



Effects of barium and cadmium on the population development of the marine nematode *Rhabditis (Pellioditis) marina*

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ABSTRACT

Offshore oil and gas drilling often involves the use of fluids containing barium and traces of other heavy metals. These may affect the environment, but information on their toxicity to benthic biota remains scant. Here, we present results of a 10-day bioassay with the marine nematode *Rhabditis (Pellioditis) marina* at different loads of barium (0–10 000 ppm nominal concentrations) and cadmium (0–12 ppm) in the range of concentrations reported from drilling-impacted sediments. Barium did not affect the fitness and population development of *R. (P.) marina* at concentrations up to 300 ppm, but did cause a decrease in population abundance and an increase in development time from concentrations of 400–2000 ppm onwards. Increased mortality occurred at 4800 ppm Ba. For cadmium, LOEC and EC₅₀ values for total population abundance were 2.95 and 8.82 ppm, respectively. Cd concentrations as low as 2.40 to 2.68 caused a decrease in the abundance of adult nematodes, indicating that assays covering more generations would likely demonstrate yet more pronounced population-level effects. Our results indicate that oil and gas drilling activities may potentially have important implications for the meiobenthos through the toxicity of barium and associated metals like cadmium.

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1. Introduction

Improved technologies for the exploration and extraction of oil and gas have caused a strong worldwide expansion of offshore drilling activities (OGP, 2003). Offshore drilling often involves the discharge of large quantities of muds into the sea and the use of drilling fluids for, among other things, cooling and lubricating the drill bit and the drill string (Berge, 1996). The discharged muds and adsorbed fluids settle onto the seafloor and accumulate to different degrees, depending on the volume and characteristics of the discharged fluid and the characteristics of the receiving environment. There are two primary types of drilling fluids: water based fluids (WBFs) and non-aqueous drilling fluids (NADFs). Concentrations of NADF compounds in the sediment immediately after the drilling discharges are typically high and may have a considerable effect on the surrounding environment and its benthic biota (Ng and Patterson, 1982), causing reductions in faunal abundance and diversity (OGP, 2003). In recent years, diesel or crude oil has been replaced as the base fluid of NADFs by low-toxicity mineral oils and

synthetic fluids (Melton et al., 2000; Pozebon et al., 2005), thus reducing direct toxicity of NADFs. However, both WBFs and NADFs contain barium as a major component (Melton et al., 2000; OGP, 2003). Barium is present mostly in the form of barium sulfate (barite), and is often contaminated with traces of several other heavy metals such as cadmium, mercury, arsenic, chromium, copper, lead, nickel, and zinc (EPA, 2005). These contaminants may long remain toxic at either lethal or sublethal levels.

Among the marine benthos, nematodes are by far the most abundant and speciose metazoans (Platt, 1984; Heip et al., 1985; Giere, 2009). Nematodes from both terrestrial and aquatic environments may be sensitive indicators of pollution (Bongers and Ferris, 1999). They are ubiquitous and persistent as a taxon in virtually all environmental conditions that can support metazoan life (Vincx and Heip, 1987). They have a rapid turnover and usually short life-span compared to most macrofauna. Moreover, their lack of pelagic life stages and their endobenthic life imply that effects of local contamination are not likely to be obscured by migrations. They can therefore be expected to demonstrate a generally fast response to pollution (Vincx and Heip, 1987).

Risk assessment efforts at population and community levels require model organisms that allow exposure and full cycle measurements throughout their reproductive and recruitment

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time windows (Chandler et al., 2004). With such model species, it is possible to assess survival, growth and reproduction, which are considered the most relevant characteristics for risk assessment at the population-level (Van Gestel and Van Brummelen, 1996). The few marine nematode species that are amenable to lab culture typically have fast growth, short generation times, fairly short longevity and a high reproductive capacity, rendering them feasible model organisms for dedicated ecotoxicological experiments (Moens and Vincx, 1998).

Rhabditis (Pellioiditis) marina is a bacterivorous nematode typical of decomposing macroalgal wrack and of sheltered permanent macroalgal holdfasts in marine and estuarine littoral environments worldwide. It is a species (complex) with a very short generation time (down to less than 3 days (Moens and Vincx, 2000)) and a fairly broad salinity and temperature tolerance (Moens and Vincx, 2000). Reproduction is obligately heterosexual; females usually deposit their eggs, but ovovivipary also occurs. Reproductive output is high (up to 600 progeny per female (Vranken and Heip, 1983)). There are four juvenile moults before adulthood is reached. Under conditions of food depletion and crowding, a metabolically less active dauer stage is formed. *Rhabditis (Pellioiditis) marina* is one of rather few marine nematode species which can easily be reared under laboratory conditions on well-defined media (Moens and Vincx, 1998), rendering it a suitable model organism to determine critical loads of various toxicants.

The aim of this work was to analyze (mostly sublethal) effects of different concentrations of barium and cadmium on the population development of the marine nematode *R. (P.) marina* under standardized laboratory conditions. Recent field studies have mainly attributed the effects of NADFs and WBFs on meiobenthic communities to a combination of hydrocarbon toxicity and anoxia (Netto et al., 2009, 2010). In this respect, WBFs are expected to yield substantially less effects on benthic fauna than NADFs. We hypothesized that the concentrations of barium and other heavy metals present both in so-called low-toxicity mineral oils and synthetic non-aqueous drilling fluids could impact benthic life at, and in the immediate vicinity of, drilling sites.

2. Materials and methods

2.1. Choice and culture of test organism

Rhabditis (Pellioiditis) marina for this study were obtained from established monospecific, agnotobiotic cultures in exponential growth. The cultures originate from the Westerschelde Estuary, SW Netherlands, and are maintained on sloppy agar (0.75%) media consisting of bacto and nutrient agar (DIFCO) in a 6 : 1 ratio, with unidentified bacteria from the habitat as food (Moens and Vincx, 1998). Stock cultures have a salinity of 25 (corresponding to the average salinity of the site from which the cultures originate) and are maintained at a constant temperature of 17 °C. They are prepared with artificial seawater (ASW) (Dietrich and Kalle, 1957) of the appropriate salinity.

