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***Caenorhabditis elegans* as a tool for Environmental Risk Assessment – emerging and promising applications for a “nobelized worm”**

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Joana Luísa Pereira is a researcher at the Department of Biology and the Centre for Environmental and Marine Studies (CESAM) University of Aveiro since her PhD in Biology, awarded in 2008. Her main research lines are within the fields of ecotoxicology and environmental risk assessment, with strong links to limnology (including under regulatory frameworks such as the EU-Water Framework Directive), extreme environments, invasive species and aquatic nuisance management and education for sustainability. She has been investing in the development of new approaches and testing platforms towards the improvement of prospective and retrospective environmental risk assessment routines, always aiming for the increase of environmental realism and/or better prediction capabilities. Pesticides, metal elements and designer chemicals have been the major contaminants focused within this prospective context. She published so far more than 60 SCI papers, collecting more than 800 citations.

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Michael Aschner serves as the Harold and Muriel Block Chair in Molecular Pharmacology at Albert Einstein College of Medicine. He served on numerous toxicology panels (Institute of Medicine, US Environmental Protection Agency, Center for Disease Control, FDA), and was a member of the Neurotoxicology and Alcohol study section (NIH). He currently serves on the SOT Council, and will soon assume the presidency of ATS. He has authored (co-authored) >600 peer reviewed manuscripts and 100 book chapters. Studies in his lab are designed to (1) shed novel mechanistic insight into metal-induced neurodegeneration; (2) identify targets for genetic or pharmacologic modulation of neurodegenerative disorders; (3) increase knowledge of the pathway involved in oxidative stress; (4) develop improved research models for human disease using knowledge of environmental sciences. *C. elegans* has been routinely used as a model at Dr. Aschner's lab.

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Declaration of Interest

The authors report no conflict of interest.

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Abstract

Caenorhabditis elegans has been an invaluable model organism in research fields such as developmental biology and neurobiology. Neurotoxicity is one of the subfields greatly profiting from the *C. elegans* model within biomedical context, while the corresponding potential of the organism applied to environmental studies is relevant but has been largely underexplored. Within the biomedical scope, the implication of metals and organic chemicals with pesticide activity (hereinafter designated as pesticides) in the etiology of several neurodegenerative diseases has been extensively investigated using this nematode as a primary model organism. Additionally, as a well-known experimental model bearing high sensitivity to different contaminants and representing important functional levels in soil and aquatic ecosystems, *C. elegans* has high potential to be extensively integrated within Environmental Risk Assessment (ERA) routines. In spite of the recognition of some regulatory agencies, this actual step has yet to be made. The purpose of this review is to discuss the major advantages supporting the inclusion of *C. elegans* in lower tiers of ERA. Special emphasis was given to its sensitivity to metals and pesticides, which is similar to that of other model organisms commonly used in ERA (e.g. *Daphnia magna* and *Eisenia* sp.), and to the large array of endpoints that can be tested with the species, both concerning the aquatic and the soil compartments. The inclusion of *C. elegans* testing may hence represent a relevant advance in ERA of chemicals, providing ecologically relevant insights towards improvement of the regulatory capacity for establishing appropriate environmental protection benchmarks.

Keywords

Caenorhabditis elegans; environmental risk assessment; neurotoxicity; pesticides; metals

Introduction

The discharge of trace elements and organic contaminants to the environment resulting from different human activities has been of utmost concern. Soil and aquatic compartments can be severely affected, as a result of direct discharge or indirect contamination following e.g. runoff and/or leaching (Petrie et al. 2014; Morrissey et al. 2015). Although the presence of contaminants in different environmental compartments does not indicate *per se* deleterious effects on organisms, cause-effect relationships may indeed exist. Consequently, the risk to human health and the environment related to contaminants presence in these compartments should be effectively assessed based on worldwide agreed upon regulatory standards. In this context, in the European Union (EU), the European Commission (EC) Regulation No 1907/2006 concerns the Registration, Evaluation, Authorization and restriction of Chemicals (REACH) based in risk assessment frameworks (EC 2018). Based on this regulation, it is likely ensured that substances, mixtures of substances and articles containing substances placed on the EU market do not cause adverse effects on human health and on the environment. This regulation originally considers all types of substances (although targeted regulation may then extend further on specific requirements), including metals and ingredients used in the formulation of pesticides, which are the two groups of contaminants

focused upon in this article. In general, trace elements are regulated by REACH, and by the EC regulation No 1881/2006 that controls maximum levels of Pb, Cd, Hg and inorganic Sn in food (European Commission 2006). On the other hand, there is specific regulation with requirements and conditions for approval of biocidal Plant Protection Products [EC regulation No 1107/2009 (EC 2009)]. These can be defined as products mainly used in agriculture to protect crops against pests, mostly pesticides, but also other products such as growth regulators and pheromones. According to the EC regulation No 1107/2009, plant protection products (hereinafter generally referred to as pesticides for readability purposes) shall have no unacceptable effects on the environment, including contamination of surface and ground waters, air and soil, and they should specifically have no adverse impact on non-target species directly or indirectly exposed to the product.

To appropriately evaluate the risk that pesticides can pose to the environment, Environmental Risk Assessment (ERA) schemes are necessary, which are based in tiered approaches intending to assess the putative adverse effects and derive the regulatory acceptable concentrations. In general, similar approaches intending to protect human health and the environment from chemical threats are followed in the USA and Canada. In the USA, the Environmental Protection Agency (EPA) assumes the regulatory enforcement since the early 1970s under some federal laws. The Toxic Substances Control Act published in 1976 controls the production, importation, use and disposal of specific chemical substances and/or mixtures. Some chemicals such as pesticides are excluded from this act. Pesticides are rather regulated by the Federal Insecticide, Fungicide and Rodenticide Act. Also specifically dedicated, the Resource Recovery and Conservation Act lists and monitors a group of eight metals (As, Ba, Cd, Cr, Pb, Hg, Se and Ag. In Canada, pesticide protection goals are managed by the Health Canada's Pest Management Regulatory Agency under the Pest Control Products Act and corresponding regulations (Pelaez et al. 2013; Canada 2018a; Canada 2018b; EPA 2018a; EPA 2018b; EPA 2018c; EPA 2018d).

As an example, the recommended framework for the ERA of pesticides in the EU, proposed by the European Food Safety Authority panel, is worthy of summarizing. For aquatic organisms in edge-of-field surface waters (Figure 1-A), four tiers (1–4) in ascending order of ecological realism and decreasing order of conservatism are used, all intending to achieve a given protection goal (EFSA 2013). Higher tiers are used when the lower tiers indicate unacceptable risk to the tested organisms by calculated risk quotients. These quotients are determined as the ratio between assessed effects and exposure likelihood, being the latter estimated on the basis of modelling approaches. In tiers 1 and 2, standard laboratorial tests with single species are used to assess effects (e.g. growth and reproduction inhibition), while an increase in the number of single species tested applies to the second tier. Tiers 3 and 4 include less standardized tests and are typically costlier, both in time and budget, since they require population/community studies (e.g. using micro and mesocosm approaches) and field studies, respectively. To evaluate the risk for in-soil organisms exposed to pesticides, the tiered approach is somewhat different (Figure 1-B). If the laboratorial studies performed in the lower tier indicate unacceptable risk, then field studies with realistic exposures are readily applicable in the highest tier (EFSA 2017). In the USA and Canada, these tiered approaches with increasing levels of refinement are also used, however the number and

identification of the tiers is slightly different (tiers 1 to 3 and tiers 0 to 3 for USA and Canada, respectively) (Canada 2018a; Canada 2018b; EPA 2018a; EPA 2018b).

