and May 2012 (Behmke et al. 2015). The authors compared these ratios with those obtained from literature from several sources and found overlap in varying degrees with those measured for ammunition, leaded gasoline, coal emissions, and zinc smelting. Comparisons of liver and bone lead concentrations in these vultures suggested that all had long-term exposure to lead, and few had significant recent exposure.

Isotopic Analysis in Feathers

To examine lead concentrations and isotope ratios in condors, Church et al. (2006) collected one retrix feather that was partially grown at the time of death from a carcass. This condor had been in the wild approximately 2.5 years before its carcass was recovered and was subsequently diagnosed with lead poisoning. Following rinsing in detergent, water, ethanol, and nitric acid, the feather was serially sampled along the length of the rachis and vane. As rinsing may not remove all of the atmospherically deposited lead from a feather, distinguishing the contribution of this exposure route, where possible, helps to better characterize lead exposure from ingestion. Since condor feathers can grow about ~5 mm per day, the 24 cm feather

was likely to have been growing 1–2 months. As condors molt on an approximate 2-year cycle (Snyder et al. 1987), this new feather would have been subject to a relatively low extent of external contamination compared to feathers formed earlier. However, it was unknown how much time elapsed between the condor's death and its discovery that may have added to the feather's exposure to the environment. Results revealed the lowest lead concentrations in the oldest part of the feather, rising sharply in the youngest part of the feather to reach values several times the original concentrations. This pattern of lead concentrations is unlikely to have arisen from external contamination, which would produce a pattern opposite to that seen here, with lead concentrations highest in the outermost, or oldest part of the feather. In addition, the increase in lead levels corresponds with a distinct change in isotopic ratios, indicating an exposure to a novel source of lead during a specific time period. These ratios are similar to those found in liver and kidney of the carcass, where exposure is limited to ingested lead. For these reasons, the source of lead measured in this feather is likely to be from ingested lead deposited in the growing feather, and not external deposition.

Notable in this feather, isotopic ratios of ^{207}Pb:^{206}Pb increased with lead levels. Ratios associated with lead concentrations below 40 ppm dry weight ranged from approximately 0.720 to 0.790, markedly different than reported ratios from environmental sources or food items reported by the authors (0.8253–0.8453). A spike to lead concentrations of 50 ppm dry weight in the rachis and 90 ppm dry weight in the vane was associated with isotopic ratios of 0.800–0.810. While these values are consistent with ratios found in ammunition, changes in isotopic ratios can also generally be associated with environmental ratios of lead as well as dietary items of condors. However, as lead shot was recovered post-mortem from the gastrointestinal tract, this direct evidence strongly suggests that exposure to ammunition contributed to elevated lead levels.

Finkelstein et al. (2010) expanded upon the work of Church et al. (2006) with more extensive analysis of lead in the feathers, blood, and tissue of six condors chosen to represent three scenarios: a well-documented lead exposure event (condors observed feeding on a carcass from which lead bullets were recovered), fatalities attributed to lead poisoning, and routine exposure monitoring. For two condors with known exposure, feathers showed a spike in lead concentrations and a drop in isotopic ratios that corresponded with the timing of the feeding event. Isotopic ratios of bullet fragments recovered from the scavenged carcass were similar to ratios in blood collected near the time of feeding. For the two condors that died from lead poisoning, feathers concentrations showed a spike at the time of poisoning that corresponds with a change in isotopic ratios. However, for one condor the isotopic ratio increased rather than declined, indicating that exposure may have derived from a source other than ammunition. In each case, isotopic ratios in blood, liver, and kidney corresponded with the acute exposure event, and ratios in bone (the site of long-term storage of lead) corresponded to pre-release ratios. These findings together indicate a change in both the concentration and source of lead. Of three feathers collected during routine monitoring, two had increased lead concentrations that corresponded with shifts in isotopic ratios, while the third gave ambiguous results,

showing decreasing lead concentrations from older to newer parts of the feather, with no corresponding change in isotopic ratios.