Rhabditis (Pellioiditis) marina is a species complex comprised of several largely cryptic species, 3 of which have been found in the Westerschelde Estuary (Derycke et al., 2006, 2008). The culture used in the present experiments belongs to the lineage Pm I (Santos et al., 2008), which is the most abundant and widespread species in the Westerschelde Estuary (Derycke et al., 2006).

2.2. Test conditions and media

Experiments were performed on sloppy bacto-agar layers in 5.5 cm diam. polystyrene Petri dishes with a final concentration of 0.75% and with a salinity of 25, maintained at 17 °C. 4 ml of agar

were added to each Petri dish. In order to avoid indirect effects, through heavy metal impacts on the growth of the unidentified bacteria which serve as food in the stock cultures, these unidentified bacteria were replaced in the experiments by *Escherichia coli* (strain K12). *E. coli* were offered as 50 µl of a frozen-and-thawed suspension with a density of 3×10^{10} cells ml⁻¹ and gently spread over the agar surface. This corresponds to an optimal food availability for *R. (P.) marina* (Santos et al., 2008), and since most *E. coli* die and burst open during the freezing and thawing, nematodes in our experiments obtained their food largely by feeding on (the remains of) dead bacteria. Transfer and growth of unidentified bacteria from the stock cultures were kept to a minimum both by thorough rinsing of the nematodes during transfer (see below), and by the use of a nutrient-poor agar medium (containing only bacto-agar). In the absence of a nutrient agar portion, the experimental agar medium had to be supplied with 100 µl l⁻¹ cholesterol, because nematodes are incapable of synthesizing sterols when their only food source is bacteria (Vanfleteren, 1980).

Doses of heavy metals (barium and cadmium) used in the present study were based on concentrations found at oil drilling sites (Rezende et al., 2002; Pozebon et al., 2005), with the lower end of our range corresponding to concentrations found in non-exploited sediments (Guthrie et al., 1979). For barium, we prepared nominal concentrations of 0, 120, 400, 2000, 3600, 4800 and 10 000 ppm using BaCl₂·2H₂O. However, Ba²⁺ reacts with sulfates in the ASW to form nearly insoluble BaSO₄. Hence, except at the highest three Ba concentrations, dissolved Ba levels in our media remained undetectable (<0.05 ppm), and all Ba was present as BaSO₄. In the media with nominal additions of 3600, 4800 and 10 000 ppm Ba, ICP-OES measured dissolved Ba concentrations of 213, 1283 and 4109 ppm, respectively. For cadmium, we prepared nominal concentrations of 0, 0.3, 1, 5, 9 and 12 ppm, corresponding to measured concentrations of 0, 0.29, 1.01, 4.80, 8.87 and 11.80 ppm (measured using ICP-OES), which were prepared with CdCl₂·2.5H₂O. In the case of Ba, we prepared the agar media by first mixing the proper amount of BaCl₂·2H₂O in ASW, and then adding and dissolving the agar powder in the Ba-containing ASW. The very low agar concentration and minimal volume shift of the ASW after dissolution of the agar ensure that deviations from the intended Ba concentrations were negligible. In the case of Cd, we prepared the Cd solutions and agar media separately. µl volumes of Cd solutions of appropriate concentration to reach the above-listed measured concentrations, were pipetted into the Petri dishes before addition of the agar medium. Proper homogenization of metals in the agar medium was then obtained by gently shaking the Petri dishes in an 8-shape while the agar was still liquid.

2.3. Experimental design

Males and gravid females of *R. (P.) marina* were manually picked up from the stock cultures and rinsed three times in sterile ASW to minimize transfer of bacteria from the stock culture. They were taken from cultures in exponential growth phase. However, this does not imply that all adults were of the same age, since such cultures contain individuals from different generations and 'cohort' development is not fully synchronous. Five males and five females were inoculated per treatment and per replicate; the animals were picked up from the ASW with a Tungsten wire needle and transferred to a drop of ASW on the agar surface without touching the agar layer. There were three replicates per treatment for the Ba treatments and four for the Cd treatments.

The population of *R. (P.) marina* was observed daily for a period of 10 days, which at the temperature, salinity and food conditions of the present experiments corresponds to two generations of *R. (P.) marina* (F1 and F2, with F0 representing the inoculum). On each

observation, we counted the total number of specimens, juveniles and adults (discriminating between males and females) as well as dead (i.e. totally immobile) individuals to determine the effects of cadmium and barium on the fecundity (i.e. number of progeny per female per unit of time), total population development, adult population development, % females, survival, and minimum development time (defined as in Moens and Vincx (2000)). Because reproduction in our microcosms could only be unequivocally attributed to the parental generation during the first five days of the experiment, our fecundity data are not representative of the total fecundity of *R. (P.) marina*.

2.4. Data analysis

For each heavy metal (Ba and Cd), effects of different concentrations were compared using repeated measures analysis of variance (ANOVA) with metal concentration and time as factors. Prior to analysis, the assumptions of normality (using the Kolmogorov–Smirnov test) and homogeneity of variances (Levene’s ANOVA-test on the deviation of scores and Bartlett’s χ^2 test) were tested, and data were $\log_{10}(x + 1)$ transformed where necessary. Pairwise *a posteriori* comparisons were performed with Tukey’s Honest Significant Differences (HSD) test. These analyses were performed using the STATISTICA 6 software (Statsoft).

