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The chronic toxicity of emamectin benzoate to three marine benthic species using microcosms

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ABSTRACT

The commercial farming of Atlantic salmon, *Salmo salar*, may require the periodic application of emamectin benzoate (EB) treatments to reduce the effects of biological pests, such as sea lice. As a result, EB is detected in sediments beneath these fish farms at considerable levels. Literature sediment toxicity data for EB for marine benthic species is only available for 10-day sediment toxicity tests, which might be too short to assess field effects. Here, we present a sediment toxicity test to determine 28-day mortality and growth effect concentrations for the non-target polychaete worm *Arenicola marina*, the crustacean *Corophium volutator* and the mollusk *Cerastoderma edule* using a marine microcosm setup. Results indicate that no concentration-dependent increase of mortality and growth rate was apparent to *A. marina* and *C. edule*. But for *C. volutator*, a concentration-dependent increase in mortality was observed, resulting in a calculated 28-d LC50 of 316 μg/kg dry sediment (95% confidence interval: 267–373 μg/kg dry sediment). There were significant effects on *C. volutator* growth rate at concentrations of 100 μg/kg dry sediment and above (NOEC = 30 μg/kg dry sediment). These observations show that *C. volutator* is more sensitive to EB than *A. marina*, which differs from results reported in previous studies. Comparison to the most sensitive NOEC (30 μg/kg dry sediment) found for *C. volutator* (organisms of 8–11 mm length), shows that the Environmental Quality Standard, derived by the Scottish Environment Protection Agency in 2017 which based on freshwater species data (NOEC = 1.175 μg/kg dry sediment), are relatively strict and is sufficiently protective for the marine species tested in this paper.

1. Introduction

Worldwide aquaculture of Atlantic salmon (*Salmo salar*) expanded rapidly during recent years. Its global aquaculture production in 2016 was more than 2 million tonnes, accounting for 4% and ranking eighth in fish aquaculture (FAO, 2018). One of the problems encountered in farming salmon is the presence of marine ectoparasites such as parasitic copepods, or sea lice, belonging to the genera *Lepeophtheirus* and *Caligus* (Caligidae: Crustacea) (Rae, 2002).

A number of physical, chemical and biological methods (Costello et al., 2001; Mayor et al., 2008; Roth et al., 1993) have been used to control sea lice in fish farms. Emamectin benzoate (EB) is the active ingredient in the veterinary medicine Slice®, which is used to control sea lice in marine cage fish. EB belongs to the avermectins group and is an insecticidal compound that comprise of two active homologues: B_{1a} ($C_{49}H_{75}NO_{13}C_7H_6O_2$: 90%) and B_{1b} ($C_{48}H_{73}NO_{13}C_7H_6O_2$: 10%) (Kuo et al., 2010; WRc, 2017).

Typical treatments of EB in aquaculture are 50 μg/kg fish biomass/day on seven consecutive days (Kuo et al., 2010; Tor, 2012; WRc, 2017). EB is readily assimilated by the salmon from food pellets, with less than 10% excreted immediately following treatment (Willis and Ling, 2003). Due to feeding efficiency, it is estimated that 5–15% of the food remains uneaten, and can thus be a point source of EB entering surrounding environments (Chen et al., 1999; Langford et al., 2014). Moreover, faecal egestion of the compound continues for an extended period of 3–4 months post-treatment. Therefore, EB may enter the marine environment either directly from surplus feed, or indirectly via faeces during the treatment and post-treatment period (Willis and Ling,

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2003). EB remains in sediments for a considerable period of time, since it's half-life is over 120 days (WRc, 2017), or even has been estimated to be 400 days in the lab and potentially years in the environment (Benskin et al., 2016). Therefore residues of EB bound to particles (food) and sediment may contribute considerably to exposure of benthic organisms to EB (Simpson et al., 2016).

For several benthic species, literature sediment toxicity data for EB was found (Table S1). From those studies, *Hediste diversicolor* appears to be least sensitive (10-d LC50 = 1368 μg/kg wet weight) and *Arenicola marina* the most sensitive (10-d LC50 = 111 μg/kg wet weight; 10-d NOEC = 56 μg/kg wet weight). In various experiments performed with the crustacean amphipod, *Corophium volutator* a similar sensitivity was found (10-d LC50 of 193 and 153 μg/kg wet weight; 10-d NOEC = 115 μg/kg wet weight). However the amphipod species *Monocorophium insidiosum*, which is taxonomically quite similar, appears to be less sensitive (10-d LC50 = 890 μg/kg wet weight). For benthic species, data is only available for 10-day sediment toxicity tests, and annelid lugworms appear to be the most sensitive species group.

EB is detected in sediments beneath Atlantic fish farms at considerable levels ranging from not detectable to 366 μg/kg wet weight (for details see discussion section). Assessment of the risks associated with the presence of EB is difficult in view of the lack of sub-lethal and chronic marine sediment toxicity assays. Following the guidelines of the Organization for Economic Cooperation and Development (OECD, 2004a, 2004b) and the U.S. Environmental Protection Agency (U.S.-EPA, 1994; 2002) for the assessment of toxicity of chemicals to marine species, we present a sediment toxicity test using marine microcosms to determine 28-day mortality and growth effect concentrations for the non-target polychaete worm *Arenicola marina*, the crustacean *Corophium volutator* and the mollusk *Cerastoderma edule*. The applicability of the proposed method and possible improvements are discussed.