If atmospheric deposition had been a source of lead in these feathers, a pattern of increasing concentrations in older parts of feathers would have been expected, as well as a corresponding isotopic signature. For most of the feathers analyzed in these studies, acute rises in concentration tended to correspond with ratios that were lower than known environmental sources. Abiotic sources (dust, snow-fed lake water, urban aeroseols, environmental lead, river water) measured in California cited by Church et al (2006) fell within the range of 0.8338–0.8453. This is similar to atmospheric concentrations measured in California cities in the 1990s (0.8418–0.8628; Bollhöfer and Rosman 2001). While this represents only a small sampling of atmospheric lead, it is noteworthy that spikes in lead concentrations measured in feathers tended to drive isotopic ratios below the ranges measured in these studies.

Key Points: Association of Lead Exposure to Spent Ammunition

Temporal association with hunting season

- Seasonality can play an important role in lead exposure as birds opportunistically forage on readily available food sources such as hunter-killed deer and offal piles.
- Many studies found an increased rate of lead exposure and mortality during hunting seasons.

Detection of ammunition

- Several studies report retrieval of lead shot or bullet fragments at necropsy, detection on radiographs, or observation of birds feeding on lead contaminated carcasses.
- The absence of a lead fragment does not negate the possibility of ammunition as the source of exposure due to the possibility that a fragment has been passed, regurgitated or completely dissolved, or because this evidence was not sought by an investigator.

Isotopic ratios in lead-exposed birds

- When used with other lines of evidence such as observations, behavioral ecology, or recovery of ingested items, measurement of isotopic ratios can provide information on the source of lead (e.g., ammunition, dietary items, paint) measured in biota or environmental samples.
- Isotopic ratios can vary for any particular source of lead and measured ranges tend to increase with sample size. Ranges from different sources can overlap.

(continued)

(continued)

- Isotopic ratios in blood of exposed birds will be similar to a known source of exposure (e.g., bullet fragment or paint chip removed from bird).
- Changes in isotopic ratios in avian tissues can reveal when a novel source of lead has been introduced into the diet. A noteworthy example is the decline in ^{207}Pb:^{206}Pb ratios associated with increased lead exposure as condors transition from captivity to the wild.
- Sampling across the length of a feather can provide a more refined view of lead exposure over time, as changes in isotopic ratios show when an elevation or decline in lead concentrations is associated with a change in the source of lead.

In a study of Andean condor feathers from the Argentine Patagonia region, Lambertucci et al. (2011) compared lead concentrations and isotopic ratios to seven types of ammunition obtained from retail stores and hunters in the study area. Molted feathers (N = 152) were collected from the base of cliffs where condors congregate to roost. Isotopic ratios from ammunition fell into two ranges, averaging 0.817 for big game hunting, and 0.857 for hare hunting. The higher isotopic ratios were indistinguishable from background ratios for this region, and most feathers with low concentrations fell within this range. While isotopic ratios in ammunition were not predictive of lead concentrations in feathers, isotopic ratios differed between feathers with high (>4 ppm dry weight) and low (<4 ppm dry weight) concentrations, indicating that elevated lead resulted from a novel, possibly anthropogenic, source of lead. While feathers were rinsed prior to analysis, the possibility of external contamination was not examined by the authors.

8 Alternate Sources of Lead as Potential Exposure Routes

While multiple lines of evidence support the conclusion that scavenging birds are exposed to lead by ingesting spent ammunition, there are numerous sources of lead that contribute to its availability on the landscape. Herein, we examine several alternate sources of lead and evaluate the likelihood of exposure for scavenging birds.