We also constructed dose-response curves by integrating the areas under the growth curves and plotting these as a function of metal concentration. We fitted a logistic exponential model of the

form $f = a * \exp(-b * x)$ through the data using SigmaPlot 10.0 (Systat Software, Inc.). In order to allow at least a rough discrimination between metal effects on the fecundity of the F0 and F1 generations, we did this separately for the first 5 days and for days 6–10 of the experiment. These areas under the curve were analyzed with one-way analysis of variance (ANOVA) after testing for the same assumptions as for the repeated measures analyses.

Following Isnard et al. (2001), we used the logistic dose-response models to estimate NOEC (no observed effect concentration), LOEC (least observed effect concentration) and EC₅₀ values. The highest metal concentration where a given response variable does not differ significantly from the control is the NOEC; the lowest metal concentration where that response variable does significantly differ from the control is the LOEC. We used the 95% confidence intervals of the means of the control to determine NOEC and LOEC. EC₅₀ was determined as the metal concentration at which a given response variable (abundance of nematodes) dropped to 50% of the highest mean (the latter invariably being either the control or the lowest metal concentration treatment).

3. Results

3.1. Barium

Barium had highly significant effects on the total population density of *R. (P.) marina* ($F = 263.80$; $P < 0.0001$; Figs. 1A and 2A), with a gradual decline of nematode densities with increasing Ba

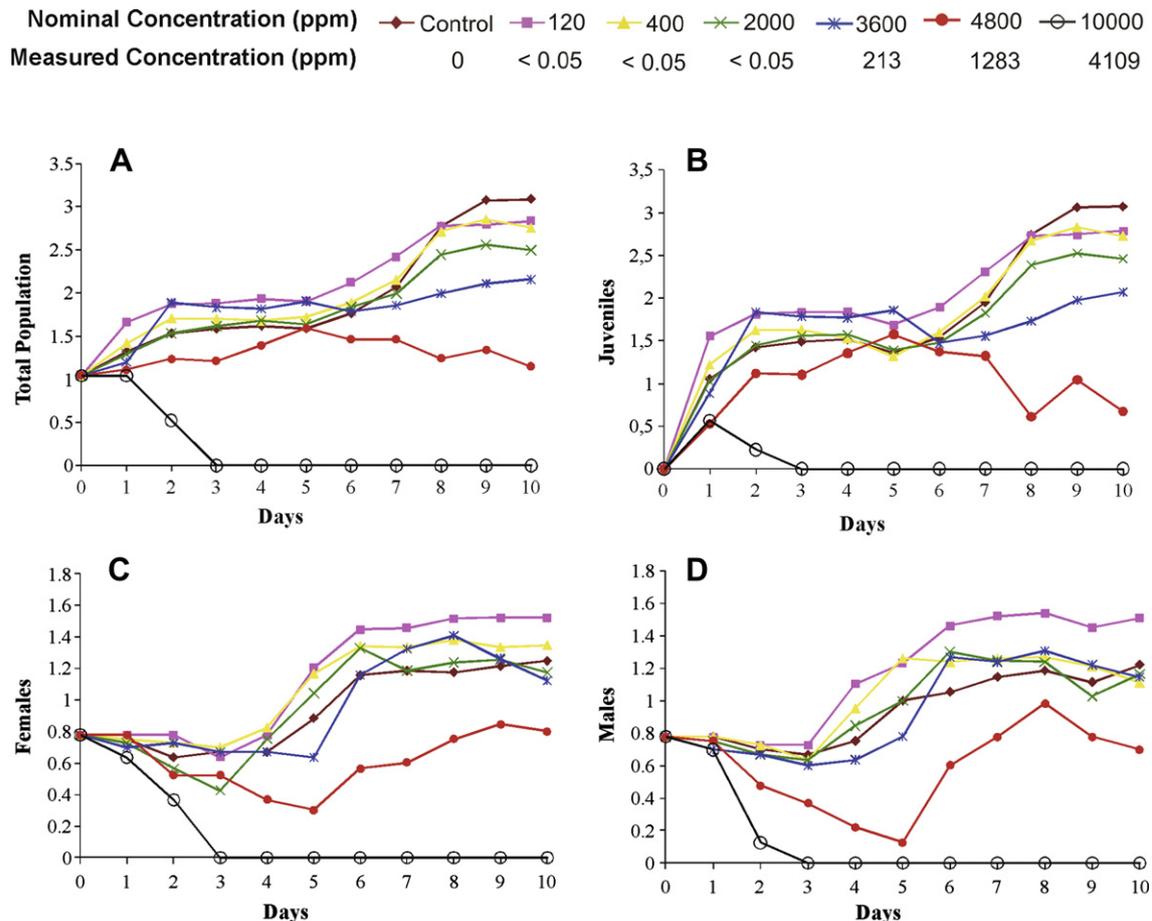


Fig. 1. Population development of *Rhabditis (Pellioditis) marina* over time at six different concentrations of barium (in ppm) and a barium-free control. A = Total Population; B = Juveniles; C = Adult females and D = Adult males. Data have been log-transformed and are means of three replicates per treatment and time.