In addition, literature data on residues observed near fish farms is compared with existing environmental quality standards and with acute toxicity data found in literature and chronic (28-day) values for toxicity established in the current study.

2. Materials and methods

A 28-day chronic multispecies sediment microcosm test with *A. marina*, *C. volutator* and *C. edule* was conducted, exposing the animals to 5 concentrations (10, 30, 100, 300, and 1000 μg a. i./kg dry sediment), a control and an acetone solvent control. There were 3 replicates for each treatment, using a total of 21 microcosms. The test was conducted in accordance with the guidelines of the Organization for Economic Cooperation and Development (OECD, 2004a, 2004b) and the U.S. Environmental Protection Agency (U.S.-EPA, 1994). Each tank (glass container, 40 × 40 × 60 cm) was filled with approximately 34 kg test OECD sediment (OECD, 2004a) to obtain a sediment depth of 15 cm, and with 30 cm overlying artificial seawater, resulting in 48 L of seawater added to each tank.

The artificial seawater was prepared according to OECD guideline 218 (OECD, 2004a). The details of the chemical composition are provided as Supporting Information (SI) in Table S2 (OECD, 2001).

The tests were performed in a temperature controlled room at 15 ± 2 °C, using a 12 h light/12 h dark regime. Throughout the study, community metabolism and nutrient concentrations were monitored weekly. Constant aeration was applied to maintain sufficiently high oxygen levels, making sure that no sediment resuspension occurred. Evaporation losses were replenished with demineralized water weekly so that salinity was maintained at 30 ± 1 g/kg throughout the study (Foekema et al., 2015).

During the experiments, test animals were fed with a commercial fish flake food (Tetra Min®) and algae *Tetraselmis suecica* three times per week. The algae were obtained from the AlgaePARC in Wageningen. Feeding rates were 3 mg of dry food per individual for *A. marina* (Diepens et al., 2015), 1.5 mg of dry food per individual for *C. volutator* (Van den Heuvel-Greve et al., 2007) and 10 million algae cells per individual for *C. edule*.

2.1. Test species

Arenicola marina: *A. marina* was collected in the Southern Wadden Sea and retrieved from 'Lobbezoo zeeaas' and were picked fresh early morning. Approximately 400 worms were maintained in two 50 L glass tanks for 5 days with continuously aerated clean artificial seawater. Animals were collected from the holding tank and 10 individuals of similar size were randomly placed into each test container. A subsample of 32 worms was taken for determining their dry weight at the start of the test. The average dry weight at the start was 5.04 ± 1.85 g. During the first 6 h, observations were made on the burying behavior of the worms. After 24 h, any dead worms on the sediment surface, or those which had not buried, were replaced with fresh ones (Allen et al., 2007; Thain and Bifield, 2002). During the experimental period, the tanks were monitored for dead worms at the sediment surface, which were recorded and removed to minimize the negative impact on the water quality. The worms were fed 10 mg of dry fish food (Tetramin) three times per week during the experiment, according to a methodology mentioned and used by other researchers as well (Bridges et al., 1997; Diepens et al., 2015).

Corophium volutator: *C. volutator* was collected with a 500 μm sieve from the top 3 cm of muddy sediment at low tide in the Oesterput, Noord-Beveland, The Netherlands. All animals were acclimated to experimental conditions for 5 days in three 10 L buckets supplied with continuously aerated clean artificial seawater and a 1 cm layer of clean artificial sediment. *C. volutator* were sieved from the holding tank and transferred to a shallow tray submerged in a separate tank filled with artificial seawater so that they could be selected more easily. This was accomplished by pouring the water from the holding tank through a 500 μm sieve, and gently washing the contents of the sieve into the tray. Fifty individuals in the size range of 3–10 mm were randomly placed into each test container (Diepens et al., 2015). A subsample of 49 amphipods was taken for determining their dry weight and photographs were taken to determine their average length at the start of the test (average length was 6.55 ± 1.17 mm). 31% of the individuals were between 3 and 6 mm, 41% between 6 and 7 mm, 18% between 7 and 8 mm 10% between 8 and 10 mm. Care was taken that no damaged animals were used in the test.

Cerastoderma edule: *C. edule* was collected at low tide near Oesterdam at harbour Rattekaai, Zeeland, The Netherlands. All animals were acclimated to experimental conditions for 5 days in three 10 L buckets supplied with continuously aerated clean artificial seawater and a 2 cm layer of clean artificial sediment. Twenty individuals of similar size were randomly placed into each test container (Diepens et al., 2015). From the pool of added specimens, a subsample of 38 mollusks was taken for determining their dry weight and shell length, height and width for their average size (average length: 17.06 ± 1.33 mm, height: 14.98 ± 1.15 mm, and width: 11.97 ± 1.05 mm). Based on the subsample 26% of the individuals were between 14.5 and 16.0 mm length, 47% between 16.0 and 18.0 mm and 26% between 18.0 and 20.0 mm. Care was taken that no damaged animals were used in the test.

2.2. Biological endpoints

The tested biological endpoints were growth and mortality. For *C. volutator,* the length (head to telson) (Allen et al., 2007) was measured from photographs. For *C. edule*, the shell length, height and width were measured with a calipers. Growth rates, in terms of length and dry weight were calculated for each replicate by subtracting the length and dry weight at the end of the experiment with those assessed on a subsample at the beginning of the experiment and divide the resulting length or weight by the test duration, i.e. 28 days.

