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Original Articles

Direct toxicity of the water-soluble fractions of a crude and a diesel-motor oil on the survival of free-living nematodes

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ABSTRACT

The water-soluble fraction (WSF) of an oil contains different classes of petroleum hydrocarbons and, despite their low potential for bioaccumulation, these can be highly toxic to biota. Nematodes are usually the most abundant and species-rich group of metazoans in marine and freshwater sediments as well as terrestrial soils and have been proposed as excellent models to monitor pollution effects. The first aim of this study was to assess the direct effects of the WSFs of a crude and a diesel-motor oil on the survival of 12 free-living bacterivorous nematode species from marine, freshwater and soil environments, and belonging to diverse taxonomic groups. The second aim was to compare the responses of these twelve test species and assess their suitability as candidate alternatives for the common model species *Caenorhabditis elegans* in future toxicity testing. The third and final aim was to test the common assumption that nematodes which are phylogenetically more closely related would exhibit more similar sensitivities than more distantly related species, and that – as is commonly stated in literature – nematodes belonging to the family Rhabditidae would be the most pollution-tolerant species. While the crude oil was a complex matrix of substances, containing many soluble compounds, the diesel-motor oil 10W40 was characterized by only few soluble substances. Nevertheless, the diesel-motor oil WSF was as toxic (or even more toxic) to some of the tested nematode species as the crude-oil WSF. This could be linked to differences in compounds interactions in each oil-WSF, or to the presence of fuel additives in the 10W40-WSF. Most species exhibited moderate to extreme mortality levels in oil treatments, and experienced an increased mortality with time. Overall, marine nematodes were more sensitive than freshwater/soil organisms, albeit with some exceptions. Species sensitivities to oil did not follow patterns of taxonomic relatedness, contradicting the idea that closely related species should intrinsically respond similarly to pollution. Rhabditidae were not generally more sensitive than other species: out of 6 species of Rhabditidae tested, only *Bursilla monhystera* was highly tolerant, while *C. elegans* and cryptic species of *Litoditis marina* were among the most sensitive taxa. Therefore, we recommend that future effect studies do not focus on a single model species but instead incorporate multiple species for a better and more robust assessment of pollutant effects.

1. Introduction

Despite recent investments in renewable energy sources, petroleum remains a major source of fuel for anthropogenic activities. For instance, in 2013 the total oil production worldwide was ca. 90 million barrels a day (EIA-US, 2016). Moreover, petroleum products have broad applications, forming part of plastics, pharmaceuticals, cosmetics and even food (Vandermeulen and Hrudey, 1987; Jones and Pujadó, 2006; Aleklett, 2012; Speight, 2015). Petroleum consists of crude oils and a variety of refined oil products (Albers, 1995). Crude oils are complex mixtures that have “light“, “medium-weight”, and “heavy” components (Michel, 1992). Light components have a higher acute toxicity due to the presence of mono-aromatic hydrocarbons (i.e. BTEX group: benzene, toluene, xylene), and a high evaporation rate associated with a low potential for bioaccumulation. Heavy components, on the other hand, pose a low acute toxicity and are almost insoluble in water, but have a higher bioaccumulation potential. Medium-weight components combine characteristics of both types, thus posing tremendous risks to the environment (Michel, 1992). The water-soluble fraction (WSF) of an oil contains different classes of petroleum hydrocarbons, mainly light and medium-weight components (Michel, 1992), and although these have low potential for environmental persistence

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and bioaccumulation, they are often highly toxic (Michel, 1992; Forth et al., 2017).

Engine oils consist of petroleum-based chemical compounds derived from crude oils, with additives such as anti-corrosion agents (Klamann, 1984). A variety of engine oils end up in the environment along with their additives, as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), requiring assessment of their environmental impacts (Madanhire and Mbohwa, 2016). However, they are generally considered by the oil industry and regulatory agencies as a class of "low environmental risk chemicals" (ATC, 2007).

In aquatic environments, many pollutants, including oil and its derivatives, tend to eventually concentrate in the sediment (Baudo et al., 1990), which harbours a vast diversity of benthic organisms (Wall, 1999; Gray and Elliott, 2009) responsible for key ecosystem functions and services (Snelgrove et al., 2014; Gamfeldt et al., 2015). Oil-derived compounds can enter rivers and seas from several sources, including ship transport, accidental spills, urban inputs (Clark, 2001) and the discharge of ballast and bilge water (Keramitsoglou et al., 2003). Thus, oil pollution can impact benthic organisms directly due to the toxicity of its WSF, and indirectly as a result of oxygen depletion when oil becomes deposited on, or buried in the substratum (Albers, 1995; Clark, 2001).