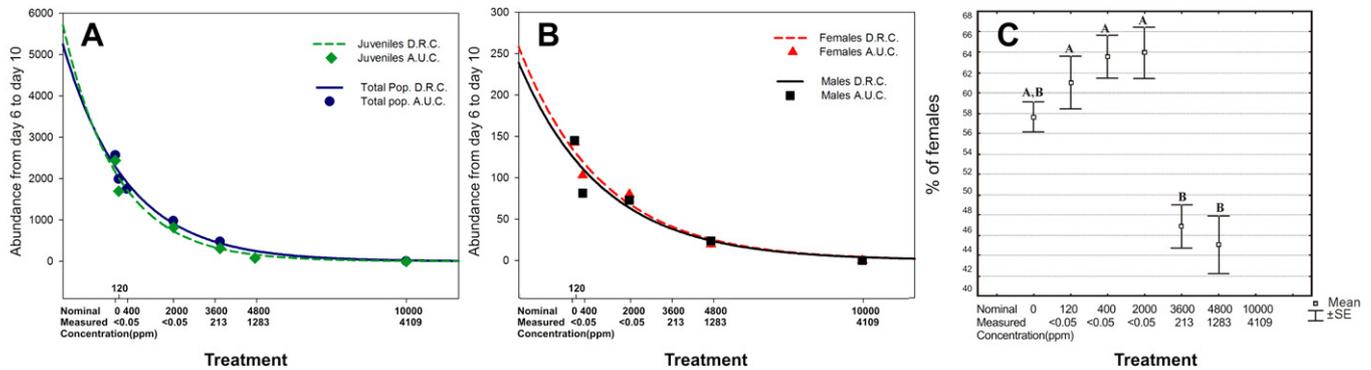


Fig. 2. Dose-response curves of abundances (A, B) and % females (C) of *R. (P.) marina* as a function of barium concentration. A = total population and juvenile abundances; B = adult male and adult female abundances; C = % females in the adult population. The dose-response curves in A and B are based on the areas under the abundance curves from day 6 to day 10. C: data on % females are time-averaged means \pm 1 sterror of four replicates per treatment.

load. The control treatment differed significantly from all other treatments except the lowest two Ba concentrations. At the highest Ba concentration, all nematodes died within 3 days after inoculation.

Adult nematodes had a very short life-span in our experiments, and the results on total population density are largely determined by the abundance of juveniles. Juveniles appeared already on the first day in all replicates and treatments (Fig. 1B), again with highly significant differences between treatments ($F = 163.69$; $P < 0.0001$). At Ba concentrations of 4800 ppm and higher, juveniles were observed after 1 day but all died shortly afterward. The daily and total fecundity of the F0-generation over the first 5 days of the experiment did not differ significantly between treatments ($F = 1.5551$; $P = 0.2319$) except at a Ba concentration of 10 000 ppm (data not shown). The fecundity of the F1 generation, estimated from the area under the total population curve in the 6–10 day interval, did show more pronounced differences between treatments ($F = 92.614$; $P < 0.0001$), with all but the lowest two Ba concentrations yielding significantly lower fecundity than the controls (Fig. 2A, ESM 1B).

Minimum development time (from appearance of first progeny till maturation of first adult) was 4–5 days for the control and the lowest two Ba concentrations, and increased significantly ($F = 8.80$; $P = 0.001$) to more than 6 days at 4800 ppm Ba (Table 1). At 3600 ppm Ba, numbers of adults already crashed from 8 days onwards. No juveniles matured at the highermost Ba concentration (10 000 ppm). Abundances of adults differed between treatments ($F = 161.98$; $P < 0.0001$), but only Ba loads of 2000 ppm and higher yielded significantly lower adult densities than the control and the

lowermost Ba concentration (120 ppm) (all $P < 0.05$). Differences between treatments were largely consistent for males and females (Figs. 1C, 1D, 2B). Abundances of adults were typically higher at 120 ppm Ba than in the Ba-free control, albeit that these differences were not statistically significant (Figs. 1C, 1D, 2B, ESM 1A).

Adult sex ratio, expressed as % females, differed significantly with Ba load ($F = 5.49$; $P < 0.01$), exhibiting a clear bias toward females in the controls and in treatments with up to 2000 ppm Ba, compared to a small bias in favor of males at still higher Ba concentrations (Fig. 2C). Up to a concentration of 2000 ppm Ba, there was a general trend of increasing % females with increasing Ba load, but this trend was not statistically significant ($P > 0.05$).

The coefficients and R^2 of the logistic dose-response model are given in Table 2. NOEC and LOEC spanned a range from 200 ppm for juveniles to 1003 ppm for adult females, whereas EC_{50} values ranged from 1057 ppm Ba for juveniles to 1865 ppm Ba for adult females. Although some adult males and no females survived at the highest Ba load, the EC_{50} value for males was slightly lower than for females (Table 3).

3.2. Cadmium

Total population densities of *Rhabditis (Pellioiditis) marina* showed only borderline significant differences between Cd concentrations ($F = 3.18$; $P < 0.05$; Figs. 3A and 4A); the only significant pairwise difference was found between the control and the 9.0 ppm Cd treatment ($P = 0.0469$).

As in the Ba experiment, total population density was largely determined by juvenile abundance. Both total and juvenile abundances were highest in the controls, but most pairwise differences were not significant (Fig. 3B, ESM 2B). Juveniles appeared from the first day of the experiment in all treatments. The daily and total fecundity over the first 5 days of the experiment did not differ between the treatments ($F = 0.8461$; $P = 0.5349$). Fecundity of the

Table 1
Minimum development time of *Rhabditis (Pellioiditis) marina* under different concentrations of barium (in ppm) (left half of the table) and cadmium (right half of the table). Data are means \pm 1 standard deviation of three replicates per treatment for Ba and four replicates per treatment for Cd. * means that no juveniles reached adulthood.

Barium	Minimum development time		Cadmium	Minimum development time	
	Mean	S.D.		Mean	S.D.
Control	5	0	Control	4.75	0.5
120	4	0	0.3	4.75	0.5
400	4	0	1.0	5	0
2000	4.67	0.58	5.0	4.75	0.5
3600	5.67	0.58	9.0	5	0
4800	6.34	0.58	12.0	5.25	0.5
10 000	*	*			

Table 2
 R^2 and values of the coefficients a and b of the logistic model $f = a \cdot \exp(-b \cdot x)$ for juveniles, adult males, adult females and total population of *R. (P.) marina*. Left half of the table: data for barium. Right half of the table: data for cadmium.