Test units were checked daily for dead animals, which were removed immediately. Death of *A. marina* was defined as lack of movement after 30 s of gentle stimulation (Diepens et al., 2015). At the end of the experiment, sediments were sieved with a 500 μm sieve and the surviving animals were counted. The missing animals were presumed to have died and decomposed during the test. All surviving animals were collected and left overnight in clean artificial seawater to empty their gut. The length of each organism was measured, after which they were dried for 24 h at 60 °C to determine dry weight (Diepens et al., 2015).

2.3. Measurement of physico-chemical properties

Throughout the test water was sampled for measurement of nutrients and chlorophyll-a content. Oxygen concentration, temperature, salinity and pH were measured weekly on days 0, 5, 12, 19, 26 with a Hach portable multi-meter (HQ 40 d) using a Luminescent Dissolved O_2 probe (LDO 101), Salinity electrode (CDC 401) and pH electrode (PHC 101). Temperature was measured with a build in sensor of the pH probe. For the analyses of nutrients, the water sample was filtered over a 0.45 μm cellulose acetate filter (Whatman Puradisc™). Analyses of the nutrients ammonium ($N–NH_4$), sum of nitrate and nitrite ($N–NO_3 + NO_2$), total soluble nitrogen (Nts) and ortho-phosphate ($P-PO_4$) were carried out by a segmented flow analyzer (Skalar®) with spectrophotometric detection, following the methods described by the Dutch Accreditation Council (L-342). Results are expressed as mg N/L and mg P/L respectively. Phytoplankton concentrations (μg/L) were measured using an Algal Lab Analyzer spectrofluorometer (Biological – Biophysical Engineering Moldaenke).

2.4. Preparation and spiking of sediments

Standard artificial sediment was prepared according to OECD guideline 218 (OECD, 2004a) containing 5% peat, 20% kaolin clay, 75% quarts sand and calcium carbonate content was used to adjust the pH.

The peat was obtained from Klasmann-Deilnmann (Zaandam, Netherlands), the raw product was air dried until all moist was evaporated and grinded until fine powder with a paricle size < 1 mm. The quarts sand (Dorsilit 8, 97% SiO2) with average grain size of 0.33 mm were acquired from Sibelco (Netherlands), without organic matter and contaminants. Kaolin clay (CAS: 1332-58-7) was purchased from VWR.

Technical grade EB (98.3% active ingredients; Sigma Aldrich, St. Louis) was used to prepare a stock solution (2 g/L) in acetone. Dilutions of the stock solution were prepared in acetone (10 ml acetone per replicate), which were used for spiking sediment at 0, 10, 30, 100, 300 and 1000 μg/kg wet weight (nominal values). Sediments were prepared and spiked on day −15 and the following preparation procedures were followed. For each replicate 1 kg peat, 4 kg kaolin, 15 kg quarts sand and 14 L ground water were used. The dry fractions of artificial sediment were spiked with 200 ml of the diluted EB solution in acetone and water in a concrete mixer for 10 min to obtain the desired EB concentration and a moisture of 40% in the final mixture. During the procedure, 240 g calcium carbonate (12 g/kg dry sediment) of chemically pure quality ($CaCO_3$, BioXtra, ≥99.0%; Sigma Aldrich, St. Louis) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5. Prepared sediments were maintained in a darkened room at 15 ± 2 °C for a period of 48 h to stabilize the substrate (OECD, 2004a). They were mixed with 2 L seawater per batch manually every three days for at least 3 times to equilibrate the test substance and evaporate the acetone. The total amount of sediment was approximately 34 kg per microcosm for each replicate. The sediment slurry was poured into the microcosms on day −6 and allowed to settle overnights. Overlying artificial seawater was added on day −1 and gently aerated for 24 h prior to introduction of the organisms on day 0. Additionally, 2 fate microcosms were prepared for each control, solvent control and treatment level, using a total of 14 fate microcosms. The volume of the jars used as fate microcosms was 1.5 L with an internal diameter of approximately 10 cm. The same height proportion of sediment and seawater was used as in the formal tests. The same experiment conditions were used as for effect microcosms, but no animals were added.

2.5. Validation of exposure concentrations

Sediment was sampled on days −13, −6, 0, 14, and 28. On days −13, −6 and 28 the sediment samples were taken from test microcosms, but on days 0 and 14 samples were taken from the additionally prepared fate microcosms, to avoid destruction of the test microcosms.

Wet sediment samples were freeze-dried for approximately 3–4 days, using a Christ Alpha 1–2 apparatus, equipped with a Vacuubrand RZ 2.5 vacuum pump. Approximately 24 g of dried sediment were transferred into a 90 ml centrifuge tube and extracted with 5 ml acetone and 50 ml methanol. After shaking by hand and 15 min of ultra-sonification, the samples were shaken for 60 min at 135 rpm (shaking stroke 25 mm). Tubes were then centrifuged at 2500 rpm (700 g-force) for 15 min at 20 °C. A small part of the acetone/methanol extract was diluted tenfold using MilliQ-water/methanol (4 : 1, V: V). The diluted sample was analyzed using HPLC combined with mass spectrometry (LC-MS).