8.1 Fishing Sinkers and Lures

Leaded fishing gear (lures, sinkers, weights) used in recreational fishing activities can be discarded in lakes or other waterways, where it may be incidentally ingested by waterbirds, most notably the common loon (*Gavia immer*). Lead poisoning from

fishing lures and sinkers can account for 10–50 % of adult loon mortality where high populations of loons overlap with recreational fishing activity (Scheuhammer and Norris 1996). Common loons submitted to the Wisconsin Department of Natural Resources for necropsy revealed that 30 % had lead poisoning, all of which had lead fishing lures or sinkers recovered from their gastrointestinal tracts (Strom et al. 2009).

Scavenging birds may be exposed to lead from these sources by feeding on waterbirds or fish that have ingested lead lures or sinkers. However, evidence of this route of exposure is limited in the scientific literature. One study in Canada found a bald eagle that had ingested a lead fishing weight (Langelier 1994, cited in Scheuhammer et al. 2003). Piscivorous species that forage in waterways are likely to be at greater risk of lead exposure from discarded lead fishing gear than scavenging raptors.

8.2 Microtrash and Other Metal Objects

The ingestion of metal objects by birds resulting in lead exposure that cannot be positively attributed to ammunition or fishing sinkers has been rarely documented. One notable case was in an endangered Mississippi sandhill crane (*Grus canadensis pulla*) that was diagnosed with lead poisoning based on necropsy findings, liver concentrations of 69–70 ppm wet weight, and an unknown lead object retrieved from the gizzard (Franson and Hereford 1994).

One scavenging species that has been documented to ingest a variety of foreign anthropogenic material, or "microtrash," is the California condor. These objects can be collected from condor nests, where adults regurgitate ingested material for the consumption by nestlings. While it has been hypothesized that condors may not collect trash until feeding nestlings (Walters et al. 2008), there have been no systematic searches of roost sites to look for evidence of regurgitated shot or microtrash during non-breeding periods (Mee et al. 2007). However, inspection of trash from nest sites shows the spectrum of materials collected by the birds.

Nests of seven condor pairs, representing 11 breeding attempts, were monitored from 2001 to 2005 (Mee et al. 2007). Nest-floor substrates were collected at the end of breeding attempts or opportunistically during nest visits using a fine-mesh window screen that allowed for collection of material greater than 1 mm^2. Of 650 microtrash items recovered, 148 (22.8 %) were metallic, including items such as bottle-tops, washers, ammunition casings, and electrical wiring (Mee et al. 2007). No objects that could be associated with alternate sources of lead (e.g., wheel weights) were specifically identified. Necropsies were performed on 13 dead nestlings from the California population between 2001 and 2009 (Rideout et al. 2012). Of these, livers were analyzed from six nestlings that were either diagnosed with trash ingestion as the cause of death, or contained microtrash. None had detectable lead concentrations despite the presence of metallic objects such as nuts and washers

(Rideout et al. 2012). Though these studies represent only a subset of condors, the analyses show no evidence of exposure to lead objects in the accumulation of microtrash.

The degree of trash ingestion by condors appears to vary between the California and Arizona populations. Trash ingestion by chicks in Arizona is rare and generally does not contribute to mortality (Walters et al. 2008). The carcass of one chick collected in 2011 contained microtrash, but it was not believed to be the cause of death (USFWS 2012). One juvenile (5 years old) and one adult (11 years old) from the Arizona population were reported to have died from trash ingestion between 1996 and 2010; neither had detectable levels of lead (Rideout et al. 2012). The geographic difference in trash ingestion has been hypothesized to be a function of foraging range and trash density and distribution. Condors in the Arizona population forage in areas that are more remote from anthropogenic activity and may contain less trash (Walters et al. 2008). Although condors of both populations have been observed ingesting metal objects, these cases are few and not a plausible explanation for the widespread lead exposure that occurs in this species.