Barium	Logistic model parameters			Cadmium	Logistic model parameters		
	a	b	R^2		a	b	R^2
Juvenile	2159.334	0.0005	95.91%	Juvenile	1636.013	0.0776	92.37%
Male	127.782	0.0003	88.17%	Male	125.657	0.0677	84.28%
Female	137.667	0.0003	95.77%	Female	143.082	0.0862	92.57%
Total pop.	2294.045	0.0005	97.06%	Total pop.	1934.604	0.0776	95.87%

Table 3

NOEC, LOEC and EC₅₀ values for juveniles, adult males, adult females and total population of *R. (P.) marina* estimated from the logistic dose-response model. Left half of the table: data for barium. Right half of the table: data for cadmium.

Barium	Observed effect			Cadmium	Observed Effect		
	NOEC	LOEC	EC ₅₀		NOEC	LOEC	EC ₅₀
Juvenile	<200	200	1057	Juvenile	<3.35	3.35	8.80
Male	<787	787	1620	Male	<2.40	2.40	6.90
Female	<1003	1003	1865	Female	<2.68	2.68	8.13
Total pop.	<302	302	1250	Total pop.	<2.95	2.95	8.82

F1 generation ($F = 1.822$; $P = 0.159$), estimated from the area under the total population growth curve from days 6 till 10, was generally lower in Cd treatments than in the Cd-free control, but there were no significant differences between the different Cd concentrations (Fig. 4A, ESM 2B).

The first F1-adults appeared in some of the replicates of the control and in the 0.3 ppm Cd treatment after the 4th day. After the 5th day, F1-adults were present in all the replicates of all the treatments, indicating that minimum development time was not significantly impacted by Cd load ($F = 0.80$; $P = 0.564$) (Table 1).

Abundance of adults in the population decreased with increasing Cd load ($F = 10.66$; $P < 0.0001$), basically separating controls + Cd concentrations of 0.3 and 1.0 ppm from all higher Cd concentrations (Fig. 4B, ESM 2A). This was most clearly evidenced by abundances of adult females (Fig. 3C) ($F = 12.90$; $P < 0.0001$), but much less so by abundances of adult males, the latter exhibiting no

significant effect of Cd in the range of concentrations tested here ($P = 0.06$) (Fig. 3D). Note that it is largely the 9.0 ppm Cd treatment where male densities deviated from the general pattern exhibited by total adult and female abundances. % females did not differ significantly between treatments ($F = 1.40$; $P = 0.27$). As a rule, the sex ratio of *R. (P.) marina* in the Cd experiment was weakly to moderately female-biased (Fig. 4C).

The coefficients and R^2 of the logistic dose-response model are given in Table 2. NOEC and LOEC varied from 2.4 ppm in males to 3.35 ppm in juveniles. EC₅₀ values ranged from 6.9 ppm Cd in males to 8.8 ppm Cd in juveniles (Table 3).

4. Discussion

Nematodes are frequently used as bioindicators of environmental disturbance and pollution in terrestrial, freshwater and marine environments. Community approaches are powerful because they provide information on the whole range of life strategies and functional groups of nematodes in a sediment (Bongers and Bongers, 1998; Bongers and Ferris, 1999; Sochová et al., 2006; Schratzberger et al., 2007). In the marine realm, intertidal and shallow subtidal nematode communities have been manipulated in micro- or mesocosm designs, allowing experimental assessment of various loads of toxicants or disturbance regimes (Sundelin and Elmgren, 1991; Austen et al., 1994; Austen and Somerfield, 1997; Schratzberger et al., 2002). Only few studies have taken a similar approach to offshore nematode communities, but these too may be amenable to incubation in fairly