Recovery of the freeze-drying and extraction procedure was checked using 3 wet sediment samples spiked at concentration levels of 75 and 750 μg/kg dry weight. The spiked samples were frozen, freeze-dried and extracted as described above for regular samples.

Chemical analyses were performed using an Agilent LC-MS 6410 employing a 4.6 mm × 150 mm Zorbax Eclipse 5 μm C18 reversed-phase column operated at 40 °C. An injection volume of 50 μL was used. Elution solvents consisted of A) 0.63 g/L ammonium formate in Milli Q water +0.1% formic acid, and B) acetonitril +0.1% formic acid. An elution gradient was used, consisting of 30% A + 70% B (0–4 min), 10% A + 90% B (4–6 min) and 30% A + 70% B (> 6 min). An electrospray ionisation source was used at a gas flow of 10 L/min, gas temperature of 350 °C, nebulizer pressure of 50 psi and capillary voltage of 3500 V. For both homologues the fragment with mass 81.9 was used as a qualifier, whereas the fragment with mass 157.9 was used for quantification. Although emamectin B_{1a} and B_{1b} homologues were quantified separately, reported values for total EB are given as the sum of both homologues (Table S3).

2.6. Statistical analyses

No-observed-effect-concentrations (NOECs) were calculated for mortality and growth in terms of length and weight data using the Williams test (Williams, 1972; $p < 0.05$) as available in the Community Analysis computer program, version 4.3.05 (Hommen et al., 1994). Prior to the analysis, the abundance, length and growth data sets were ln ($ax + 1$) transformed for a better approximation of a normal distribution of the data. For the determination of a and the rationale behind the transformation the reader is referred to Van den Brink et al. (2000). The calculation of LC50 values was performed by means of log-logistic regression using GenStat 11th edition (Lawes Agricultural Trust 2009; VSN International Ltd., Oxford, UK) (Rubach et al., 2011).

3. Results and discussion

3.1. Validation of exposure concentrations

Recovery of 65.6 ± 0.78% was found for EB and this value was used to correct measured values. The limit of quantitation (calculated as 3x the limit of detection) was 1.5 μg/kg dry sediment for both the B_{1a} and B_{1b} homologue. The levels of EB (sum of the B_{1a} and B_{1b} homologues) measured in controls and solvent controls were always below the limit of detection.

The levels of EB measured in spiked sediment samples were highly

Table 1
Summary of mortality and growth endpoints established for *A. marina*, *C. volutator* and *C. edule* exposed to EB.

Effect	Organism	Endpoint (28-day)	Value (µg EB/kg dry weight sediment)
Abundance	*A. marina*	LC50/LC10	> 1000
		NOEC	≥1000
	C. volutator	LC50/LC10	316/155
		NOEC	100
	C. edule	LC50/LC10	> 1000
		NOEC	≥1000
Growth	*A. marina*	NOEC	≥1000
	C. volutator	NOEC	30
	C. edule	NOEC	≥1000

variable on day −13 (61 ± 32% of nominal levels, 21 effect microcosms) and day −6 (137 ± 62% of nominal, 21 effect microcosms), but were close to intended concentrations on day 0 (110 ± 10% of nominal, 5 fate microcosms), day 14 (116 ± 7% of nominal, 5 fate microcosms), and day 28 (88 ± 17% of nominal, 21 effect microcosms). Over days 0–28, the average level of EB measured in the sediment samples corresponded to 112 ± 44% of the intended levels. Effect levels (LC50, NOEC) were calculated using nominal concentrations. The concrete mixer worked efficiently and all the sediments were mixed with it for 10 min, the high variation found on days −13 and −6 may have been due to an incomplete sorption so that some EB was still in the water phase in those days and reached the sediment only after 15 days.

3.2. Effect concentrations of the three test species

Results for biological endpoints are summarized in Tables 1 and 2; results are discussed in more detail in the following sections.

3.2.1. Mortality

Mortality resulting from exposure to EB is shown for all 3 species in Fig. 1 and Fig. S1. For *A. marina* (Fig. S1a) mean mortality in the controls and solvent controls was 23.3% and 13.3% respectively. No concentration-dependent increase of mortality was apparent. For *C. volutator* (Fig. 1) mean mortality in the controls and solvent controls was relatively high at 41.3% and 31.7% respectively. Nevertheless, a concentration-dependent increase in mortality was observed, resulting in a calculated 28-d LC50 of 316 µg/kg dry weight (95% confidence interval: 267–373 µg/kg dry weight), a 28-d LC10 of 155 µg/kg dry weight (95% confidence interval: 93–257 µg/kg dry weight) and a 28-d NOEC of 100 µg/kg dry weight. For *C. volutator*, the higher length classes were affected at lower concentrations, with a lowest NOEC of 30 µg/kg dry weight for the highest length class (Table 2). For *C. edule* (Fig. S1b), the mean mortality in the controls and solvent controls was high at 60.0% and 65.0% respectively. No concentration-dependent increase in mortality was apparent.

3.2.2. Growth rate

For *A. marina* dry weight decreased over the course of time in a similar fashion for all treatment levels, including the controls and solvent controls (Fig. S2). No significant treatment-related effect on dry weight was observed.

For *C. volutator* the growth rate calculated for dry weight (Fig. 2a) and length (Fig. 2b) decreased with increasing concentrations of EB. Almost all of the mud shrimps died in the highest treatment level (1000 µg/kg dry weight), for which no growth rate was calculated. There were significant effects on growth rate at concentrations of 100 µg/kg dry weight and above (NOEC = 30 µg/kg dry weight).