8.3 Paint

Ingestion of lead-based paint from buildings has been documented as a source of lead exposure for birds. Perhaps the most widespread and studied instance of this exposure occurred on Midway Atoll, where lead poisoned Laysan albatross (*Phoebastria immutabilis*) chicks were associated with proximity to historical buildings and discovered with paint chips in the proventriculus (Sileo and Fefer 1987; Work and Smith 1996). Finkelstein et al. (2003) performed lead isotope analysis on blood lead from asymptomatic and symptomatic chicks on Midway, as well as from soil and paint chips found in or near the nests of sampled chicks. While there was wide variation in isotopic composition of samples in both blood and paint, ratios from blood of chicks exhibiting droopwing, a sign of lead toxicosis, were significantly related to the ratios of lead in paint chips collected from nests. No relationship was found between isotopic ratios of blood and soil samples. Few other examples exist of birds exposed to lead through this route. In an isolated incident, a group of 13 captive sandhill cranes (*Grus canadensis*) exhibited signs of lead poisoning after being moved to a facility that was later discovered to have lead-based paint on the walls, though this event occurred indoors with a non-wild population (Kennedy et al. 1977).

Lead-based paint from an inactive fire lookout tower has been identified as a source of lead exposure for a subset of California condors (N = 5; Finkelstein et al. 2012). These cases were confirmed both by observations of condors roosting on or near the tower, and by matching isotope ratios between blood of these condors and paint chips sampled from the tower (Finkelstein et al. 2012). Lead-based paint can have a wide range of lead isotope ratios (Rabinowitz and Hall 2002; Finkelstein et al. 2003). However, by matching isotopic ratios from blood of lead-exposed birds

to the specific source suspected of this exposure based upon condor movements and behavior, it was possible to narrow the range of isotopes to the paint used on the roosting tower. Outside of this somewhat unique exposure pathway, there are no other clear pathways for condors to ingest lead-based paint that are likely to elude detection, considering that condors are highly studied and tracked through both radio transmitters and visual observation (Cogan et al. 2012). Condor association with fire lookout towers in central California is a rare occurrence, as indicated by tracking data (Finkelstein et al. 2012), and reports are not available of condors associating with other historical structures. Frequent exposure to lead-based paint appears implausible in consideration of known behavior. In addition, no examples are available from the scientific literature of birds achieving elevated lead concentrations from chronic low-level exposure to various sources of lead-based paint; each example has been tied to a known source providing the exposure (i.e., buildings on Midway, fire lookout tower).

8.4 Mine Tailings

Lead mines exist throughout the nation, with the largest concentration located in southeastern Missouri, known as the "Old Lead Belt". The process of extracting lead from the earth produces waste tailings that are typically deposited on the landscape if environmental clean-up or storage measures are not followed or in place. Lead tailings have polluted water and sediment in lakes and rivers, and soil in terrestrial habitats. Lead exposure to wildlife can occur when there is direct contact with the tailings dermally or by ingestion, or by consumption of prey items exposed as such.

One of the best known examples of lead poisoning to waterfowl from mine waste is in the Coeur d' Alene River basin in northern Idaho (Chupp and Dalke 1964). Lead from mining and smelting activities in the Coeur d' Alene River system in northern Idaho was associated with mortality in tundra swans (*Cygnus columbianus*) that ingested lead-contaminated sediment and plants (Blus et al. 1991). A more recent publication found that lead-contaminated sediment was the cause of mortality in 77 % of 285 waterfowl (mostly tundra swans) found sick or dead from 1992 to 1997, with an additional 7 % of deaths attributed to lead poisoning from ingested lead shot (Sileo et al. 2001).

Waterfowl are thought to be the most common species exposed to lead from mine waste because of their physiology and foraging behavior; however, passerines can be at risk through ingestion of soil and soil-ingesting invertebrates. Hansen et al. (2011) investigated lead exposure from mine wastes and soil ingestion rates of three species of ground-feeding songbirds, American robin (*Turdus migratorius*), Swainson's thrush (*Catharus ustulatus*), and song sparrow (*Melospiza melodia*), at the Coeur d'Alene River basin. The highest soil ingestion rates and the highest blood lead concentrations were found in the American robin at the most contaminated sites. At these sites, 18 % of 204 American robins had severe poisoning