Depending on the specific assessment context, as well as on the type of pesticide, different model organisms are recommended. For example, to evaluate the impacts of pesticides with an herbicidal mode of action at edge-of-field surface waters, the EFSA panel recommends the use of specific aquatic and sediment-dwelling organisms. The crustacean *Daphnia sp.* (OECD 2004a) and the fish *Oncorhynchus mykiss* (e.g. OECD 1992) are recommended when the focus is on acute toxicity. To assess chronic toxicity EFSA propose the use of a green algae such as *Raphidocelis subcapitata* (OECD 2011a), an additional non-green alga such as the diatom *Navicula pelliculosa* (OECD 2011a), a macrophyte such as *Lemna sp.* (OECD 2006), again *Daphnia sp.* (OECD 2008) and *Chironomus riparius* larvae (OECD 2011b) or the oligochaete *Lumbriculus sp.* (OECD 2007a) (EFSA 2013). Currently, to evaluate the impact of pesticides for in-soil organisms (EFSA 2017) the lower tier mostly considers a selection of invertebrate model species such as the earthworms *Eisenia fetida* or *Eisenia andrei* (ISO 2008; OECD 2015), the collembolans *Folsomia candida* or *Folsomia fimetaria* (ISO 1999; OECD 2009), the mite *Hypoaspis aculeifer* (OECD 2004b) and one microorganism-mediated process (N transformation) test (OECD 2000).

Specifically, regarding soil-directed risk assessment, it has been recognized that the standard organisms that have been recommended for testing might not be the most sensitive species, while groups such as the mycorrhiza, nematodes, terrestrial gastropods, enchytraeids and macrodetritivores are poorly represented (e.g. Bongers & Ferris 1999; De Vaufléury et al. 2006; EFSA 2017; Mallmann et al. 2018). In fact, the addition of a test with mycorrhizal fungi was recently proposed (EFSA 2017). Additionally, the nematode species *Caenorhabditis elegans* has been recognized as a meaningful add-on to the ecotoxicological test battery in pesticides ERA at least in the EU (EFSA, 2017), considering the availability of standard test guidelines to assess both acute (ASTM International 2014) and chronic (ISO 2010) toxicity. In fact, *C. elegans* and other nematodes have been widely presented as suitable ecological indicators for monitoring water (Boyd & Williams 2003a; ISO 2010) and soil (Peredney & Williams 2000; Höss et al. 2009; ASTM International 2014) quality. This nematode species should be included in test batteries used for the evaluation of pesticide impacts both regarding the water and soil compartments (specifically in lower ERA tiers), as detailed in the guideline ISO 10872 (ISO 2010) standardizing chronic tests with *C. elegans* in aqueous medium, sediments and soil (Figure 1-A and B). This guideline is designed for assessing chronic effects on growth, fertility and reproduction of the nematode, while the guideline ASTM E2172–01 (2014) supports acute toxicity assessment in soil (Figure 1-B), using lethality as an endpoint. Still, *C. elegans* has yet to be routinely included in ERA test batteries. Although no standard guideline to assess behavioral ecotoxicology and/or neuro-ecotoxicology is currently available for *C. elegans*, this organism could also be used under this context, as demonstrated by previous studies. For example, computer tracking methods were used to feasibly assess behavioral changes in the nematode as a response to exposure to different contaminants (e.g. Boyd et al. 2000; Dhawan et al. 2000; Cole et al. 2004). Others studies went further when addressing alterations in the locomotive behavior by investigating associations with dysfunction of the nervous system (Negga et al. 2012; e.g. Fagundez et al. 2015). The European Food Safety Authority, the Organization for Economic Co-operation

and Development and the US Toxic Substances Control Act already recognized that there is a need to develop standardized protocols using models alternative to vertebrate animals (; EFSA 2009; EPA 2019) to assess effects of thousands of chemicals present in industrial, agricultural and consumer products in a time- and cost-efficient manner (Fritsche et al. 2015; e.g. Fritsche 2017; Bal-Price et al. 2018). A well-studied organism like *C. elegans* is at the forefront as candidate model in this context, especially to replace rodent testing.

Since 1960s that *C. elegans* has been a relevant experimental model organism in fields such as developmental biology and neurobiology. Its remarkable course in this context made it a nobelized worm once several Nobel Prizes have been awarded to researchers for discoveries based on *C. elegans*: Sydney Brenner, Robert Horvitz, and John Sulston earned the 2002 Nobel Prize in Physiology or Medicine; Andrew Fire and Craig Mello were awarded in 2006 with the Nobel Prize in Physiology or Medicine (Fire 2007; Mello 2007). Several characteristics, such as its easy maintenance in the laboratory, presence of a neuronal system with neuronal signal pathways and molecular networks shared with mammals, and the availability of its complete genome sequence, explain the growing and wide use of this organism as a model (Corsi et al. 2015). The use of *C. elegans* for research in medical and related fields is conspicuous, while its use in environmental sciences remains seldom and its potential is far from being recognized. An detailed search regarding topics related to the biomedical and environmental sciences fields in the scientific literature confirmed this picture (see Table S1 for details), showing that there are still few ecotoxicological studies with *C. elegans* (around 60 documents) compared to those developed within the scope of the biomedical sciences (more than 300 documents). It is notable that the studies related to neurodegenerative diseases are clearly collecting the highest relative frequency.

This review was built on the understanding that *C. elegans* utility in the biomedical fields does not extend to disciplines more strictly linking to environmental sciences, although both human health and ecological risk assessment are critical and mandatory to regulate the environmental safety of chemicals such as trace elements and pesticides. The overarching aim here was to collect relevant information about the studies that have been performed with this nematode, concerning their general purpose and specificities such as the contaminants, test platforms, test duration and endpoints assessed. Through this approach, we intended to show the range of applications available for the nematode towards clarification of different environmental research questions and its value as an add-on to ERA test batteries. Our attention was centered on pesticides and metals as major groups of contaminants of environmental concern.

Pesticides are chemicals specifically designed to affect biological targets in order to increase agricultural yield and, consequently, ensure the survival of human populations worldwide. Metals have been widely surveyed in different environmental compartments and bear the potential to bioaccumulate and biomagnify through the food web. Specific and tight environmental regulation is used worldwide to control these two groups of contaminants. Consistently, the literature review pointed out to these two classes as being the most commonly used in studies with *C. elegans*. A comparison of sensitivity to metals and pesticides was then done between the nematode species and two model invertebrates frequently used in ERA, in order to clarify its relative sensitivity and the applicability as an

add-on to the ecotoxicological toolboxes supporting regulatory efforts. For these comparison purposes, the crustacean species *Daphnia magna* and the earthworm *Eisenia fetida* were selected, given their representativeness in surface waters and soil, respectively (Figure 1). The scope and aims of this review are addressed following a structured line of reasoning. First, *C. elegans* is characterized as a model organism, while the features supporting its use in environmental sciences are presented, with special attention being given to the neurotoxicity studies that can represent an important plus to ERA test batteries. Then, the versatility of this organism is exposed, elucidating on the possibilities for testing at different levels of biological organization and using diverse test platforms. Finally, the suitability of including *C. elegans* in ERA is addressed by appraising its relative sensitivity to the selected two classes of contaminants.

Features promoting *C. elegans* as a model species

Caenorhabditis elegans is a nematode of worldwide distribution in the wild. It is a small sized organism, with adults and its newly hatched larvae being 1 and 0.25 millimeters long, respectively. *C. elegans* can be isolated from organic-rich garden soils, compost and rotting vegetable matter since these substrates contain bacteria and small eukaryotes that the nematode can use as a food resource (Corsi et al. 2015; Frézal & Félix 2015). The organism can also be recovered from snails (e.g. *Helix aspersa*), since associations of this nematode species with terrestrial gastropods and other small soil organisms are common (Caswell-Chen et al. 2005; Schulenburg & Félix 2017). More rarely, *C. elegans* can also be found living in aquatic ecosystems (Majdi and Traunspurger 2015; Rasch et al. 2016; Abu Khweek and Amer 2018). It was described in the waterfront of saprobic rivers in Germany, in freshwater habitats in Italy and in aquatic biofilms in France (Kiontke 2006; Rasch et al. 2016). In fact, this species is abundant in microbe-rich environments (Schulenburg & Félix 2017). In the laboratory, the organism is normally cultured in agar plates with an added lawn of the bacterium *Escherichia coli* as food. In soil habitats, *C. elegans* has an important role in nutrient cycling and, consequently, in maintaining agricultural production, its sustainability and environmental quality, similarly to other soil invertebrates (Traunspurger et al. 1997; Höss et al. 1999; Lavelle et al. 2006).