For *C. edule* the growth rate calculated for dry weight and length, as well as growth rates calculated for height and width (Fig. S3), were highly variable and did not show significant treatment-related effects.

Table 2
Summary of NOECs calculated based on abundance values of *C. volutator* within different length classes as assessed at the end of the experiment. The mean length at the start was 6.55 (± 1.17) mm.

Length	Endpoint (28-day)	Value (µg EB/kg dry weight sediment)
3–6 mm	NOEC	≥1000
6–7 mm	NOEC	300
7–8 mm	NOEC	30
8–11 mm	NOEC	30

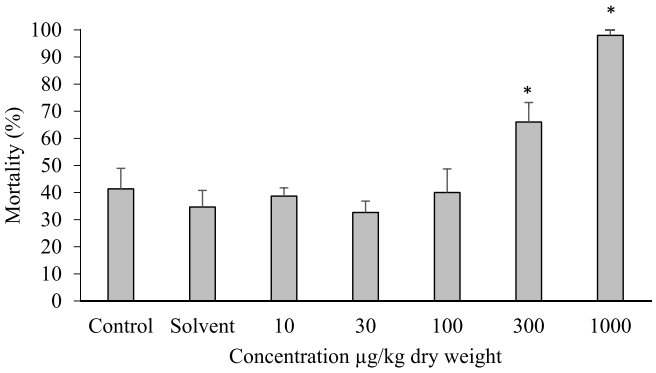

Fig. 1. Percentage lethality of *C. volutator* during exposure to EB for 28 days. Sediment concentrations are nominal concentrations."*"show differences ($p < 0.05$).

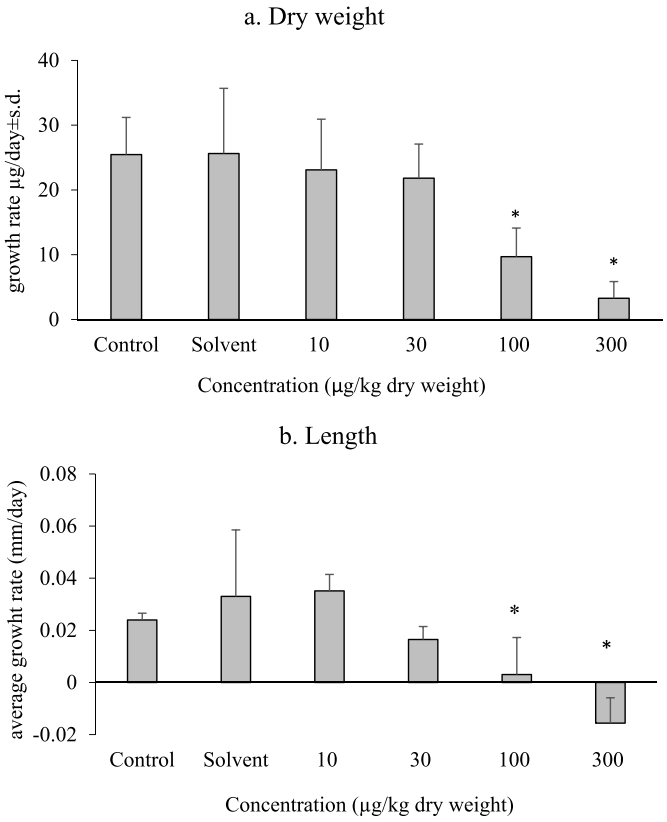

Fig. 2. Growth of *C. volutator* exposed to EB-spiked sediment for a period of 28 days: (a) Growth in terms of dry weight increase; (b) growth in length. No growth rate was calculated for 1000 µg/kg dry weight, since almost all of them died at this exposure level. "*"show differences ($p < 0.05$).

3.2.3. Arenicola marina

For *A. marina*, the results show that this species is able to tolerate high concentrations of EB, and can thus be regarded as not very sensitive to this substance. This is consistent with findings of Mayor et al. (2008) who reported an LC50 of 1368 μg/kg wet weight for *Hediste diversicolor* and McBriarty et al. (2018) who reported no treatment-related mortality of *Nereis virens,* two polychaetes similar to *A. marina*. However, WRc (2017) found a 10-d LC50 of 110 μg/kg wet weight, which made *A. marina* the most sensitive sediment dwelling species investigated for EB (WRc, 2017). Information about organic matter, carbon content, clay content of the sediment and the other exposure conditions is missing from the WRc (2017) report and was also not provided in the original study, which makes a direct comparison difficult. Meanwhile, in our tests the abundance and dry weight of *A. marina* decreased over the 28-d exposure period in the controls as well as in the treated groups (Fig. S1a and Fig. S2). The water quality indicators, pH, dissolved oxygen, etc. Indicated good water quality (Figs. S4–S12). Some casts were initially seen on the surface of the sediment, but at the end of the experiment most of these were probably buried and not many new casts were seen on the surface. Apparently, the worms did not consume the offered food. Moreover, the sediment containing only 2% organic carbon and may have supplied insufficient nutrition to the worms as well. These factors probably combined to cause insufficient feeding of *A. marina* in the long-term toxicity tests, resulting in mortality and weight loss in controls and treatments. The lack of feeding activity could also have resulted in lower exposure to the sediment bound EB. Similar findings were reported by McBriarty et al. (2018) testing the effect of EB on the polychaete *Nereis virens* in EB-treated sand with low organic carbon content. It was reported that food ration had a strong influence on observed toxicity in a similar kind of polychaeta *Neanthes arenaceodentata* (Bridges et al., 1997). Our result at least suggests that more attention should be paid to the impact of food on chronic toxicity testing and further investigations on how to prevent the possible impact of insufficient feeding on the outcome of long-term toxicity studies with *A. marina* are required.