(>29.4 ppm dry weight), 52 % had clinical poisoning (17.6–29.4 ppm dry weight), and 24 % had subclinical poisoning (5.9–17.6 ppm dry weight). Soil ingestion accounted for almost all of the species' exposure to lead. Beyer et al. (2013) analyzed lead concentrations in soil, earthworms, and songbirds at lead mining and smelting sites in southeast Missouri's Lead Mining District. Lead concentrations in soil sampled from three mining/smelting sites ranged from 1000 to 3000 ppm dry weight, and directly correlated with lead concentrations in earthworms. For six species of songbirds analyzed at the lead mining/smelting sites, lead concentrations were greater in blood by a factor of 8, in liver by a factor of 13, and in kidney by a factor of 23 compared to the reference site. American robins, which are known to feed on earthworms, had lead concentrations up to 1000 ppm dry weight in kidney.

The likelihood of scavenging birds to directly ingest mine tailings is low. However, secondary exposure from dietary sources has been documented, though rarely. For example, blood samples collected from Eurasian eagle owl chicks (*Bubo bubo*) between 2003 and 2007 were significantly higher at a site contaminated from an abandoned mine than at a reference site, suggesting dietary exposure to lead (Gómez-Ramírez et al. 2010). In the Coeur d'Alene River basin, samples collected from adult and nestling osprey (*Pandion haliaetus*), American kestrels (*Falco sparverius*), northern harriers (*Circus cyaneus*), red-tailed hawks (*Buteo jamaicensis*), great horned owls (*Bubo virginianus*), and western screech-owls (*Otus kennicotti*) contained lead concentrations (Henny et al. 1991, 1994) suggesting these individuals had foraged on lead-contaminated fish and other wildlife (Henny et al. 1991, 1994). However, no effects to behavior, reproduction, or survival were detected in the lead-exposed raptors. The authors suggest that raptors have traits that can reduce their potential for accumulating critical levels of lead which is primarily stored in bones of prey species (Henny et al. 1994). For birds exposed via secondary consumption, lead that is biologically incorporated by prey may be less bioavailable due to partitioning into bone (Custer et al. 1984). In a study of American kestrels fed cockerels that were experimentally treated with lead, kestrels accumulated concentrations in tissues, but exhibited no effects on survival, body mass, or hematological endpoints (Custer et al. 1984). The authors concluded that biologically incorporated lead alone was unlikely to cause lead poisoning.

8.5 Shooting Ranges

Shooting ranges, either military or for recreation, are a source of spent lead ammunition and soil contamination. There are approximately 3000 military shooting ranges and 9000 non-military sites in the United States (USEPA 2005). Birds foraging in these areas or in wildlife habitats adjacent to the shooting ranges can be directly exposed by the uptake of spent shot incidental to grit ingestion, or by ingesting contaminated soil or food. A study investigating the toxicity of lead in soil collected from a small arms-range found that rock doves accumulated lead in the blood, tissues, and feathers when dosed with soil, with an accompanying increase in erythrocyte protoporphyrin at blood concentrations >0.5 ppm (Bannon et al. 2011).

Species most likely to be at risk through direct ingestion of spent lead or contaminated soil on or near shooting ranges are waterfowl, mourning doves, and other species that forage on the ground or use grit to help aid in digestion (i.e., quail, grouse, pheasant). Lead poisoning has been documented in northern pintails that foraged in a tidal meadow contaminated with lead from a trap and skeet shooting range (Roscoe et al. 1989). Greater lead exposure was found in passerines that foraged on the ground near a small-arms range as compared to those in a wildlife habitat a distance from the range (Vyas et al. 2000). The likelihood of scavenging birds that normally feed on live or dead animals to directly ingest contaminated soil or spent lead shot from the ground is low. Scavengers could become exposed through consumption of prey items that have directly ingested spent lead shot or contaminated soil from shooting ranges; however, no examples of this resulting in lead poisoning were found in the literature.