The nematode was first proposed by Brenner (1974) as a genetic model in developmental biology and neurobiology fields. Over time, it has been contributing to answer several scientific questions, such as those related to the understanding of different molecular mechanisms in mammals (Yuan et al. 1993; Hengartner & Horvitz 1994; Horvitz 1999; Corsi et al. 2015). Nowadays, this species has developed into an important model for biomedical research, mainly in the functional characterization of novel drug-target interactions and in the identification of genes associated with particular pathways of toxic response or modes of toxic action, which may relate to specific diseases, e.g. the Alzheimer disease (Kaletta & Hengartner 2006). For example, *C. elegans* has been assuming a relevant role in the understanding of disorders such as Alzheimer's disease and Parkinson's disease, Diabetes type 2 and depression (Kaletta & Hengartner 2006; Harrington et al. 2010; Lublin & Link 2014), while supporting the discovery of drugs to be used in the development of therapy approaches (Lublin & Link 2014; Habchi et al. 2016). On the other hand, the use of *C. elegans* as an experimental model for ecotoxicity testing and ERA purposes has been

relatively recent (over the past two decades) (Sochová et al. 2006; Höss et al. 2008; Höss et al. 2011; e.g. Hagerbaumer et al. 2015), and its applicability has been by far less explored compared to the other research fields (see table S1).

Table 1 depicts a summary with the strengths and limitations of *C. elegans* as an experimental model organism in biomedical research, as well as an appraisal of the features that can concomitantly be valuable for promoting the worm as a model in ERA. The promotion of this nematode species as a laboratory model in medical fields stems from several internal and external attributes that have been making the research with this species particularly fruitful. The knowledge of its complete genome sequence bearing conserved gene sequences, and the peculiar sharing of signaling pathways and cellular machinery for DNA replication and repair between *C. elegans* and humans (Table 1) are worth noting in this context. These are key attributes for the investigation of the modes of toxic action and disease pathways, which are frequently similar between humans and this invertebrate model (Cole et al. 2004; Kaletta & Hengartner 2006; Leung et al. 2008; Destefani et al. 2017). The available mutant and transgenic *C. elegans* strains (Table 1) are also very useful to mechanistically clarify modes of action, oxidative stress pathways, DNA damage patterns and neurodegeneration (Corsi et al. 2015; Hunt 2016). As an organism with metabolically active digestive, reproductive, endocrine, sensory and neuromuscular systems (Table 1), *C. elegans* responds as a functional multicellular and multi-systems unit in the toxicity assays. Furthermore, the logistics involved in culturing and testing with *C. elegans* is relatively easy to handle, which mostly derives from the species biological and physiological characteristics (Table 1). The nematode has a life cycle that extends only for 3.5 days when incubated at 20 °C (Figure S1). The organisms of this species are mostly self-fertilizing hermaphrodites, meaning that only one animal is needed to populate an entire plate. Only 0.1–0.2 % of the population produced by self-fertilization are males as consequence of the rare meiotic non-disjunction of the X chromosome, with most of the tests being carried out with hermaphrodites even though tests with males can also be done depending on the research hypothesis. As integrated in ERA, tests with hermaphrodites would thus be the most straightforward option following, with the advantage of supporting comparison to previous data, but sex-specific effects can also be tested particularly at higher ERA tiers where population and community studies are typically considered for a better and more direct insight on ecological responses (see Figure 1). *C. elegans* can be cultured within a large interval of temperature, ranging from 12 °C to 25 °C, meaning that its growth rate can be controlled by manipulating the temperature within this range. *In-vivo* toxicity assays with the organism can be performed within a short time period and using simple equipment. Moreover, they are cheaper and faster than the tests available with rodent models (Hunt 2016). All mentioned features are also valuable in the environmental sciences field, specifically regarding integration in ERA approaches.

The successful application of *C. elegans* as an experimental model depends also on an accurate perception of its limitations so that the experimental design can be properly tuned to the tested hypothesis with no uncontrolled interferences leading to uncertainty or feasibility problems (Table 1). For example, above 25 °C, the organisms become sterile, while shorter exposures to higher temperatures increase the production of males (Corsi et al. 2015), which may not comply to the desired experimental conditions; in extreme cases,

poorly controlled culture practices favor the development of “dauer” larva, which are stress resistant alternatives to the common third larval stage (L3) (Hunt 2016).

Extending on *C. elegans* potential for broad-scope environmental studies: lessons learnt from human neurotoxicology

Shared neurophysiological components, cell functions, genes and pathways involved in DNA repair, as well as the mechanisms that are activated when the DNA is not repaired (e.g. cell cycle arrest and apoptosis), have made *C. elegans* an invaluable model to decipher important aspects of neurodegeneration and/or neurotoxicity (for specific cases in which the neurodegeneration is caused by toxic substances). As detailed in the previous section, *C. elegans* has been frequently used in a human neurotoxicology context (Cole et al. 2004; Benedetto et al. 2009; Harrington et al. 2010; Farina et al. 2014; Bailey et al. 2018). In contrast, studies investigating the neurotoxicity of contaminants in *C. elegans* under the environmental assessment scope are recent and scarce. It would be particularly useful to clarify the neurotoxicity of environmental compounds that were specifically designed to target the nervous system of biological pests. For example, organophosphates and carbamates are two sub-groups of insecticides specifically designed to inhibit irreversibly or reversibly the enzyme acetylcholinesterase (AChE), respectively. AChE inhibition interrupts the hydrolysis of the neurotransmitter acetylcholine, leading to its accumulation at the nerve synapses or neuromuscular junctions (Fukuto 1990). These insecticides have been shown to elicit adverse effects in non-target organisms such as earthworms (e.g. lethality, behavior alteration), which ultimately can be translated into decreased soil fertility and plant performance considering the ecological role of these organisms in agroecosystems (Reinecke & Reinecke 2007; Pereira et al. 2010; Pelosi et al. 2014). Similarly, the neurotoxicity of some trace elements [e.g. Hg and manganese (Mn)] to wildlife should not be disregarded, considering their occurrence in the environmental compartments. For example, Hg emissions were estimated to increase (Streets et al. 2009) and therefore neurotoxic effects in aquatic and soil organisms deserve attention.

The homology of the mammalian nervous system and other organisms support the hypothesis that the neurotoxicity of several contaminants as already well demonstrated in mammals could be also recorded in other groups of animals. ERA is an appropriate context where this hypothesis can be tested. There are general guidelines designed for neurotoxicity risk assessment both in USA and EU, but they have human health as the main focus (EPA 1998; OECD 2007b). The testing of the neurotoxic potential of chemicals is not mandatory by legislation of these countries and, when performed, it is generally based on the effects to adult rodents. Therefore, in the EU, both EFSA and OECD agreed that there is a need of developing a more standardized *in vitro* battery of tests to address the effects of chemicals on the nervous system for the final assessment of human toxicity (Fritsche et al. 2015; Fritsche 2017; Bal-Price et al. 2018). In summary, *C. elegans* has potential to be used in neurotoxicity assessment, not only in a human health context but also in an environmental health context as already recognized in other studies (Anderson et al. 2004; Cole et al. 2004; Fagundez et al. 2015).

The nervous system of hermaphrodite *C. elegans*, which has a total of 302 neurons, is subdivided in the pharyngeal nervous system with 20 neurons and the non-pharyngeal nervous system with 282 neurons (Figure 2) (Leung et al. 2008; Hunt 2016). Most of these neurons have a simple structure, presenting only one or two neurites (or processes), and most of these neurites cannot be distinguished as axons or dendrites because they both receive and transmit signals (Corsi et al. 2015). The use of *C. elegans* transgenic strains expressing fluorescent proteins in specific neurons allows *in vivo* visualization of virtually any desired neuron (Leung et al. 2008). The neurotransmitters found in the nematode and their main functions are summarized in Table 2. All these neurotransmitters except octopamine, as well as the neuronal signal pathways and molecular networks, are common to vertebrates (Leung et al. 2008; Hunt 2016). This includes neurotransmission, neuronal development and neuronal death or survival processes. Information obtained with *C. elegans* in studies evaluating alterations in these biological mechanisms can be used to interpret and infer regarding vertebrate systems based on the similarity the two groups for these processes. This possibility is paramount nowadays given the worldwide recognition of ethical principles that should rule animal experimentation and ensure the welfare of vertebrates. Alternative and complementary testing with *C. elegans* fits the replacement principle of the 3R's philosophy, largely recommended in human and ecological risk assessment (e.g. Madden et al. 2013; Sneddon et al. 2017).