3.2.4. Corophium volutator

Two previous studies on EB reported 10-d LC50 values for *C. volutator* of 153 and 193 μg/kg wet weight, respectively (Mayor et al., 2008; WRc, 2017), which corresponds quite well to the nominal 28-d LC50 of 316 μg/kg dry sediment reported here (which corresponds to 190 μg/kg wet sediment assuming 40% water content of the sediment). But considering the difference of the experiment duration (28 days vs 10 days), the results in this research suggests that *C. volutator* shows no increased sensitivity beyond day 10 (Mayor et al., 2008; WRc, 2017).

Previous studies used sediment with somewhat higher (4%) organic carbon content (Mayor et al., 2008). Allen et al. (2007) suggest that inconsistent results between sediment ecotoxicological studies may arise because of differences in the concentrations of organic material in the test sediments, affecting the bioavailability of the active ingredients. Table 3 shows the variation in experimental conditions between various studies. It is quite possible that the relatively low organic carbon content of the sediment used decreased the bioavailability of (sorbed) EB and enhanced the tolerance of *C. volutator* in our experiment.

Besides, it was observed that the chemical altered the size distribution, since the abundance in higher length classes was significantly lower at higher concentrations (Table 2). This could indicate that larger organisms are more sensitive, but the observed effects can also be a result of decrease in growth rate and/or reproduction. Allen et al. (2007) did not observe an increase in the mortality with length in a long-term sublethal whole sediment tests with *C. volutator* and ivermectin. Normally, early life stages of many species are more sensitive to toxicants than later developmental stages (Rand et al., 1995; WRc, 2017). *C. volutator* is a slender animal, up to 11 mm long (Neal and Avant, 2006), and this population has two generations per year, with

Table 3
Sediment ecotoxicological studies for EB in *C.volutator*.

test duration (days)	LC50 Concentration	pH	Sediment	seawater	salinity (‰)	T	size of micro-cosms	mutil or single species	chemical	sediment/ water	number of animals per coms	light	feed	organic matter of the sediment	reference
10 days	153 μg/kg wet weight	not reported	natural	natural	33	15.0 ± 0.1 °C	Perspex, 2.355 L	single	Slice® was provided courtesy of J. McHenery, Schering-Plough.	8cm/ 20 cm	30	12:12	not reported	4%	Mayor et al. (2008)
28 days	316 μg/kg dry weight	8.0 ± 0.5	artificial	artificial	26.74 ± 0.69	15.0 ± 0.1 °C	glass, 96 L	mutil		15cm/ 30 cm	50	12:12	feed with fish feed and agle once/week	2% ± 0.5%	this research
10 days	193 μg/kg wet weight	not reported	not reported	not reported	not reported	not reported	not reported	not reported	not reported	not reported	not reported	not reported	not reported	not reported	WRc, 2017, 2000;

one generation born in May to mid–June and growing to reproduce and give rise to a second generation in August (Wilson and Parker, 1996). This difference can be explained that the higher length classes of *C. volutator* are at breeding period making them more sensitive to EB, however, more research is needed to find the exact mechanism as inhibited growth may also be a probable cause of the observed effects.

3.2.5. *Cerastoderma edule*

C. edule shows no treatment-related effects in mortality and growth rate (Table 1, Fig. S3). Many studies have shown that mollusks are less sensitive to insecticidal compounds than crustaceans and polychaetes. Collier and Pinn (1998) and Grant and Briggs (1998) found that crustaceans and polychaetes are more susceptible to ivermectin than mollusks. Lobster and shrimp were the most susceptible species to azamethiphos in laboratory-based acute toxicity tests, while bivalves such as scallops and clams were unaffected (Burridge and Haya, 1998; Haya et al., 2005). This confirms that mollusks are less sensitive to EB than crustaceans and polychaetes. At present, there is no report on the toxic effects of EB on *C. edule* or any other bivalves. Since *C. edule* is one of the most widely distributed species in the North-Atlantic Ocean, research in this field should be expanded. Mortality and growth rates are not sensitive endpoints, but an interesting phenomenon is that 3 juveniles were found in one replicates of the 10 μg/kg dry weight treatment, 2 and 7 juveniles were found in two replicates of 30 μg/kg dry weight treatment. This may indicate that reproduction may be a useful endpoint for long-term toxic effects for *C. edule*, as was earlier found in outdoor mesocosms (Foekema et al., 2015). The applicability of this endpoint in smaller indoor microcosms however requires further work and longer studies to verify.

