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Differential heavy-metal sensitivity in two cryptic species of the marine nematode *Litoditis marina* as revealed by developmental and behavioural assays

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A B S T R A C T

Heavy metals are persistent pollutants, the concentrations of which have increased in many natural environments as a result of anthropogenic activities. Accumulation of heavy metals in coastal benthic habitats is common. Nematoda are the most abundant and species-rich phylum in these benthic habitats, and shifts in their assemblage composition are considered good indicators of environmental change. At the same time, single-species assays can be highly informative of the organismal and population-level effects of pollution. *Litoditis marina* is a marine nematode inhabiting decaying macroalgae in the littoral zone of coasts and estuaries; it represents a cryptic species complex, and local co-occurrence of several cryptic species is common. Impact studies often assume that phylogenetically related nematodes exhibit similar sensitivities to disturbance, and commonly used biomonitoring indices often utilize scores defined at the level of guilds or families. Nevertheless, studies on other phyla have shown that even closely related taxa such as the cryptic species analysed in this study, may differ in their tolerance to pollution. Here we test the effects of Cu and Pb on the growth, fecundity and behaviour of two cryptic species of *L. marina*, PmII and PmIV, in single-species assays. Pb is a neurotoxicant and we expected that it would strongly affect behaviour (measured here as taxis to food). Increased Cu concentrations cause oxidative stress and can interfere with osmoregulation and endocrine pathways; Cu was therefore expected to impact more on growth and fecundity. Both cryptic species of *L. marina* were sensitive to heavy-metal pollution, but PmIV was substantially more sensitive than PmII, especially to copper. In contrast to our expectation, copper yielded the strongest effects on all endpoints, including behaviour. Lead yielded more discrepant results, inhibiting reproduction but stimulating growth and taxis to food at low (PmII) to intermediate (PmIV) concentrations. This study demonstrates that also in nematodes, closely related species, such as the cryptic species PmII and PmIV analysed in this study, can substantially differ in their response to contaminants, necessitating species-level rather than family or guild-based analyses of pollution effects. This is further supported by a comparison with literature data on the sensitivity of other nematode species to the same heavy metals. This comparison highlights that sensitivity to pollution can vary equally strongly between as within nematode families and guilds, and thereby challenges many guild- or taxonomic-relatedness-based approaches in impact studies using nematodes.

1. Introduction

Human activities have immensely increased the concentrations of pollutants in natural environments, with accumulation often occurring in the benthos of coastal areas. Even low concentrations of contaminants can yield significant effects on organisms that may in turn affect ecosystem health (Fleeger et al., 2003; Boyd and Williams, 2003; Höss and Williams, 2009). Substance-specific effects are linked to the mode of action of a toxicant and therefore require substance-specific endpoints for their assessment (Höss and Williams, 2009). For example,

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hormone-regulated processes (e.g. growth, reproduction) can change under the influence of endocrine disrupting substances (Höss and Weltje, 2007), whereas neurotoxicity can induce changes in behaviour (e.g. motility (Anderson et al., 2001) and chemotaxis (Chen et al., 2014)). Therefore, focusing on a single (for instance mortality) or a few often related endpoints (e.g. growth and reproduction) may give partial and/or biased appraisals of the effects of contaminants on organisms. A combination of different endpoints may provide a more complete and comprehensive picture.

Heavy metals are conservative pollutants, i.e. not subject to microbial decomposition (Clark, 2001). Because of their wide use in several anthropogenic activities, concentration levels of many metals in the environment are above natural levels, representing an environmental threat (Clark, 2001; Bradl, 2005). Copper (Cu) is an essential metal to most organisms (Holm et al., 1996; Solomon et al., 1996), but in elevated doses it is known to interfere with osmoregulation (Brooks and Mills, 2003) and oxidative stress pathways (Lushchak, 2011), and can act as an endocrine disruptor (Handy, 2003). Lead (Pb) is a non-essential metal, widely used in industry (Clark, 2001), but also a known neurotoxicant that can yield severe effects on living organisms (Williams and Dusenbery, 1990; Verstraeten et al., 2008). Because neurobiological alterations may provoke behavioural changes, it is expected that behavioural endpoints will be more sensitive to toxicity of neuro-contaminants such as Pb (Anderson et al., 2004) than developmental endpoints like growth and reproduction. Vice versa, because of its role in oxidative stress and endocrine disruption, Cu may yield more severe effects on developmental endpoints than on organismal behaviour.