Validated testing platforms with *C. elegans* and endpoints at different levels of biological organization

A literature survey of the studies performed within neurotoxicology with *C. elegans* was done in order to provide an overview of the endpoints that have been assessed to address different research questions. These studies are based on different types of laboratorial assays assessing lethal and sub-lethal effects resulting from acute and chronic exposures to specific toxicants (e.g. Bailey et al. 2018). They regard mostly effects at the sub-cellular and cellular levels and, more rarely, the whole-organism. Examples of studies performed at different levels of biological organization are explored below.

Specifically considering sub-cellular effects (molecular endpoints), the neurotoxic potential of metals and pesticides in *C. elegans* has been investigated based on assays intending to measure the activity of acetylcholinesterase, oxidative stress or changes in gene expression. Cole et al. (2004) concluded that *C. elegans* can be a good model to the first-round screening of organophosphate-induced mammalian neurotoxicity, since cholinesterase inhibition occurred for 5 of 8 compounds tested, with an agreement between the ChE response of mammals and the worm in 7 out of 8 compounds. The occurrence of oxidative stress and mitochondrial inhibition have been signalled as relevant mechanisms of toxic action for some metals and associating to human neurological diseases (e.g. Parkinson's disease), by evaluating mitochondrial function and/or quantification of reactive oxygen species (ROS) in *C. elegans* (e.g. Bailey et al. 2016; Todt et al. 2016): specific parameters such as oxygen and ATP concentrations and proton gradient integrity were used to assess the status of mitochondrial function after exposure; actual ROS quantification (e.g. superoxide, hydrogen peroxide, hydroxyl radical) and/or the monitoring of the up-regulation of genes encoding for enzymes with antioxidant activity, namely glutathione-S-transferase, superoxide dismutase,

catalase and glutathione peroxidase were used to assess oxidative stress. Oxidative stress is widely studied in environmental assessment, being understood as both an early warning and a sensitive response of the soil and the aquatic biota to contamination challenges (e.g. Bernard et al. 2015; Brix et al. 2017; Wang et al. 2018; Qi et al. 2018). *C. elegans* can hence provide consistent information in this context as a nematode representative *per se* that can be compared/integrated with that provided by other ecological indicators. Furthermore, and importantly, the environmental assessment field can benefit from a well-developed testing platform allowing a detailed discrimination of common mechanisms of toxic action by environmental contaminants.

For example, to evaluate changes in the gene expression, modified strains of *C. elegans* are available, e.g. CL2166 strain (Bailey et al. 2016; Todt et al. 2016; e.g. Bailey et al. 2018). These strains are genetically modified such that a green fluorescent protein (*gfp*) gene is fused with a nuclear localization sequence driven by the promoter for the enzyme gene. These strains show increased fluorescence in the presence of oxidative stress that can be promoted by neurotoxicant substances (e.g. glyphosate, Mn). Microarray analyses (e.g. Custodia et al. 2001) and reverse transcription polymerase chain reaction (e.g. Zeng et al. 2017) are other techniques that have also been used for this purpose. Chronic exposure of *C. elegans* to concentrations of the fungicide Mn/Zn ethylene-bis-dithiocarbamate within the application range recommended by the manufacturer (thus relevant levels from the occupational exposure point of view), resulted in increased oxidative stress, namely mitochondrial Complex I inhibition with concomitant hydrogen peroxide production (Bailey et al. 2016). Similar results were obtained with glyphosate-containing pesticides as Bailey et al. (2018) verified that chronic exposure of *C. elegans* to a glyphosate-containing herbicide (TouchDown®, Syngenta) resulted in mitochondrial inhibition and hydrogen peroxide production. Fagundez et al. (2015) verified that acute exposure to Fe increased ROS levels in *C. elegans*, and Novillo et al. (2005) verified a change in the gene expression patterns as a consequence of a short-term exposure to Cd. Basically, this exposure activated the expression of genes encoding stress-responsive proteins, such as *mtl-2*, member of the metallothionein family, and *cdr-1*, inducible for lysosomal protein (Novillo et al. 2005). Mechanistic approaches such as those exemplified above are highly valuable in environmental assessment to disentangle adverse outcome pathways of contaminants in the biota that may explain or predict escalating ecological effects. In this sense, and again, the toolbox available for *C. elegans* testing can be a valuable alternative or complement to current omics-based approaches currently used for the purposes with standard model organisms (e.g. Kim et al. 2015; Simões et al. 2019).

The number of studies focused on physiological and morphological endpoints in *C. elegans* is small, but results have provided important insights on the consequences of neurotoxic compounds at the morpho-functional level. For example, they helped in the evaluation of neurodegenerative effects, mostly related with exposure to pesticides (Meyer & Williams 2014; Todt et al. 2016; e.g. Bailey et al. 2018) and metals (e.g. Benedetto et al. 2009; Chen et al. 2013; Farina et al., 2014). Negga et al. (2011) verified that acute and chronic exposures of *C. elegans* to a glyphosate-containing herbicide and a Mn/Zn-ethylene-bis-dithiocarbamate fungicide lead to the formation of gaps between the nervous system and the inner cuticle, as well as to a thinning of the nerve cord. More recently, the authors confirmed

that the exposure of the nematode to the same pesticides resulted in neurodegeneration of GABAergic and DAergic neurons (Negga et al. 2012). Stanwood et al. (2009) verified that chronic exposures to Mn can result in neurodegeneration of GABAergic and DAergic neurons within the basal ganglia, as well as Fagundez et al. (2015) observed that acute exposures to Fe resulted in morphological changes in dopaminergic and cholinergic systems.

Behavioral assays can detect alterations in the neuronal function that can be compromised by molecular and cellular alterations. Zeng et al. (2017) showed that the synthetic pyrethroid deltamethrin affects the locomotion (trashing rate, body bend frequency), foraging ability and egg-laying of *C. elegans*. The exposure to sub-lethal concentrations of the organophosphorus insecticide monocrotophos provoked paralysis (loss of wriggling movements) as well as decrease of the brood size of the worms (Leelaja & Rajini 2013). Moyson et al. (2018) evaluated the single and mixture effects of different metals, namely Cu, Cd and Zn on behavior (locomotion – average crawling speed and trashing behavior; chemosensation – chemotactic index, drop test) and lethality of the nematode. The authors observed that Cu was the most toxic metal, with higher mortality rates and reduced locomotion, and that some mixtures (Zn-Cu, Cu-Cd, and Zn-Cu-Cd) can increase the toxicity compared to what was observed in single exposures, decreasing the locomotion and increasing the lethality of the nematode. Fagundez et al. (2015) have shown that an acute exposure to Fe induced alterations in the survival, lifespan and brood size in *C. elegans*.

Overall, *C. elegans* has been widely used for testing the neurotoxicity of organic compounds and trace elements with the final aim of addressing human health effects. These research efforts have been focused on molecular, morphological, physiological, behavioral and lethal endpoints, as summarized in Figure 3. Given the potential of *C. elegans* as highlighted previously, we specifically argue on the establishment of the nematode as a model organism for neuro-ecotoxicology assessment within lower tiers of ERA (Figure 1), comprising laboratorial tests (and endpoints) as illustrated in Figure 3. Additionally, *C. elegans* can be a good model also for studies involving higher levels of biological organization, namely in population and community studies (e.g. Haegerbaeumer et al. 2016) as required in higher ERA tiers (Figure 1), but further research is needed to confirm its suitability in this context.

***C. elegans* as a suitable test model in environmental assessment studies**

As an inherent consequence of the genetic, physiological, and toxicological studies with *C. elegans* (see above), many and diverse molecular and biomedical tools are available and can be exploited for environmental assessment purposes (Menzel et al. 2009). Table 1 identifies the features of *C. elegans* as an experimental model that can also apply to ERA. This organism represents an advantage compared to other organisms already used in ERA given its suitability to be used in the assessment of neurotoxic substances (e.g. Cole et al. 2004; Negga et al. 2012); as well as serves as a model for both aquatic and soil ecosystems, mainly associated to microbe-enriched environments and occupying an intermediary position between microorganisms and higher trophic levels (Hagerbaumer et al. 2015). Microbe communities can act (1) as food themselves or as food supplier, processing substrate material to make it accessible to the nematode; (2) as part of its microbiome; (3) as parasites and pathogens; and (4) possibly as competitors. The organism can use macroscopic

invertebrates such as slugs, snails and isopods as hosts or vectors to disperse into new habitats. Other nematode species and also small invertebrates can compete with *C. elegans* in the wild, and mites, collembola and fungi are its main predators (Schulenburg & Félix 2017).