Nematodes are usually the most abundant and species-rich metazoan taxon of the benthos in marine (Heip et al., 1985; Moens et al., 2013), freshwater (Traunspurger, 2002) and terrestrial sediments (Yeates, 2003; Sochovà et al., 2006). Nematode assemblages typically comprise species with different feeding types and life strategies, as well as different levels of tolerance to changes in environmental conditions (Bongers and Ferris, 1999; Höss and Traunspurger, 2003; Moens et al., 2004). Because of their limited mobility, nematodes are unable to escape pollution events (Bongers et al., 1991; Schratzberger et al., 2003; Höss et al., 2006). Additionally, nematodes are in direct contact with their surrounding (micro)habitat, both through ingestion and due to the permeability of their cuticle to many chemicals (Bongers and Ferris, 1999; Bird and Bird, 1991), and may react in diverse ways to contaminants (Sochovà et al., 2006). All those reasons justify the use of nematodes as sentinels for toxicity impact studies. The analysis of nematode assemblage structure may be particularly powerful in revealing *in situ* effects of pollutants (Bongers and Ferris, 1999; Danovaro et al., 2009). However, not only is this a time-consuming task requiring substantial taxonomic expertise, it often fails to pinpoint exact mechanisms underlying the observed responses (Höss and Williams, 2009). Moreover, establishing proper dose-response relationships from analyses at the assemblage level is fraught with difficulty (Höss and Traunspurger, 2003). Hence, an important role remains for species-specific assays under controlled laboratory conditions.

The choice of a proper target species for monospecific laboratory assays is a crucial aspect in ecotoxicology (Sochovà et al., 2006). The soil bacterivore *Caenorhabditis elegans* is the best-known nematode species used in toxicity testing, among other reasons due to its ease of culture, its short generation time and high fecundity, the vast amount of available literature (Williams and Dusenbery, 1990; Boyd and Williams, 2003a; Frézal and Félix, 2015), and due to the availability of internationally recognized standardized ecotoxicological assay protocols (ISO/DIS, 2010). However, its occurrence is limited to specific (organically enriched) soil habitats, and it does not naturally occur in truly aquatic environments (Frézal and Félix, 2015), despite being a test species for soil and freshwater environments. Moreover, there is hitherto no marine nematode species for which well standardized ecotoxicological test protocols are available. Other nematode species, including the genus *Plectus* and the genera *Monhystera* and *Litoditis*, have been proposed as alternative test sentinels for freshwater and marine environments, respectively (Vranken et al., 1984, 1985; Hägerbäumer et al., 2015; Monteiro et al., 2018). Similarly to *C. elegans*, these species are bacterivores and are relatively easy to culture in the laboratory (Kammenga et al., 1996a; Moens and Vincx, 1998).

Closely related species and/or species with similar life-cycle or morphological traits, often belonging to the same trophic guild, are commonly assumed to exhibit similar responses to disturbance events (Bongers et al., 1991). Conversely, Höss et al. (2011, 2017) encountered large differences in pollution response among several species belonging to the genus *Eumonhystera*. Likewise, Monteiro et al. (2018) reported significant differences in sensitivity to heavy-metal pollution between two cryptic marine nematode species, belonging to the *Litoditis marina* cryptic species complex.

The aim of this study was therefore to assess the direct effects of the WSF of two types of oil on the survival of a range of bacterivorous free-living nematode species from soil, freshwater and marine habitats. The two oils differed in their composition: while the crude oil was a complex matrix of substances, containing many soluble compounds, the diesel-motor oil 10W40 was characterized by a very small fraction of soluble substances. Therefore, we hypothesized that the crude-oil WSF would produce greater effects on nematode survival than the diesel-motor oil WSF. A second aim was to compare the responses of the test species and assess their suitability as candidate alternatives for the common model species *C. elegans* in future toxicity testing. We hypothesized that nematodes which are phylogenetically more closely related would exhibit more similar sensitivities when compared to more distantly related species.

2. Material and methods

2.1. Cultivation of nematodes

Nematode cultures were maintained in Petri plates with agar media (Moens and Vincx, 1998; Muschiol et al., 2009). New nematode cultures were prepared from stocks prior to each mortality assay in order to have healthy and active young adult nematodes for the experiments. For the soil and freshwater species, the agar medium of the stock cultures was made up with distilled water, while for the estuarine/marine species, agar was prepared with artificial sea water (ASW, Dietrich and Kalle, 1957) with a salinity of 20. The agar concentration and composition of the cultivation medium varied among species according to established best practices in the laboraties of the authors of this paper. While for most species, a combination of bacto and nutrient agar was used (Moens and Vincx, 1998), some freshwater and soil species were cultivated on bacto agar only (Table 1), following the specifications for cultivation from the ISO protocol 10872 (ISO/DIS, 2010) for *C. elegans*, which includes growing a small aliquot of a bacteria (*Escherichia coli* OP50) suspension (grown overnight in Luria Bertani media) on the agar surface prior to nematode incubation.

While nematode stock cultures were raised in agar media, the toxicity assays used gellan gum instead of agar because of some particular properties: unlike agar, a semi-fluid consistency can be achieved with gellan gum (Brinke et al., 2011), allowing nematodes to easily move tridimensionally, thus avoiding that animals remain at the surface or at the bottom of experimental vessels (as is often the case when using agar and liquid media, respectively) and hence also reducing the risk that nematodes are not properly exposed to the contaminants and/or suffer oxygen depletion.

Nematode species used as test sentinels were isolated from marine, freshwater or soil. Their approximate generation time under optimal cultivation conditions as well as growth media specifications are listed in Table 1.

2.2. Contaminants

The extraction of the soluble compounds of the two oils followed the methodology of Tsvetnenko and Evans (2002). In short, this involves mixing one part of oil with ten parts of water on a rotary shaker (190 rpm) at a temperature of 27 °C for 24 h. Both phases (oil and water) were subsequently separated with a separation funnel. We