3.2.6. *Water parameters*

During the study water temperature in all microcosms varied from 14.0 to 16.5 °C (Fig. S4). Concentrations of DO were never below 6.9 mg/L, and the average DO (8.9 mg/L) was lower in the highest treatment than the controls on day 12 with a NOEC of 300 μg EB/kg dry weight sediment (Table 4; Fig. S5). The salinity in all microcosms decreased on the fifth day and then increased on the 12th day, followed by a slight decrease over the following days. The average salinity was higher in the treatments than the control and the highest value was observed in the highest treatment (27.9 on day 0 and 27.4 on day 12) with a NOEC of 300 μg EB/kg dry weight sediment (Table 4; Fig. S6). In the first 20 days, pH showed a gradual increase in all treatment levels, followed by a downward trend in the last week. Compared to controls, the pH was lower in all treatments on day 19 with a NOEC of 10 μg EB/kg dry weight sediment (Table 4; Fig. S7). The concentration of chlorophyll-a increased gradually in all treatment levels except for a decrease in the controls during the last week. The final concentration in all treatment levels ranged between 6.90 μg/L and 8.73 μg/L was approximately 3–5 times higher than observed on day 0 (Fig. S8). Compared to the controls, chlorophyll-a was lower in the highest treatment level on all sampling days, except on day 0 (NOEC ≥ 1000 μg/kg dry weight) and day 5 (100 μg/kg dry weight; Table 4). Concentrations of ammonium increased in the first 12 days in all the treatment levels and then began to decrease in the following days (Fig. S9). Also, the total amount of nitrate and nitrite increased consistently in all treatment levels on day 12 and later, the highest concentration (3.73 mg/L) was measured on day 26 in the second highest treatment level (300 μg EB/kg dry weight sediment) (Fig. S10). Total soluble nitrogen and orthophosphate continuously increased throughout the experiment (Figs. S11 and S12). For none of the nutrients a significant treatment-related effect was observed (Table 4).

In the present study, the effects found on water quality parameters chlorophyll-a, dissolved oxygen and pH exposed to EB were indirect. With the feeding continues, the nutrient contents of ammonium, nitrate and nitrite, total soluble nitrogen and ortho-phosphate in the microcosms were all gradually increased, this promoted the bloom of phytoplankton and consequently a gradual increase of chlorophyll-a in all treatment levels. But acute and chronic ecotoxicity data showed that algae are also sensitive to EB (96-h EC50 = 7.2 μg/L; WRc, 2017), so we found that the chlorophyll-a was consistently affected, although the effect size is minimal, from day 0 onwards. The significant reduced dissolved oxygen on day 12 from 300 μg EB/kg dry weight sediment was most likely related to the combination of *A. marina* mortality and treatment-related increased *C. volutator* mortality, as the decaying tissues of worms and mud shrimps consumed the dissolved oxygen. The pH of the microcosms water was affected by the response of the phytoplankton community. As the decreased CO_2 consumption related to the lower algae density led to the reduced pH level of the water column from the 10 μg EB/kg dry weight sediment on day 19 that became significantly lower than in the controls.

3.3. Environmental occurrence and quality standards

The occurrence of EB in sediments in the vicinity of fish cages has been reported for several locations (Table 5). Some of the concentrations reported by Boxall et al. (2004) for sediments collected near Scottish fish farms are clearly above the 28-d NOEC for large *C. volutator* (30 μg/kg dry weight), and may even exceed the 28-d LC50 for that species. However, most of the reported concentrations are below the 28-d NOEC for *C. volutator*, indicating that these concentrations would most likely pose only a small risk to that species if it occurred at that location. Although, the indirect functional changes in chlorophyll-a, dissolved oxygen and pH following EB exposure were found in this study, these changes may be influenced by the size of the microcosms and the lack of water replacement, and can, therefore, not directly be translated to the field situation. Whether the reported concentrations of EB in sediments in the vicinity of fish cages could lead to long-term effects needs more studies using larger scale systems and/or field observations.

WRc (2017) recently proposed Environmental Quality Standards (EQS) for EB, which updated the EQS values derived by SEPA in 1999. According to EC Guidance Document No. 27 only long-term PNECs are considered appropriate for sediment (European Commission, 2011). Using the lowest available NOEC for sediment organisms (1.175 μg/kg, based on emergence in a 28-d study on the freshwater midge larvae *Chironomus riparius*), and applying an assessment factor of 100, a long-term PNEC of 0.01175 μg/kg dry sediment is derived. This value was used to set the 'far-field' chronic EQS (EQS-AA) at 0.012 μg/kg dry sediment (WRc, 2017), and the 'near-field' acute EQS (EQS-MAC) intended for protection of sediment reworkers below cages of 0.12 μg/kg dry sediment.

Applying the same method to the most sensitive NOEC found for *C.*

Table 4
The No Observed Effect Concentrations (NOECs) for water quality endpoints expressed in terms of nominal single-dose of EB concentrations (μg/kg dry weight sediment) measured on each sampling day (Williams test; $p < 0.05$). See Figs. S4–S12 for the results for all parameters.

Endpoint	Sampling days				
	0	5	12	19	26
Salinity	300 (+)	>	300 (+)	>	>
Dissolved oxygen	>	>	300 (−)	>	>
pH	>	>	>	10 (−)	>
Temperature	>	>	>	>	>
Chlorophyll-a	>	100 (−)	300 (−)	300 (−)	300 (−)
Ammonium	>	>	>	>	>
Nitrate and nitrite	>	>	>	>	>
Total soluble nitrogen	>	>	>	>	>
Oortho-phosphate	>	>	>	>	>

> = no significant effect (NOEC≥ 1000 μg EB/kg dry weight sediment); significant decrease (−) and significant increase (+) compared to solvent.