Although relatively scarce and scattered, there are ecotoxicological studies in the literature with this organism, further supporting its utility as a valuable environmental indicator (Cioci et al. 2000; Roh et al. 2006; Ma et al. 2009). These studies are summarized in Tables 3 and 4. The 48 studies presented in table 4 assessed the toxicity of different contaminants (e.g. metals, pesticides, microplastics) to *C. elegans* and the 5 studies summarized in table 4 evaluated the toxicity of contaminated environmental matrices (soils and sediment) to the organism. The suitability of *C. elegans* to be successfully maintained in agar, soil, sediments and aqueous media supports the variety of setups used in the tests (e.g. Mutwakil et al. 1997; Black & Williams 2001; Höss et al. 2008). The worm's applicability to both acute and chronic studies, intending to assess both lethal but also sub-lethal endpoints at different levels of biological organization is also evident (Tables 3 and 4). Growth (eg. Höss et al. 2001), reproduction (eg. Boyd & Williams 2003a), gene expression (eg. Anbalagan et al. 2012), locomotor and feeding behavior (Jiang et al. 2016), endocrine disruption (eg. Custodia et al. 2001) and enzyme activity are among the sub-lethal endpoints more frequently used in ecotoxicological studies performed with *C. elegans*. Neurotoxicity has been occasionally evaluated in an environmental context through endpoints such as locomotive and feeding behavior and expression of specific genes (Tables 3 and 4). It is noteworthy that most, if not all these endpoints are relevant in the environmental assessment context. Lethality, growth and reproduction of standard organisms (see 'Introduction') are frequently included as testing endpoints in lower ERA tiers (e.g. EFSA 2013). Behavioural endpoints reflecting in contamination avoidance and feeding inhibition are commonly used as early warning cues of exposure to noxious contaminants (e.g. Castro et al. 2018; de Santo et al. 2019; Gainer et al. 2019; Vidal et al. 2019), often reasoning neurotoxicological interference (e.g. Pereira et al. 2010; Bicho et al. 2015; Castro et al. 2018). Enzyme activity and endocrine disruption markers are also common endpoints both regarding the soil (Ibrahim et al. 2019) and the aquatic biota (Windsor et al. 2018; Li et al. 2016). Whether the responses of *C. elegans* compare to those by other model species typically used in ERA will be appraised in the following section of this manuscript.

The objectives and scope of the ecotoxicological studies that have been carried out in *C. elegans* are quite diverse (Tables 3 and 4). For example, Black and Williams (2001) assessed the mortality of *C. elegans* and of the amphipod *Hyalella azteca* when exposed to soil and sediment contaminated after a release of trace elements from a mine waste lagoon to the Hungarian Tisza river (acute toxicity; whole-organism level). The authors reinforced the ease and utility of *C. elegans* in soil toxicity tests, which, on the one hand, involves the use of lower quantities of soil compared to other soil toxicity tests with other organisms and, on the other hand, allows to retrieve large sets of information. Tejeda-Benitez et al. (2016) evaluated the toxicity profiles of freshwater sediments contaminated by metals (Co, Ni, Cu, Zn, Cd, Hg and Pb) through endpoints such as survival, locomotion, growth (wild-type strain) and changes in gene expression (GFP transgenic strains) of *C. elegans*, thus addressing acute and chronic toxicity, as well as the sub-cellular and whole-organism levels.

Tominaga et al. (2002; 2003) studied the effects of different substances and compounds (steroid hormones, alkylphenol and organotin compounds) in the reproduction of over multiple generations of *C. elegans* in agar plates (chronic toxicity; whole-organism level) and showed that the effects can persist over generations, highlighting the nematode as a good model for multigenerational studies and epigenetic effects.

The contaminants that have been tested in ecotoxicological studies with the nematode species are also diverse. For example, Höss et al. (2011) analyzed the environmental hazardous potential of the protein Cry3Bb1, which is produced by a genetically modified maize and presents insecticidal properties against the coleopteran *Diabrotica virgifera*, using *C. elegans* as a non-target representative. The authors observed that even though the Cry3Bb1 exerted dose-dependent inhibitory effects on growth and reproduction of the nematode, the concentrations found in crop soil were four orders of magnitude below the toxicity threshold, indicating low hazardous potential at least to *C. elegans*.

Transgenic strains of *C. elegans* can be used for mechanistic ecotoxicity assessment through integrating the response of different biomarkers. For example, Tejeda-Benitez et al. (2016) evaluated changes in the gene expression of *C. elegans* as a response to the exposure to freshwater sediments contaminated by metals, using several transgenic nematode strains (*hsp-6::gfp*, *hsp-16.2::gfp*, *hsp-70::gfp*, *sod-1::gfp*, *sod-4::gfp*, *gpx-6::gfp*, *mtl-2::gfp*, *mtl-1::gfp*, *gst-1::gfp*, *cyp-34A9::gfp*). They detected changes in the expression of the *mtl-2* gene (metallothioneins-related gene), indicating contamination by metals, as already expected; antioxidant gene *sod-4*, indicating possible presence of compounds responsible for the generation of ROS; and metabolism-related genes *sod-4* that have been frequently activated by the presence of pesticides and other xenobiotics. Chu and Chow (2002) evaluated the mortality caused by trace elements and compared the results between the wild-type nematode and a transgenic strain (KC136), carrying a heat shock promoter driven GFP reporter gene. They concluded that the transgenic strain revealed the same sensitivity to metals that the wild-type, meaning that the transgenic KC136 could be used to early and rapidly detect pollution by metals *in situ* (Wah Chu & Chow 2002; Tejeda-Benitez et al. 2016).

Although most of the tests with *C. elegans* have been performed using a single-species approach, these organisms are referred as suitable to integrate more holistic test approaches such as those based in microcosm and mesocosm experiments (Bone et al. 2015; Hagerbaumer et al. 2015). For example, Haegerbaeumer et al. (2016) used *C. elegans* in a microcosm study containing sediment, along with other nematode species, to investigate direct and indirect effects of Zn on natural nematode assemblages. They concluded that testing the toxicity of Zn under more realistic exposure conditions revealed a species sensitivity ranking comparable to field observations, confirming that assessments at these higher levels of biological organization represent a more sensitive approach to realistically address the effects of the chemicals. It also can render more realistic the assessment of environmental interference in the response to chemical challenges. For example, Collin et al. (2016) showed that natural organic matter alters the toxicity of sulfurized Ag nanoparticles to *C. elegans* since its dissolution is influenced by the organic matter. The use of the

nematode species in microcosm and mesocosm studies would also be an important asset for assessment at higher tiers of ERA (EFSA 2013).

Comparative sensitivity between *C. elegans* and standard ERA models

C. elegans has been shown to be highly sensitive to different environmental contaminants, namely to some metals and pesticides (see Table S2 for a summary list denoting sensitivity). Despite this has been generally argued, studies directly comparing its sensitivity with standard laboratorial test organisms are still scarce. While the ecological representativeness of the organism, knowledge on its physiology and the availability of suitable testing platforms are key features of a valuable candidate to integrate ecotoxicological test batteries for ERA purposes, its sensitivity is of no lesser importance given the relevance of the precautionary principle in environmental assessment (e.g. EFSA 2013). This motivated us to run a comparative analysis, confronting the responses of *C. elegans* to different contaminants with their counterpart recommended models in ERA focusing on soil (*E. fetida*) or aquatic (*D. magna*) ecosystems. The raw results of these analyses are available in supplementary tables S2–S4, while figure 4 provides a graphical comparative view of sensitivity to metals, as detailed later in this section.