Key Points: Alternate Sources of Lead as Potential Exposure Routes

- Numerous sources of lead contribute to its availability on the landscape, including lead fishing tackle, microtrash, lead-based paints, mining operations, and shooting ranges.
- There are few documented cases of lead poisoning to scavenging birds from alternate exposure routes as compared to lead ammunition.
- These exposure pathways cannot be entirely ruled out as potential sources of lead to scavenging birds; however, foraging habits and behavioral traits of scavenging birds make these improbable as sources of widespread exposure.

9 Toxicity of Alternative Metals Used in Ammunition

The use of alternative metals in ammunition has been proposed, and in some cases implemented (e.g. within the range of the California condor), as a means of breaking the exposure pathway of scavenging birds to lead, provided that ammunition derived from these metals is non-toxic (i.e., does not cause toxicity or death when ingested by migratory birds; USFWS 2013a).

9.1 Non-toxic Shot Approval Process

To determine the types of ammunition that are non-toxic and safe to use for waterfowl hunting, a comprehensive testing method was developed for the registration of alternative shot and shot coatings (Rattner and Morehouse 1994). The protocol, formalized and implemented in 1997, requires manufacturers to abide by a

three-tiered test method in order for the USFWS to consider approval of any proposed non-toxic candidate material (USFWS 1997). Tier 1 requires existing data to be compiled on (1) the physical and chemical characterization of the shot or coating, (2) any existing ecological risk assessments and toxicity information of the candidate material, and (3) available tests that determine the effects on the reproduction of waterbirds. Tier 2 requires erosion rate testing and acute toxicity testing on mallards, invertebrates, and early life stage vertebrates to assess potential impacts on waterfowl habitat. Tier 3 requires chronic exposure tests to mallards under adverse environmental conditions to determine effects on reproduction. Based on the Tier 1 information, the USFWS can approve or deny the candidate material or require further testing in the Tier 2 and Tier 3 requirements (USFWS 1997).

Based on toxicity testing and results from the shot approval process, the USFWS established a maximum environmentally acceptable level of lead in shot as <1 % (USFWS 1995), and steel was the first non-toxic shot approved following the three tiered testing requirement (USFWS 1999). To date, the testing has resulted in 12 approved non-toxic shot types, including different combinations of tungsten, bismuth, tin, iron, copper, and nickel (Table 5), illustrating that there are suitable alternatives to lead that present limited threats to waterfowl and species that consume waterfowl (USFWS 2013a). The testing requirements remain the current method used to approve the registration of non-toxic shot for hunting purposes (USFWS 2013b). While these protocols do not include testing of other forms of ammunition (e.g., bullets), results from studies performed under the approval process can provide data on the toxicity of metals to birds treated under similar conditions that may be suitable for extrapolation to other ammunition types.

Table 5 Shot types approved as nontoxic for waterfowl hunting in the United States

Approved shot type[a]	Percent composition by weight
Bismuth-tin	97 bismuth, and 3 tin
Iron (steel)	Iron and carbon
Iron-tungsten	Any proportion of tungsten, and ≥1 iron
Iron-tungsten-nickel	≥1 iron, any proportion of tungsten, and up to 40 nickel
Tungsten-bronze	51.1 tungsten, 44.4 copper, 3.9 tin, and 0.6 iron, or 60 tungsten, 35.1 copper, 3.9 tin, and 1 iron
Tungsten-iron-copper-nickel	40–76 tungsten, 10–37 iron, 9–16 copper, and 5–7 nickel
Tungsten-matrix	95.9 tungsten, 4.1 polymer
Tungsten-polymer	95.5 tungsten, 4.5 nylon 6 or 11
Tungsten-tin-iron	Any proportions of tungsten and tin, and ≥1 iron
Tungsten-tin-bismuth	Any proportions of tungsten, tin, and bismuth.
Tungsten-tin-iron-nickel	65 tungsten, 21.8 tin, 10.4 iron, and 2.8 nickel
Tungsten-iron-polymer	41.5–95.2 tungsten, 1.5–52.0 iron, and 3.5–8.0 fluoropolymer