As a group, Nematoda present several characteristics that justify their use in ecotoxicological research (Bongers and Ferris, 1999; Höss and Williams, 2009). Nematodes are usually the most abundant and species-rich taxon of the benthos, which is often the ultimate fate of chemicals that contaminate aquatic bodies. Due to their limited mobility, nematodes are unable to escape from pollution effects (Bongers et al., 1991). They also present different life strategies and different levels of tolerance to changes in environmental conditions (Bongers and Ferris, 1999; Höss and Traunspurger, 2003). Such differential tolerances may affect the outcome of species interactions (Martinez et al., 2012, 2016) and may therefore cause changes in community composition. As a consequence, analyses of change at the community level have commonly been interpreted in the context of environmental stress or pollution, including heavy-metal pollution (Korthals et al., 1996; Austen and McEvoy, 1997; Austen and Somerfield, 1997; Gyedu-Ababio et al., 1999; Mahmoudi et al., 2007). Different concentrations of both heavy metals used in our study, Cu and Pb, affected the structure of estuarine nematode assemblages, and species responses varied, with the appearance of either tolerant or sensitive indicators (Austen et al., 1994; Mahmoudi et al., 2007). *Oncholaimus campylocercoides*, for instance, has been classified as a species tolerant to low and medium Pb doses (< 1.45 mg/g sediment dry weight), but it is sensitive to higher Pb concentrations (i.e. 2.1 mg/g sediment dry weight). *Calomicrolamus honestus*, by contrast, was eliminated at all Pb treatments, including concentrations as low as 0.76 mg/g (Mahmoudi et al., 2007). Caution is due when trying to extrapolate such sensitivity levels to other settings, since the sediment type will influence heavy-metal bioavailability: sand assemblages, for instance, are usually more vulnerable than mud communities due to the binding of heavy metals by the organic matter in muddy sediments (Wolanski and Elliot, 2016).

In addition, some nematode species have been used as model organisms in single-species toxicity assays (Höss and Williams, 2009). The most popular and well established model species is the soil rhabditid *Caenorhabditis elegans* (Traunspurger et al., 1997; Leung et al., 2008). It is the only nematode species used so far in internationally recognized standardized single-species toxicity assays, either from the American Society for Testing and Materials (ASTM, 2001) or from the International Organization for Standardization (ISO/DIS 10872, 2010).

Litoditis marina (Sudhaus, 2011), formerly known as *Rhabditis marina* (Bastian, 1865) or *Pellioditis marina* (Andrássy, 1983), is a cosmopolitan morphospecies which is mostly found on decaying macroalgae in the littoral zone of coasts and estuaries (Inglis and Coles, 1961), where it may play a significant role in organic matter decomposition (Urban-Malinga et al., 2008). This species displays tolerance to a broad salinity and temperature range (e.g. Moens and Vincx, 2000). However, this alleged broad tolerance to environmental conditions may be partly biased in view of the discovery of substantial cryptic diversity within *L. marina*. The morphospecies comprises a complex of at least ten different cryptic species (Derycke et al., 2008a, 2008b). Sympatric occurrence of two or more species at local scales is common (Derycke et al., 2006). Recent studies have shown that those species differ in their autecology (De Meester et al., 2011, 2015), feeding preferences (Derycke et al., 2016) and dispersal behaviour (De Meester et al., 2012) and may differentially affect organic matter decomposition (De Meester et al., 2016). Nevertheless, none have assessed whether they differ in their tolerance to pollution. Differences in sensitivity to pollutants between cryptic species have already been reported for earthworms (Andre et al., 2010) and for a number of aquatic invertebrates, including other oligochaetes (Sturmbauer et al., 1999), polychaetes (Bach et al., 2005; Linke-Gamenick et al., 2000; Reynoldson et al., 1996) and copepods (Rocha-Olivares et al., 2004). It is crucial to consider such differences when members of cryptic species complexes are included in environmental monitoring surveys or when they are used in bioassays. In addition, any significant differences in sensitivity of such closely related species could potentially challenge the widespread use of biomonitoring indices defined at the level of nematode families, under the assumption that related nematode species display similar tolerances to various kinds of disturbances (Bongers and Ferris, 1999).

The first aim of this study was to assess the effects of copper and lead contamination on two cryptic species of *L. marina* using behavioural, developmental and reproductive endpoints. We hypothesized that effects of the neurotoxicant Pb would be better revealed by behaviour, whereas those of Cu would be better illustrated by growth and fecundity. Secondly, we compared the sensitivities of these two morphologically (nearly) identical species and hypothesized that these would not differ given the close phylogenetic relatedness and morphological as well as ecological similarity of these two species.

2. Material and methods

2.1. Nematode cultures

Stock cultures of two cryptic species of *Litoditis marina* (PmII and PmIV) were initially raised from specimens collected from decaying macroalgae at the Belgian coast (Blankenberge) for PmII and at lake Grevelingen in The Netherlands for PmIV. Lake Grevelingen is a former estuary of the rivers Rhine and Meuse, but since 1971 a non-tidal brackish-water lake. The stock cultures were maintained under controlled laboratory conditions at a constant temperature of 20 °C on sloppy agar plates (1% bacto and nutrient agar in a 4:1 ratio, prepared with artificial sea water (ASW) with a salinity of 25 and pH 7.5–8, buffered with TRIS-HCl at a final concentration of 5 mM), with unidentified bacteria as a food source (Moens and Vincx, 1998). Fresh nematode cultures were prepared for each experiment. To avoid food depletion and to stimulate growth and reproduction, a few drops (30–50 μL) of a suspension of frozen-and-thawed *Escherichia coli* (K-12 strain, 3×10^9 cells/mL, ASW, cholesterol 0.1%) were added to the stock plates from which nematodes were harvested for toxicity assays.

2.2. Test substances and concentrations

The same four molar toxicant concentrations were used for both copper and lead: 10, 40, 80 and 240 μM (Table 1), the higher three in the behavioural and the lower three in the growth and reproduction