Peredney et al. (2000) showed that *C. elegans* was more sensitive to trace elements contamination than other model soil organisms frequently used for ecotoxicological assessment purposes, namely *E. fetida* and *F. candida*. The authors compared effective concentrations obtained following a 24-h exposure of *C. elegans* with equivalent benchmarks obtained following tests lasting for up to 14 days with *E. fetida*. The dramatic shortening of test length with equivalent or more environmentally protective outcomes is an additional argument in favor of considering *C. elegans* testing within ecotoxicological batteries for risk assessment purposes. Höss et al. (2009) also assessed the toxicity of soils contaminated by trace elements and organic compounds to the nematode, and compared to the results obtained in tests with other test organisms (*F. candida*, *Eisenia andrei* and *Brassica rapa*). They reported that *C. elegans* was, in most cases, the most sensitive species to soils contaminated with PAH, compared to *E. fetida* and *F. candida*, based on the reproductive endpoint. Exceptions to this general pattern revealed equivalent sensitivity to *F. candida*, or sensitivity close to that of the two other organisms. Other organic contaminants such as quinoline, acridine, phenazine, 1,10-phenanthroline and toxaphene were found to be similarly or occasionally less lethally toxic to *C. elegans* than *E. fetida* and *F. candida* (Sochová et al. 2007). Daam et al. (2011) showed that nematodes in general were more sensitive to two fungicides containing Cu than *E. fetida*.

Considering the tests performed in soil medium since 2000, a sensitivity comparison between *C. elegans* and the earthworm *E. fetida* (tables S2 and S4) was found fruitful for Cu. This was the combination selected because *E. fetida* is a recommended model soil organism in ERA and, importantly, there is data available for both cases with comparable units, endpoints, test medium and test type. A graphical comparison between EC₅₀ Cu values (effective concentrations of Cu causing 50% of effect on the tested species) obtained for both species shows that, considering the endpoint lethality, *C. elegans* was more acutely sensitive to Cu than the earthworm (Figure 4-A). This is in agreement with the conclusions

of Daam et al. (2011). Based on reproductive effects following chronic exposures to Cu, the sensitivity was similar for the two organisms (Figure 4-B). Still, the use of the nematode instead of the earthworm in risk assessment approaches targeting Cu would be more advantageous both considering acute and chronic assays, in the latter case due to the relevant shortening of the test length. It is noteworthy that the use of only *E. fetida* as environmental indicator for the soil compartment regarding contamination by Cu may not be appropriate, since concentrations of Cu that are safe for *E. fetida* can compromise the short-term survival of *C. elegans*.

Figure 4 (C, D) shows a comparison between the sensitivity of *C. elegans* and *D. magna* regarding 24 h-LC₅₀ and 48 h-LC₅₀ values (lethal concentrations causing mortality of 50 % of the tested organisms, 24 h and 48 h after the beginning of the test, respectively) for different metals, namely Cu, Pb, Zn, Cd, Ni, Cr, Hg and Mn. Following 24 h of exposure, *D. magna* was generally more sensitive than *C. elegans* to all metals. The lower and higher differences in sensitivity occurred for Cu and Cd, respectively. On the other hand, the sensitivity to Cu, Pb and Cr was very similar for both organisms following 48 h of exposure, with *C. elegans* being apparently more sensitive than *D. magna* to Hg (Figure 4-D). In fact, *D. magna* was generally more sensitive to metals, when compared to *C. elegans*, suggesting that the inclusion of the nematode in test batteries may not be a valuable add-on to the aquatic risk assessment of these specific metals, especially in lower tiers where lethality can be inspected. However, the set of data available for comparison is very limited, preventing robust conclusions in this context. Moreover, *C. elegans* actually represents a different functional group in aquatic ecosystems, which may render its inclusion in test batteries valuable *per se*.

Ecotoxicological studies with pesticides for *C. elegans*, *E. fetida* and *D. magna* are very scarce (Tables S2–S4), severely limiting any attempt to conclude on the differential sensitivity of organisms; for chlorpyrifos, *D. magna* feeding at 24 h (average EC₅₀ = 0.450 ± 0.066 mg/L) was more sensitive than *C. elegans* locomotive behavior at 4 h (EC₅₀ = 1.750 mg/L) (see tables S2 and S3; Anderson et al., 2004; Loureiro et al., 2010), although the endpoints were inherently different since filtration in *Daphnia* is a more passive process than *C. elegans* locomotion (McMahon 1965; e.g. Corsi et al. 2015). Still, *C. elegans* has been proven to be very sensitive to some pesticides (e.g. carbamates, organophosphates) in neurotoxicological studies within the human toxicology field.

Conclusions and future perspectives

The contribution of *C. elegans* to scientific advances in the medical science field is unequivocal, namely by supporting many important findings ranging from genetics, molecular biology to physiology and neurotoxicology. Although *C. elegans* can be very useful as a model to other fields of research, namely those related with environmental sciences, its applicability has yet to materialize. Nevertheless, several studies have highlighted the potential utility *C. elegans* might have in ecotoxicological studies with trace elements and pesticides. Notably, standardized protocols for toxicity tests with *C. elegans* in several environmental matrices are already in place. These observations provided the foundation for the current review, for supporting the integration of *C. elegans* into

ecotoxicology test batteries within ERA frameworks. As described above, *C. elegans* may be used both in acute and chronic studies, which are requirements of the lower tiers of ERA, representing a more efficient model than standard soil organisms currently used in these procedures (e.g. *E. fetida* for soil ERA). Tests with *C. elegans* can have a shorter duration (both for acute and chronic exposures) than those performed with other organisms, while presenting a higher or equivalent sensitivity to several toxicants. Moreover, effects of environmental contaminants can be assessed in the nematode at different levels of biological complexity (from sub-cellular and cellular levels to whole-organism), commensurate with the utility of the nematode as an established model in neurotoxicology.

Finally, it is worth emphasizing that the nematodes group has yet to be integrated as a model organism represented in ERA ecotoxicological test batteries. This vision has been corroborated by at least one expert group reporting to a European regulatory agency, namely EFSA, which has considered *C. elegans* as a potentially relevant model organism to be used in this context (EFSA 2017). This review supports the assertion that *C. elegans* is a valuable model to be considered for ERA, particularly for lower tiers targeting the soil compartment. Studies investigating the overall potential of the organism in microcosm and mesocosm approaches are necessary to further support the utility of *C. elegans* as a model for higher tiers of ERA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Tiered approach used in the environmental risk assessment of PPP for aquatic organisms in edge-of-field surface waters (A) and for in-soil organisms (B). All the tiers intend to achieve a defined specific protection goal (SPG). The nematode species *Caenorhabditis elegans* could be included in tiers 1 and 2 of the risk assessment regarding aquatic organisms (A) and in the lower tier and intermediate tier A of the risk assessment regarding soil organisms (B), using standard guidelines: ISO 10872 (ISO, 2010) (A) or ASTM E2172–01 (ASTM, 2014) and ISO 10872 (ISO, 2010) (B). In soil (part B), the toxicity assessment includes both tests with fauna and mycorrhiza and a microorganism-mediated process test. OECD N202 is the available guideline to perform the acute immobilization test with *Daphnia* Sp. (OECD, 2004a); OECD N201 to perform the growth inhibition test with freshwater alga and cyanobacteria (OECD, 2011a) and OECD N226 from 2004 to perform the reproduction test in soil with the mite *Hypoaspis (Geolaelaps) aculeifer* (OECD, 2004b). SSDs is the acronym for Species Sensitivity Distributions. The information used to build this figure was retrieved from EFSA reports (EFSA, 2013; EFSA, 2017).

Figure 2. Division and classification of the neurons throughout the pharyngeal and non-pharyngeal nervous subsystems in *C. elegans* hermaphrodites. Adapted from McVey et al. 2012.

Figure 3.

Summary of the sub-lethal and lethal endpoints that have been assessed in the laboratorial studies performed with the model organism *C. elegans* in an attempt to assess the neurotoxic potential of specific substances/mixture of substances. Endpoints are displayed by an increasing order of biological complexity.

Figure 4.