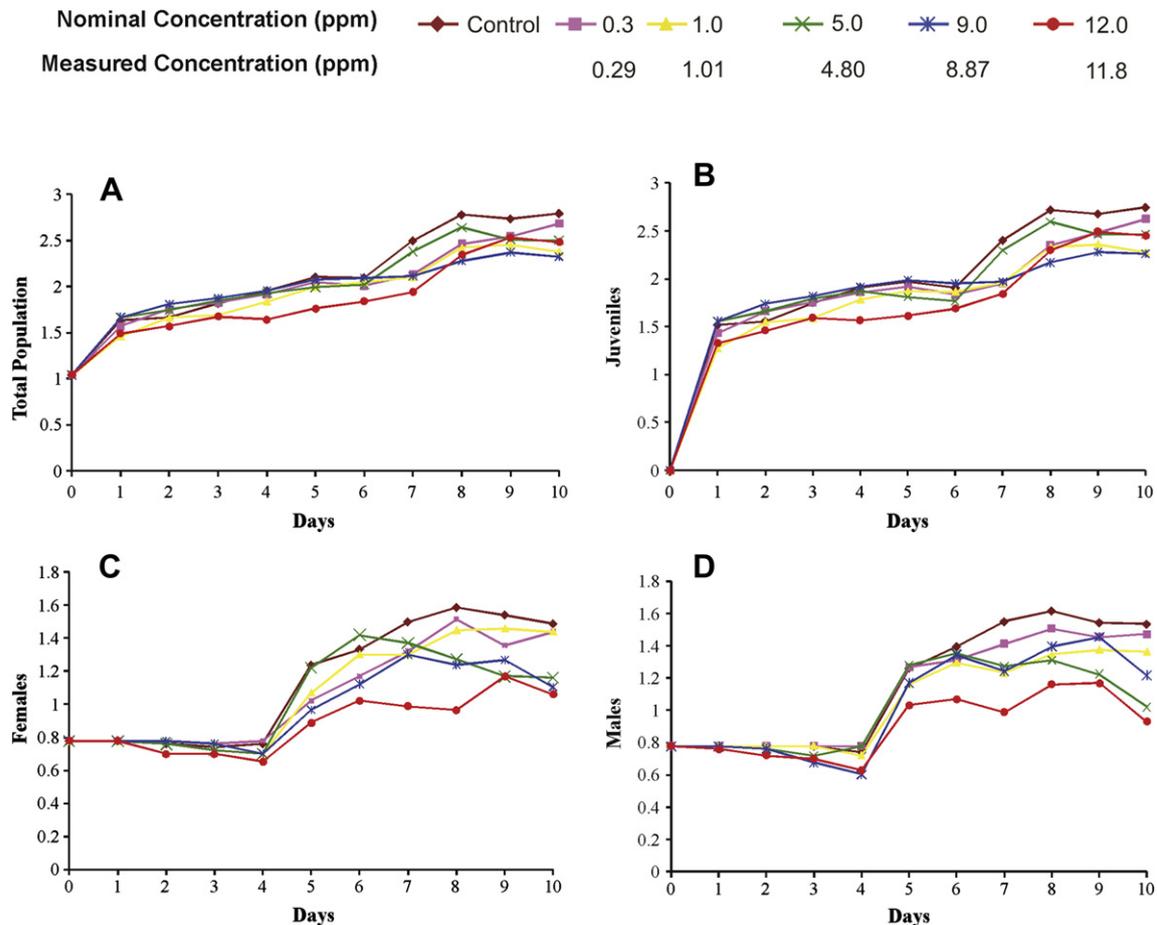


Fig. 3. Population development of *Rhabditis (Pellioiditis) marina* over time at five different concentrations of cadmium (in ppm) and a cadmium-free control. A = Total Population; B = Juveniles; C = Adult females and D = Adult males. Data have been log-transformed and are means of four replicates per treatment and time.

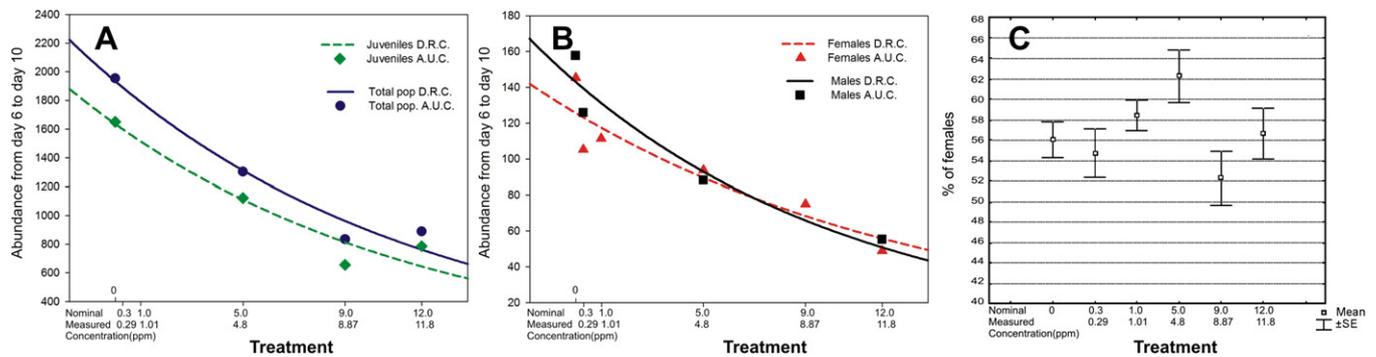


Fig. 4. Dose-response curves of abundances (A, B) and % females (C) of *R. (P.) marina* as a function of cadmium concentration. A = total population and juvenile abundances; B = adult male and adult female abundances; C = % females in the adult population. The dose-response curves in A and B are based on the areas under the abundance curves from day 6 to day 10. C: data on % females are time-averaged means \pm 1 sterror of four replicates per treatment.

simple microcosms for several weeks (Austen and McEvoy, 1997; Gallucci et al., 2008). However, because of the complexity of benthic nematode communities and the multitude of trophic and non-trophic relationships they have with other foodweb components, the processes responsible for the outcome of community level approaches usually remain hidden. Therefore, experiments on individual nematode species are required to obtain a better understanding of the effects of toxicants or other disturbances on marine nematodes, and hence to be able to make more accurate predictions.

Unfortunately, there have hitherto been no successful attempts at rearing any nematode species from shelf or deep-sea sediments in permanent culture on well-defined media. *Rhabditis (Pellioiditis) marina* is a nematode from intertidal environments and one of few marine representatives of the family Rhabditidae, to which belong, among others, *Caenorhabditis elegans* (Anderson et al., 2001; Höss et al., 2002; Ura et al., 2002; Höss and Traunspurger, 2003) and *Pellioiditis pellio* (Eveland and Fried, 1990), species which have been used successfully in ecotoxicological assays in soil and freshwater environments. Rhabditidae in general appear to have a high tolerance to a variety of pollutants, and our results and conclusions on the sensitivity of nematodes to Ba and Cd should therefore be considered conservative, even though sensitivity differences of nematodes to heavy metals may not always be very pronounced and do not relate to life-history strategy (Kammenga et al., 1994). As illustrated in a recent study on sublethal Cd toxicity in *R. (P.) marina*, the use of this species in bioassays allows a combination of population growth-related parameters and genetic diversity (Derycke et al., 2007), rendering it a particularly interesting marine model organism for dedicated toxicological tests (Breitholtz et al., 2006).

4.1. Effects of barium

Barium at concentrations up to 400 ppm did not significantly affect the population development of *R. (P.) marina*, even though the logistic model predicts that concentrations as low as 302 ppm may affect juvenile and total population abundance. At Ba concentrations of 2000 ppm and higher, abundances of juveniles and adults were negatively affected, in agreement with predicted LOEC values for adults between 400 and 2000 ppm. Ba loads above 3600 ppm impaired both juvenile and adult survival. The precision of our LOEC and EC₅₀ estimates is, of course, affected by the absence of treatments with concentrations intermediate between 400 and 2000 ppm, but modeled LOEC and EC₅₀ values ranged between 200 (for juveniles) and 1003 ppm (for adult females) and between 1057 (for juveniles) and 1865 ppm (for adult females), respectively.