Table 5
Summary of EB concentrations levels in sediments near intensive salmon aquaculture activities. Abbreviation: n.d. = not detected, d.w. = dry weight.

country	Concentrations (μg/kg wet weight)	sample location	reference
Scotland	n.d. – 366	within 25 m	Boxall et al. (2004)
	n.d. - 75.1	25 m away	Boxall et al. (2004)
Norway	2–6.5 (d.w)	within 25 m	Langford et al. (2014)
Chile	2.2–8.38 (d.w)	within 25 m	Tucca et al. (2017)
	9.97 (± 1.7) (d.w)	25 m away	Tucca et al. (2017)
Canada	0.051–35	within 25 m	Ikonomou and Surridge (2013)
	2.73	within 25 m	McHenery and Mackie (1999); Bright and Dionne (2005)
	0.62	25 m away	McHenery and Mackie (1999); Bright and Dionne (2005)

volutator (organisms of 8–11 mm length), i.e. 30 μg/kg, a "near-field" sediment EQS of 3 μg/kg (dry sediment) and a "far-field" sediment EQS of 0.3 μg/kg (dry sediment) can be derived. These values are between the old (SEPA derived) EQS values (7.63 μg/kg wet sediment and 0.763 μg/kg wet sediment) and new EQS values (0.12 μg/kg dry sediment and 0.012 μg/kg dry sediment). This may suggest that the new EQS-values, which were calculated based on freshwater species data, are relatively strict and are sufficiently protective for the marine species tested in this paper.

Nonetheless, long-term sub-lethal effects may still be apparent at concentrations far below those causing 50% mortality of species used for routine bioassays. Inconsistent results between studies reporting similar experiments, different sensitivities of endpoints, indirect functional changes in physico-chemical parameters and interesting reproduction phenomenon of *C. edule* highlight the need for more research into how experimental conditions and the physiochemical properties of the test toxicant affect the repeatability of sediment bioassays.

3.4. Outlook: possible improvements to the test protocol

The high control mortalities and variation in both control and treatments mortalities indicate that this preliminary test design is not optimal. In contrast to previous studies (McBriarty et al., 2018), all animals used in the current study were collected from natural locations. Moreover, there were differences in the composition of the test sediment and water in the previous and current studies. In this study, formulated sediment and seawater were used according to OECD 2004a, 2004b, whereas natural sediment and seawater were prepared in previous studies (Allen et al., 2007; Kuo et al., 2010; Mayor et al., 2008; McBriarty et al., 2018). Allen et al. (2007) mentioned that differences in the properties of the sediments used for spiking are likely to affect mortalities and growth rates. Culture stress may be an influence in a long-term sediment test (Robinson et al., 1988). The low control growth rates and variable treatment growth rates also occurred in the longer-lasting laboratory test with *C. volutator* and *A. marina* (Allen et al., 2007). In a test using the amphipod *E. estuaries*, the temperature was gradually increased to 15 ± 1 °C (at a rate of 1 °C per day) and the amphipods were slowly acclimated to a salinity of 28 parts per thousand (ppt) (at a rate of 5 ppt per day) over 12 d (Kuo et al., 2010). McBriarty et al. (2018) suggested that when the test start, the test worms (*Nereis virens*) would better burrow into the substrate rapidly and remained with their burrow except when food was present, at which point they would partially emerge to feed. Therefore the worms (*Nereis virens*) were allowed to acclimate to laboratory holding conditions for at least 30 days (McBriarty et al., 2018). Using good culture facilities, supplied with clean sediment and continuous replenished seawater, and extending the acclimation period to longer times, e.g. 12 days, should minimize culture stress and reduce control mortalities to acceptable levels (< 20%). Extending the number of replicates to 5 per treatment level should help in better estimating the variation between replicates (Kuo et al., 2010; Mayor et al., 2008). Due to a sub-optimal test design, no definitive conclusions could be made on the suitability of *A. marina, C. volutator* and *C. edule* for sublethal toxicity testing and further developmental work is necessary.

Additionally, Atlantic salmon have been commercially cultured for decades, and the steady growth of this industry means more and more EB will applied in the farm with other range of chemotherapeutic treatments (Mayor et al., 2008). Due to a sub-optimal test design, and comparison with previously published LC50 experiments demonstrates the need for further standardization of methods, particularly with regards to the multi-species tests, acclimated methods and the composition of the sediments.

Compliance with ethical standards

-The authors declare that they have no conflict of interest.
-Research involving human participants and/or animals: This article does not contain any studies with human participants or vertebrate animals performed by any of the authors
-Informed consent: Informed consent was obtained from all individual participants included in the study.

CRediT authorship contribution statement

Bo Cheng: Writing - original draft, Conceptualization, Investigation, Data curation, Formal analysis, Methodology. **Jasper Van Smeden:** Investigation, Data curation. **John Deneer:** Writing - review & editing. **Dick Belgers:** Investigation, Data curation. **Edwin Foekema:** Conceptualization, Methodology. **Ivo Roessink:** Conceptualization, Investigation, Data curation, Methodology. **Arrienne Matser:** Investigation, Data curation. **Paul J. Van den Brink:** Conceptualization, Funding acquisition, Writing - review & editing, Project administration, Formal analysis, Methodology.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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