Comparative view on the sensitivity to several environmental contaminants of *C. elegans* and that by two standard organisms used in ecotoxicological test batteries within environmental risk assessment frameworks, *Eisenia fetida* and *Daphnia magna*, representing soil and aquatic ecosystems, respectively. At the top panel, comparison of (A) acute EC₅₀ values (effect: lethality following 24 h exposure for *C. elegans* and 14 days for *E. fetida*), and (B) chronic EC₅₀ values (effects: reproduction following 7 days for *C. elegans* and 28 days for *E. fetida*). These effective concentrations were selected from laboratorial studies performed in soil (see tables S2 and S4; Boyd et al., 2010; Jonker et al., 2004b; Spurgeon et al., 1994; U.S. Environmental Protection Agency ECOTOX database, 2018). At the lower panel,

comparison of (C) 24 h-LC₅₀ and (D) 48 h-LC₅₀ values found for exposures of *C. elegans* and *D. magna* to metals. These LC₅₀ values were selected from laboratorial studies performed in aqueous media (see tables S2 and S3; Altındag et al., 2008; Boyd and Williams, 2003a; Dhawan et al., 2000; Ferreira et al., 2008; Graves et al., 2005; Sorvari and Sillanpää, 1996; Wah Chu and Chow, 2002), and when several values were available for a given element, average between available values and corresponding standard deviation were used to obtain the plotted information.

Table 1.

Strengths and limitations of *C. elegans* as an experimental model organism. [ERA] is a code added to identify those features that prominently apply also to Environmental Risk Assessment.

Strengths	Limitations
<ul style="list-style-type: none"> • Rapid and productive life cycle (≈ 3 days generation time and ≈ 300 progeny per hermaphrodite worm – primary form)^{1,2} [ERA] • Organism transparency allowing a good visualization of internal structures under a microscope^{1,2} [ERA] • Small body size (around 1 mm long) and relatively simple physiology and anatomy^{1,2} • Easy and relative inexpensive maintenance in the lab, including to feed experiments¹ [ERA] • Non-hazardous to lab workers² [ERA] • Exists primarily as a self-fertilizing hermaphrodite^{1,3} • Simple synchronization of organisms by isolation of newly hatched larvae or by treatment of gravid adults with bleach, with posterior isolation of the eggs¹ [ERA] • Sexual crosses can be easily performed³ • Complete genome sequence available¹ [ERA] • Good conservation of genes (60–80 % homologue genes), signaling common pathways and cellular machinery for DNA replication and repair between <i>C. elegans</i> and humans^{1,2,4} • Mutant and transgenic <i>C. elegans</i> strains are readily available for many genes² [ERA] • Reliable and reproducible DNA mutations are easily inducible³ • A small-scale <i>C. elegans</i> larval growth assay can be conducted in less than a week² [ERA] • Possibility of long-term cryopreservation¹ • Presents neuronal, motor, digestive, reproductive and endocrine systems² • Shows endocrine signaling and sensory/behavioral responses to stimuli² [ERA] • It is a well understood model organism, namely regarding its genetics, physiology, morphology, neurology and cell signaling pathways^{1,2} [ERA] 	<ul style="list-style-type: none"> • Become sterile at temperatures above 25 °C¹ • Not all metazoan genes are found in its genome • No <i>C. elegans</i> cell culture lines are commercially available¹ • Experimental manipulation of individual tissues can be difficult due to its small size¹ • Absence of some molecular pathways of mammals² • Absence of several mammalian organs such as eyes, lungs, heart, kidney and liver² • Absence of adaptive immunity² • The relatively impermeable cuticle limits toxicants internalization via direct dermal absorption² [ERA] • Small, unwanted changes in culture conditions, namely in temperature, salt and nutrients concentrations can alter assay results² [ERA] • Bad culture practices can result in altered gene expression patterns and accumulation of “dauer” larvae and males² [ERA]

¹ Corsi et al., 2015

² Hunt, 2016

³ McVey et al., 2012

⁴ Leung et al., 2008

Table 2.

Identification and clarification of the major functions of the different neurotransmitters reported in *C. elegans*.
Adapted from McVey et al. 2012.

Neurotransmitters	Inhibition	Promotion
Octopamine	Egg laying Pharyngeal pumping	—
Tyramine	Egg laying Pharyngeal pumping Spontaneous reversal	—
Dopamine	Food-dependent forward movement	Slow movements in the presence of food High-angle turning Learning behaviors
Serotonin	Locomotion Defecation	Pharyngeal pumping Egg laying
GABA	Muscle contraction	Muscle relaxation Defecation
Acetylcholine	—	Muscle contraction

Table 3.

Summary of ecotoxicological studies performed with *C. elegans* since 2000 where the nematode was found responsive to imposed toxicant challenges. The contaminants tested are indicated by grouping into major classes; in addition, the medium used, endpoints assessed and test length are given for each study, along with notes or conclusions as found relevant within the specific focus of the present review.

Contaminants	Medium	Endpoints	Length	Notes/conclusions	References
<i>Metals</i>					
Cd, Cu, Ni, Pb, Zn	Soil	Lethality	24 h	The soil type used in the ecotoxicological tests with <i>C. elegans</i> can influence the results	(Boyd & Williams 2003b)
Cu-Zn, Cu-Cd, Cd-Pb (mixtures)	Soil	Growth	1-week	The simple additivity model underestimated mixture effects on reproduction and body length of <i>C. elegans</i> .	(Jonker, Sweijen, et al. 2004)
Zn, Cd, Hg, Cu, Fe, Cr, As, Al, Ni, Mn, Pb	Soil pore water	Gene expression	40 h	<i>C. elegans</i> GFP-reporter strains can be very useful in lower tier ecotoxicological screening	(Anbalagan et al. 2012)
Cu	Soil Aqueous medium	Lethality, reproduction, movement, feeding behavior	24 h; 72 h; 24 h; 24 h	<i>C. elegans</i> exhibited intermediate sensitivity compared to two other nematode species	(Boyd & Williams 2003a)
Cu	Aqueous medium Soil	Lethality, behavior	24 h; 24 h	<i>C. elegans</i> carrying a GFP-expressing element responded similarly to the wild-type N2 strain	(Graves et al. 2005)
Cd	Aqueous medium Soil	Growth	72 h	--	(Höss et al. 2001)
Cu, Cd, Zn, Pb, Ni	Soil	Lethality	24 h	<i>C. elegans</i> and <i>Eisenia fetida</i> have a similar response to metals	(Peredney & Williams 2000)
Cu, Cd	Agar	Reproduction, larval growth, lifespan	36 h; 32 h	--	(Kurauchi et al. 2009)
Cd, Cu	Agar	Reproduction, larval growth, lifespan	36 h; 32 h	--	(Harada et al. 2007)
Cd-Cu	Agar	Reproduction, length of the juvenile period, length of the reproductive period, body length	≈13 days	--	(Jonker, Piskiewicz, et al. 2004)
Al, Cu, Pb, Zn, Cd	Aqueous medium	Lethality, behavior	24 h; 24 h	A computer tracking system was successfully used to measure several behavioral parameters in <i>C. elegans</i>	(Dhawan et al. 2000)
Cu, Zn, Cd, Cr	Aqueous medium	Growth, reproduction, behavior (head thrash frequency, body bend frequency, feeding), enzymatic quantification	24 h; 72 h; 24 h; 24 h; 24 h	Considering simplicity, accuracy, repeatability and costs of the experiments, feeding inhibition is a good sub-lethal endpoint for assess metal toxicity using <i>C. elegans</i>	(Jiang et al. 2016)