[a]Coatings of copper, nickel, tin, zinc, zinc chloride, and zinc chromate on approved nontoxic shot types also are approved

9.2 Toxicity of Alternative Metals

The tiered testing protocol for the approval of non-toxic ammunition provides comparable data on the toxicity of different metal alloys to mallards. In general, the results of these tests found combinations of tungsten, tin, bismuth, iron, and copper to provide limited threats based on a number of health metrics. Studies found no adverse effects on mallards dosed with bismuth-tin and steel (Sanderson et al. 1997), tungsten-iron, tungsten-polymer, and steel (Kelly et al. 1998), and tungsten, tin, and bismuth (Ringelman et al. 1993). Additionally, Sanderson et al. (1997) found no effects on blood chemistries, body and kidney weight, livers, gonads, or gizzards for mallards dosed with bismuth-tin and steel. However, mild impairment of bile flow was observed in mallards in a study that tested tungsten alloys (tungsten-iron and tungsten-polymer; Kelly et al. 1998). Ringelman et al. (1993) suggested that there is no uptake of tungsten, tin, or bismuth to mallard tissues, and study results found no changes in 23 hematology and blood parameters measured. For toxicity tests with iron, mallard mortality rates were low and weight losses were not significantly different from control groups (Irby et al. 1967). Locke et al. (1967) investigated the histopathological response of mallards dosed with uncoated and coated iron shot and also found limited effects. Mallards fed iron, molybdenum coated iron, and zinc coated iron developed hemosiderosis of the liver (Locke et al. 1967).

The mixture of tungsten-tin-bismuth has been tested in scavenging birds, with similar results as those found in the mallard toxicity tests. Risebrough (2001) tested a composite of tungsten-tin-bismuth on turkey vultures trapped from the wild. Tungsten and bismuth did not accumulate in tissues, although tin concentrations increased in the blood. There was no change in body mass, hematology, plasma biochemistries, or histopathology between dosed and control vultures. Krone et al. (2009) investigated the toxicity of brass and zinc to Pekin ducks (*Anas platyrhynchos domestica*) and found no mortality or organ alterations; however, the ducks dosed with zinc showed a higher weight loss compared to ducks dosed with brass, and zinc showed the highest solubility in duck gizzards.

Birds are generally tolerant to metallic copper, and multiple dosing studies found no mortality or clinical signs of toxicity and limited biochemical effects (Bellrose 1965; Irby et al. 1967; Locke et al. 1967; Krone et al. 2009; Bannon et al. 2011; Franson et al. 2012). Toxicity tests on mallards with metallic copper found no mortality in the dosed group (Irby et al. 1967). Locke et al. (1967) investigated the histopathological response of mallards dosed with copper shot, finding no lesions in tissues, no renal acid-fast inclusion bodies, and no hemosiderin in the liver. Bellrose (1965) tested different weights of single copper shot and dosed mallards lost 15–20 % of their initial weight by day 35 but recovered by day 110. The mallards in the copper tests were given doses over the limit of what they would normally be exposed to in the wild and several times the dose used in previous lead shot tests, suggesting that the quantities of normally ingested copper in the wild would result

in lesser effects. Bannon et al. (2011) investigated the toxicity of copper to rock doves that were exposed to small arms-range soil contaminated by spent ammunition from copper jacketed bullets with a lead core, reporting no copper retention in the tissues or any adverse effects. Similarly, Krone et al. (2009) investigated the toxicity of copper to Pekin ducks and found no mortality or organ alterations in the dosed group. Additionally, the ducks dosed with copper did not show weight loss even after 4 weeks of shot retention in the gizzard.