Because dead juveniles decomposed fairly quickly in our microcosms, our counts do not allow reliable direct estimates of juvenile mortality. The reason(s) for the lower population densities at 2000 ppm Ba therefore remain unclear. Fecundity of the F₀-generation was not significantly affected by Ba at 2000 ppm, but the increasing gap in population density with time between lower and higher Ba loads suggests that fecundity of later generations (F₁, F₂...) may have been affected by barium. Santos et al. (2008) also warned that treatment effects on the F₀ and its progeny may be obscured by the (beneficial) culture history of the F₀ adults. Because generations rapidly start to overlap in these microcosms, it is not possible to discriminate strictly between effects on subsequent generations. This would require establishing novel microcosms with adults harvested from the treatments immediately after they have matured. At Ba concentrations exceeding 2000 ppm, the lower overall population abundance results from a combination of increased mortality and delayed maturation of adults. Based on the ANOVA analyses of population growth and abundance, juveniles and adults had a comparable sensitivity to Ba. However, based on the dose-response model, it is clear that both LOEC and EC₅₀ are substantially lower for juveniles than for adults, in line with the idea that juveniles are the most sensitive life stages (Kammenga et al., 1996, 1997).

Sex ratio was consistently female-biased, except at Ba concentrations of 3600 ppm and higher. EC₅₀s calculated from our logistic dose-response model nevertheless do not support the idea that males are less sensitive to Ba than females, rather the contrary.

Empirical studies on the effects of barium on invertebrates have hitherto been few. Ba at a concentration of 5000 ppm caused 100% mortality of the oligochaete *Enchytraeus crypticus* (Kuperman et al., 2002), which is very similar to the present results on *R. (P.) marina*, and the LC₅₀ value for juveniles was 947 ppm. The earthworm *Eisenia foetida* was unable to produce cocoons at Ba concentrations above 500 ppm (Simini et al., 2002). Ba concentrations of less than 10 ppm already impacted reproduction of *Daphnia magna*, whereas concentrations between 10 and 100 ppm caused behavioral as well as lethal effects in daphnids (Biesinger and Christensen, 1972; Khangarot and Ray, 1989) and in crayfish (Boutet and Chaisemartin, 1973). Developmental effects in the bivalve *Mytilus californianus* even occurred at < 1 ppm Ba (Spangenberg and Cherr, 1996). By contrast, Berge (1996) analyzed the impact of muds from drilling fluids on the benthic macrofauna, but found no effects on the recruitment of larvae nor on the establishment of benthic communities at Ba loads of 700–1100 ppm. A field study in the Campos Basin in Brazil indicated a fast recolonization of drill-disturbed sediments, dominated by opportunistic burrowing and tube-building polychaetes. Within 3 months after disturbance,

macrofaunal abundances exceeded those from the pre-drill period, whereas diversity initially decreased but returned to pre-drill values within 22 months (Santos et al., 2010). Meiobenthos from the same area showed more pronounced and longer-lasting effects, communities of nematodes taking more than 2 years to recover to pre-disturbance conditions. These effects were, however, not obvious from abundance or diversity data, but only from community composition (Netto et al., 2010). In the same area, nematode communities at deeper drilling sites did recover to pre-disturbance conditions within 1 year, probably as a result of much lower hydrocarbon concentrations (Netto et al., 2009).

Next to a strongly differential sensitivity of different taxa to Ba, part of the large discrepancies between the studies listed above may be related to the bioavailability of different Ba compounds. Barite, for instance, has an extremely low solubility in water, in contrast to Ba chloride, and pathways for uptake of the former would likely be limited to ingestion, whereas the latter may both be ingested orally and absorbed through the cuticle (Howell and Smith, 1985). Among benthic organisms, deposit-feeders are thus more likely to ingest substantial levels of barite from drilling fluids than more selective feeders. Barite used in drilling fluids typically has an average particle diameter of 44 μm (WHO, 2001), probably excluding oral uptake in a majority of nematodes in offshore sediments. However, up to 30% of barite particles in NADFs has a diameter $\leq 6 \mu\text{m}$ (WHO, 2001), suggesting that a substantial fraction is at least physically amenable to uptake by many marine nematodes, and may be assimilated (Howell and Smith, 1985). McCauley and Washington (1983) suggest that soluble barium compounds (like barium chloride) and/or barium compounds that yield a dissociated barium ion (like barium sulfate) in an acid gut may be similarly absorbed. Nematodes can internalize a range of small particles in gut cells which contain lysosomes, with activity of enzymes such as acid phosphatase (Deutsch, 1978; Munn and Munn, 2002), suggesting that suitable conditions for dissociation of barium ions and subsequent assimilation of barium may be present. Hence, while the bioavailability of barite to nematodes in aquatic sediments is likely less than that of barium chlorides at identical Ba concentrations, barite too is available to meiobenthic infauna.

Concentrations of barium in marine waters and sediments generally increase with depth, but based on the present results and the reported Ba concentrations in 'natural', non-impacted marine sediments (WHO, 2001; Pozebon et al., 2005), Ba at 'natural' levels would only have minor effects on the population growth of *R. (P.) marina*. Nevertheless, some of these concentrations already exceed the LOEC for juvenile and total population abundance. However, maximal Ba concentrations in sediments 1 month as well as one year after oil drilling activity exceeded 4500 ppm (Pozebon et al., 2005), which is well above EC₅₀s found in the present study. Ba migration from discharged drilling muds is, however, limited (Pozebon et al., 2005), suggesting that the effects of drilling discharges remain strongly localized.