Contaminants	Medium	Endpoints	Length	Notes/conclusions	References
Cd, Hg, Cu, Pb, Cr, Ni, Al, Co, Zn, Mn (single and mixtures)	Aqueous medium	Lethality	96 h	<i>C. elegans</i> KC136 strain allows rapid monitoring of environmental hazards	(Wah Chu & Chow 2002)
Pb, Cu, Cd	Aqueous medium	Movement, feeding, growth, reproduction	4 h; 24 h; 72 h	Differential sensitivity among endpoints for any metal after 4 h but not after 24 h	(Anderson et al. 2001)
Cu, Pb, Al	Aqueous medium	Locomotor behavior	4 h	--	(Anderson et al. 2004)
Cd	Aqueous medium	Gene expression	4 or 7 days	--	(Novillo et al. 2005)
Cu	Aqueous medium Soil	Maturity index	24 h	The maturity index responds to metal pollution and thus may serve as an indicator of soil health	(Bongers et al. 2001)
Zn	Sediment Aqueous medium	Reproduction, lethality	96 h; 48 h	--	(Haegerbaeumer et al. 2016)
Zn	Sediment Aqueous medium	Abundance, species richness	6 months	Inhibitory effects of Zn on abundance and species richness occurred only in the higher concentration (100 mg Zn/Kg d.w. soil)	(Haegerbaeumer et al. 2016)
Organic compounds					
Atrazine, PCB52, fluoranthene, lansoprazole	Aqueous medium	Reproduction, gene expression	96 h	--	(Menzel et al. 2005)
Carbendazim	Agar	Reproduction, length of the juvenile period, length of the reproductive period, body length	≈ 13 days	--	(Jonker, Piskiewicz, et al. 2004)
Pentadecafluorooctanoic acid, dichlorvos, sodium dodecyl sulfate	Agar	Reproduction, larval growth, lifespan	36 h; 32 h	--	(Kurauchi et al. 2009)
Sodium dodecyl sulfate	Agar	Reproduction, larval growth, lifespan	36 h; 32 h	--	(Harada et al. 2007)
4-Nonylphenol	Aqueous medium	Growth, reproduction	72 h	Growth and reproduction of <i>C. elegans</i> were enhanced at concentrations of 66 and 40 µg/L of 4-Nonylphenol	(Höss et al. 2002)
Glyphosate, paraquat, 2,4-dichlorophenoxyacetic acid, cypermethrin, carbendazim, dichlorvos, endosulfan, diuron, chlorpyrifos	Aqueous medium	Gene expression, feeding	40 h; 28 h	Transgenic GFP-reporter strains may be useful to assess stress patterns regarding gene expression in response to pesticides	(Anbalagan et al. 2013)
Quinoline, acridine, phenazine, and 1,10-phenanthroline, toxaphene, hexachlorobenzene, short-chain chlorinated paraffins	Soil Agar Aqueous medium	Mortality	24 h; 48 h	Recommended inclusion of <i>C. elegans</i> in the battery of tests for ERA of persistent organic pollutants in soil	(Sochová et al. 2007)
Carbendazim, carbendazimiprodione	Soil	Growth	1-week	--	(Jonker, Sweijen, et al. 2004)
Progesterone, estradiol, cholesterol	Aqueous medium	Gene expression	4 or 7 days	--	(Novillo et al. 2005)

Contaminants	Medium	Endpoints	Length	Notes/conclusions	References
DMSO, acetone, ethanol, mebendazole, levamisole, chlorpyrifos	Aqueous medium	Locomotor behavior	4 h	--	(Anderson et al. 2004)
Cholesterol, estrogen, testosterone, progesterone	Aqueous medium	Vitellogenin expression	3–4 days	<i>C. elegans</i> may be a useful laboratorial and field model for screening endocrine disruptors	(Custodia et al. 2001)
Cholesterol, 4-nonylphenol, p-octylphenol, triphenyltin chloride, tributyltin chloride	Agar	Reproduction, fecundity	120 h	<i>C. elegans</i> fecundity and reproduction rates can be good endpoints to assess effects of alkylphenols and organotin compounds	(Tominaga et al. 2002)
17 β -Estradiol, Bisphenol, Tributyltin Chloride	Agar	Number of germ cells	6 days	<i>C. elegans</i> can be a good model to determine the reproductive toxicity of contaminants	(Hoshi et al. 2003)
Atrazine, fluoranthene, diethylstilbestrol, β -naphthoflavone, clofibrate	Aqueous medium	Gene expression	48 h	Gene expression of <i>C. elegans</i> induced by contaminants can be a good tool to detect responses at a transcriptional level	(Reichert & Menzel 2005)
Bisphenol A	Aqueous medium	Growth, locomotion behavior, lifespan, lipofuscin accumulation, stress-related genes expression, population size	10 d	Chronic exposures of <i>C. elegans</i> to Bisphenol A caused adverse effects in all endpoints measured, including population size	(Zhou et al. 2016)
Bisphenol A	Aqueous medium	Growth, reproduction, locomotion behavior, stress-related genes expression	96 h x4	<i>C. elegans</i> was successfully used to conduct multigenerational testing	(Zhou et al. 2016)
Vinyl chloride	Aqueous medium	Survival, reproduction, stress-related gene expression	72 h	Reproduction and gene expression were the most sensitive endpoints	(Nam & An 2010)
Chlorpyrifos	Aqueous medium	Lethality, stress-related gene expression, acetylcholinesterase activity, growth, reproduction, development	24 h; 24 h; 24 h 24 h; 96 h	--	(Roh & Choi 2008)
Insecticidal Cry3Bb1 protein	Soil Aqueous medium	Growth, reproduction and gene expression	96 h; 96 h; 16 h, 24 h and 48 h	--	(Höss et al. 2011)
Mixtures of metals and pesticides					
Cu-carbendazim, Cu-carbendazimprodione	Soil	Growth	1-week	--	(Jonker, Sweijen, et al. 2004)
Cu-carbendazim	Agar	Reproduction, length of the juvenile period, length of the reproductive period, body length	≈ 13 days	--	(Jonker, Piskiewicz, et al. 2004)
Nanoparticles					
ZnO-NPs	Aqueous medium	Growth, locomotive behavior, metabolic ATP level, oxidative stress	72 h; 72 h; 66–67 h; 72 h	Sub-lethal metabolic and locomotive endpoints of <i>C. elegans</i> allow assessing	(Huang et al. 2017)

Contaminants	Medium	Endpoints	Length	Notes/conclusions	References
Ag-NPs	Agar	Survival, growth, reproduction, stress-response gene expression	24 h; 24 h; 72 h; 24 h	ecotoxicity of nanoparticles in aquatic environments	(Roh et al. 2009)
ZnO-NPs, Al ₂ O ₃ -NPs, TiO ₂ -NPs	Aqueous medium	Lethality, length, number of eggs, offspring per worm	24 h; 5 d; 5 d; 5 d	--	(Wang et al. 2009)
CeO ₂ -NPs, TiO ₂ -NPs	Agar	Stress- response gene expression, growth, fertility, survival	24 h	--	(Roh et al. 2010)
Ag-NPs	Aqueous medium	Growth	24 h, 48 h, 72 h	--	(Bone et al. 2015)
Sulfidized Ag-NPs	Aqueous medium	Lethality	24 h	--	(Collin et al. 2016)
Ag-NPs	Soil	Locomotive behavior; bioaccumulation	65 h; 24h and 48h	<i>C. elegans</i> can be an effective organism to rapidly screen and assess the impacts of metal nanoparticles on soil environment	(Yang et al. 2017)
Microplastics					
Polyamides, polyethylene, polypropylene, polyvinyl chloride, polystyrene particles	Agar	Survival, body length, reproduction, intestinal damage, oxidative stress	2 d	Microplastic particles induced effects in all endpoints measured in <i>C. elegans</i>	(Lei et al. 2018)

Summary of ecotoxicological studies performed with *C. elegans* since 2000 in matrices collected from the field. Only studies where the nematode was responsive to the imposed toxicant challenges where included and the contaminants burdening the tested matrices are indicated. Basic test features (medium, endpoints, test length) are given, along with notes or conclusions as found relevant within the specific focus of the present review.

Table 4.

Tested matrices	Contaminants	Test medium	Endpoints	Test length	Notes/conclusions	References
Contaminated freshwater sediments	Co, Ni, Cu, Zn, Cd, Hg, Pb	Aqueous extract of the sediments	Survival, growth, locomotion, gene expression	24 h; 48 h; 24 h; 4 h; 24 h	<i>C. elegans</i> testing allowed to identify patterns of toxicity along Magdalena river	(Tejeda-Benítez et al. 2016)
	Several metals and organic pollutants (e.g. Hg, Zn, As, Cr, tributyltin, petroleum hydrocarbons)	Freshwater sediments	Reproduction, gene expression, endocrine disruption	96 h; 48 h	Gene expression in <i>C. elegans</i> should not replace conventional aquatic monitoring techniques, but can be a good complement	(Menzel et al. 2009)
	Several metals and organic pollutants (e.g. polycyclic aromatic hydrocarbon, Cd, Cr)	Soil	Fertility, growth and reproduction	96 h	<i>C. elegans</i> was more affected by organic than by metal contaminants	(Höss et al. 2009)
Contaminated soils	Cd, Cu, Zn, Pb	Soil	Lethality	24 h	—	(Black & Williams 2001)
	Di(2-ethylhexyl)phthalate	Soil	Mortality, growth, reproduction, stress-related gene expression	24 h	Growth, reproduction and stress-related gene expression were the most responsive endpoints	(Roh et al. 2007)