Tests of metallic copper on raptors and scavenging species found similar results. Franson et al. (2012) tested copper shot on captive raised American kestrels and found no mortality or clinical signs of toxicity in the dosed birds. In addition, biochemistries, hematocrit values, and copper concentrations in the kidney and plasma did not differ between dosed and control birds. Copper concentrations in liver were greater in dosed birds, and were correlated with metallothionein concentrations. The authors suggested that birds sharing similar regurgitation frequency as American kestrels are not likely to be adversely affected by copper ingestion. Risebrough (2001) investigated the toxicity of copper to turkey vultures that were trapped from the wild. The vultures were fed food dosed with copper at a level that was considered equivalent to the ingestion of two copper bullets. The study found no mortality; however, the copper levels were greater in the livers of dosed vultures compared to those in the control group.

Although metallic copper does not seemingly present a health threat to birds, the salts of copper have been found to exhibit toxicity in selected cases. Kobayashi et al. (1992) investigated a die-off of free-ranging mute swans with high copper concentrations in liver tissue, and attributed the deaths to copper poisoning of unknown source. Henderson and Winterfield (1975) recorded two cases of copper poisoning in Canada geese; copper sulfate used as an algicide was the suggested cause.

Tissue concentrations of copper can vary widely among individuals and species, and can reach seemingly elevated levels without detectable effects to individuals. For example, copper concentrations in the livers of California condors ranged from 2.2 to 531 ppm wet weight at necropsy (Rideout et al. 2012). None of these birds were diagnosed with copper toxicosis. In American kestrels dosed with copper shot the mean liver concentration was 20.7 ppm dry weight (Franson et al. 2012). Multiple studies report much higher concentrations in liver, notably mute swans and common eiders. The copper concentration in the livers of three mute swans found dead in Mamaroneck Harbor, New York, ranged from 1562 to 5857 ppm dry weight, while concentrations in two captive mute swans were much lower, 64 and 121 ppm dry weight (Molnar 1983). The copper contamination was suspected to be from antifouling paint released in water through the scraping of vessel hulls. Clausen and Wolstrup (1978) and Kobayashi et al. (1992) also detected high concentrations in mute swans, and other studies found copper concentrations of over 1000 ppm wet weight in the livers of common eiders (Norheim and Borch-Iohnsen 1990; Hollmén et al. 1998; Franson et al. 2000; Stout et al. 2002). Because of this variability, interpretation of copper concentrations in tissue should be done with caution, as seemingly elevated levels do not necessarily signify adverse effects.

Key Points: Toxicity of Alternative Metals used in Ammunition

- A comprehensive testing method exists in the United States to ensure alternative metals used to hunt waterfowl do not cause sickness and death when ingested by migratory birds. These data can be extrapolated to ammunition used for other forms of hunting.
- The non-toxic shot approval process has resulted in the approval of suitable alternatives to lead that present limited environmental threats.
- Copper, the primary alternative currently used in bullets, exhibits low toxicity to birds in its metallic form.

10 Conclusion

Although there are multiple sources of lead in the environment, scientific evidence points to spent lead ammunition as the most frequent cause of lead exposure and poisoning in scavenging birds. Numerous lines of evidence support this conclusion, including the extent of lead ammunition currently used for hunting and its tendency to fragment, the behavioral ecology and physiology of scavenging birds, their susceptibility to lead as exhibited in controlled dosing studies, the diagnosis of lead poisoning by well-established tissue thresholds and clinical signs, the recovery of ingested lead fragments or shot from exposed birds, observations of birds feeding on contaminated carcasses, isotopic analyses relating tissue concentrations to ammunition, patterns of mortality coincident with hunting seasons, and the lack of abundant evidence for other sources of lead. While few cases may exhibit all of these lines of evidence, there are numerous documented cases of lead poisoned scavengers in the literature and many are supported by one or more lines of evidence. Lead can be replaced in ammunition by alternative metals that are currently available and present limited environmental threats. Scientific literature shows spent lead ammunition to be the primary pathway for widespread lead exposure to scavenging birds in the United States. Reducing this route of exposure will result in the greatest alleviation of mortality and other adverse effects to these species from lead in the environment.

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