4.2. Effects of cadmium

Based on the ANOVA's on population growth and abundances of *R. (P.) marina* at various Cd concentrations, Cd in the concentration range tested here did not impact total population abundance. Concentrations above 1 ppm Cd did have a negative effect on the abundance of adult females, which in the longer run should inevitably lead to effects on the population-level. Based on the dose-response model, however, Cd concentrations as low as 2.95 ppm may impact total population abundance, and concentrations between 8 and 9 ppm Cd may decrease population abundance by more than 50%. These model results are in agreement with a recent study where a *R. (P.) marina* population isolated from the same

locality as that used in the present study exhibited effects of Cd on population development: 10 ppm Cd caused substantial adult as well as preadult mortality; at 3 ppm Cd lower total as well as adult densities were found, but only at salinities which deviated from the typical range of salinities at the site of origin (Derycke et al., 2007). That study, however, did not find evidence that Cd effects would become more pronounced with time of exposure, nor that Cd at concentrations up to 3 ppm would affect the genetic diversity of *R. (P.) marina*. Growth and fecundity of the soil nematode *C. elegans*, which belongs to the same nematode family as *R. (P.) marina*, were significantly impacted at Cd concentrations ≤ 5 ppm (Popham and Webster, 1979), and EC₅₀ values for movement, feeding, growth (all after 24 h exposures) and reproduction (after 72 h) of this species ranged between 14 and 20 ppm Cd (Anderson et al., 2001). These values are about twice as high as our EC₅₀ values for *R. (P.) marina* population growth, but this discrepancy may be largely explained by the different exposure duration. Lowest observed effect concentrations for growth and individual reproduction in *C. elegans*, however, were 50–100 times lower (Traunspurger et al., 1997). Considering the comparatively high tolerance of rhabditid nematodes to various toxicants, these results indicate that even fairly low concentrations of Cd may have effects on the demography and population development of free-living nematodes. However, Kammenga et al. (1994) did not find substantial differences in the tolerance of 12 different soil nematode species to Cd, and could not corroborate the idea that rhabditid nematodes have a higher tolerance than other taxa. LC₅₀ for juvenile mortality and LOEC values for juvenile development in *R. (P.) marina* were, however, much higher: 77 and 50 ppm, respectively (Vranken et al., 1985), illustrating nicely that bioassays of short duration and with mortality as the main response variable tend to substantially underestimate population-level effects of toxicants (Vranken et al., 1985; Traunspurger and Drews, 1996).

The lowest Cd concentrations affecting population development and abundance in *R. (P.) marina* in the present study and in Derycke et al. (2007) are in the range of values found in mud from drilling fluids (2–5 ppm, Berge, 1996). When assuming a similar bioavailability of Cd to bacterivores in sediment as on agar, this suggests that Cd contamination of drilling fluids may have measurable sublethal effects on nematode populations. Such effects may become more important when other environmental factors also deviate from a 'normal' situation, i.e. from the environmental history of the nematodes prior to their exposure to toxicants (Millward and Grant, 1995), suggesting that local adaptation in nematodes is substantial. The Cd effects on *R. (P.) marina* observed by Derycke et al. (2007), for instance, were most pronounced when salinity deviated from typical local values. Of course, Cd toxicity depends on its free ion activity and should therefore increase with decreasing salinity (Blust et al., 1992), but the Cd effects on *R. (P.) marina* were pronounced at both low and elevated salinities (Derycke et al., 2007). In addition, combinations of different toxicants, in this case particularly the combination of different heavy metals in drilling fluids, may well affect nematode populations above levels expected based on assays with single toxicants (Vranken et al., 1988; Beyrem et al., 2007). Finally, the availability of Cd and other metals to nematodes increases with sediment concentrations of both dissolved and particulate organic matter (Höss et al., 2001) and varies with a variety of physicochemical properties, suggesting that similar metal loads in different sediment types may cause differential effects on nematode populations (Tatara et al., 1998; Peredney and Williams, 2000).

4.3. Responsive traits

Juvenile mortality has been suggested to be a particularly sensitive life-cycle trait in bacterivorous nematodes under various

types of pollution (Kammenga et al., 1996, 1997) as well as under food shortage (Schiemer, 1982). Our data provide only equivocal support for the contention that juveniles would be more sensitive to heavy metal pollution than adult nematodes: LOEC and EC₅₀ values for Ba were indeed lower for juvenile than for adult *R. (P.) marina*, but for Cd this was not the case. Metals may enter nematodes both through feeding and through the cuticle (Howell and Smith, 1985). In view of their higher surface-to-volume ratio, cuticular absorption should be comparatively more important in juveniles. However, especially first- and second-stage juveniles may still largely thrive on energy reserves from the egg (Santos et al., 2008), so their feeding activity is likely comparatively less than in adults. Furthermore bioaccumulation may become more pronounced with age (WHO, 1992). Indeed, heavy metal (e.g. Cd, Zn and Cu) concentrations twice those of ambient sediment have been recorded in nematodes (Fichet et al., 1999).

In the present study, total and adult population density best reflected the effects of heavy metals on nematode fitness. Individual life-cycle traits such as development time and fecundity, generally did not exhibit such pronounced treatment effects, except at high Ba loads. This is perhaps surprising, because these traits have been shown to respond significantly to changes in environmental factors like temperature and salinity, but may at least in part be explained by the effects of culture history of the F0 adults (Santos et al., 2008 and references therein). A population assay focusing mainly on counts of total and adult nematodes may thus be a fairly simple and adequate tool to assess population-level effects of heavy metals on nematodes. However, an accurate understanding of the results of such an assay may still require more detailed information on particular life-cycle traits.

5. Conclusions

Free-living marine nematodes as a higher taxon have been reported to be highly resistant to metal pollution (Heip et al., 1984). Our results nevertheless demonstrate that oil and gas drilling activities may have significant effects on the benthic meiofauna through the toxicity of barium and associated metals like cadmium. In view of the high stress tolerance of *R. (P.) marina* compared to many other nematodes, it is likely that yet lower doses of these heavy metals would affect a substantial part of offshore nematode communities. In the absence of cultures of species from shelf or deep-sea sediments, *R. (P.) marina* is a suitable model organism for the assessment of dose-effect relationships and more detailed demographic, population-genetic and fitness analyses.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.marenvres.2011.07.003.

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