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Short-Term Effects of the Anti-sea Lice Therapeutant Emamectin Benzoate on Clam Worms (*Nereis virens*)

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Abstract The polychaete *Nereis virens* occurs commonly in marine sediments, is widely distributed, and is a popular bait species, as well as a potential replacement for wild-caught fish in commercial fish feed preparations. It is being considered as a potential co-extractive species for culture in integrated multi-trophic aquaculture operations. However, it is not known whether pesticides or drugs used to treat sea lice on farmed salmon, such as emamectin benzoate (EB), would adversely affect cultured or wild worms, because these compounds may persist in the environment. To determine the potential effects of EB to *N. virens*, bioassays were performed wherein worms were exposed in sand for 30 days to a concentration of 400 µg/kg dw (nominal). While no treatment-related mortality occurred, significant decreases in worm mass and marked behavioral changes (lack of burrowing) were observed in EB-treated sand compared with controls. These lab-based observations suggest a potential hazard to worms at sites where EB treatments have occurred.

In New Brunswick, Canada, the primary form of marine finfish aquaculture is the monoculture grow-out of Atlantic salmon (*Salmo salar*) (Pinfold 2013; GNB 2010). Recent research and development on the incorporation of other commercially valuable species into these farms, known as integrated multi-trophic aquaculture (IMTA), has been undertaken on both coasts of Canada (Barrington et al. 2009). The goal of this production technique is to use additional species to incorporate the organic and inorganic wastes generated by salmon aquaculture and, in turn, reduce the environmental impacts associated with traditional aquaculture monocultures (Barrington et al. 2009). One species of interest is the benthic invertebrate known as the clam worm (*Nereis virens*), which is valuable both to the sport fishing industry as bait and as a potential source of oils and nutrients for commercial fish feed (Olive 1999). In an IMTA system, *N. virens* would be held in containers beneath salmon cages where they would feed on solid wastes leaving the cages. In addition, because these worms construct and maintain burrows, they would provide a bioturbative service, which could help to alleviate the anoxic conditions that can develop underneath cages (Hargrave 1994).

A problem often encountered by salmon farmers is infestations of ectoparasites commonly referred to as sea lice (Roth et al. 1993). These infestations are treated with pesticides or drugs that are released directly into the marine environment during and after treatment (Burridge et al. 2010). Emamectin benzoate (EB) is the active ingredient in the antiparasitic drug SLICE™. It is delivered as an in-feed additive at a dose of 50 µg (EB)/kg (fish) for 7 consecutive days (Burridge et al. 2004). Fish faeces containing EB as well as uneaten EB-treated food sink to the bottom, becoming incorporated into sediments around cage sites (Telfer et al. 2006). Once in sediments, EB is moderately persistent. A study by Benskin et al. (2016) suggests a minimum half-life of 404 days for this chemical. The use of anti-louse treatments at aquaculture sites can lead to significant potential for chemical inputs to affect nontarget organisms (Burridge et al. 2010), both wild fauna and those currently being

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considered for IMTA. For example, Van Geest et al. (2014) reported that the pesticide formulation AlphaMax® was not lethal to *N. virens*, but it did affect behaviors of this species during lab exposures. We present the results of a 30-day sediment bioassay to determine the effects of EB on the survival, growth, and behavior of *N. virens*.

Materials and Methods

Bioassay

N. virens of the same age class (< 1 year) were purchased from the University of Maine's Center for Cooperative Aquaculture Research (CCAR) facility in Franklin, Maine. These worms were raised at CCAR in silica sand before purchase, transported to the St. Andrews Biological Station, New Brunswick, and placed in holding tanks containing silica sand (SiO_2; Ottawa Sand, Fisher Scientific). Previous studies in which marine invertebrates were exposed to aquaculture pesticides, including EB, were conducted using silica sand as a substrate (Mayor et al. 2008). Similar conditions were used in the current study. Worms were held in flow-through conditions under a constant photoperiod of 12 h light and 12 h dark. The holding water was from Passamaquoddy Bay, New Brunswick (salinity ~ 30 ppt, temperature = 13 ± 0.5 °C) and was filtered, degassed, and aerated. Worms were allowed to acclimate to laboratory holding conditions for at least 30 days. Preliminary studies showed that worms could be held under laboratory conditions for several months at least. Worms would burrow into the substrate rapidly and remained with their burrow except when food was present, at which point they would partially emerge to feed. On a weekly basis, worms were hand-fed a mixture consisting of commercial salmon pellets (2 mm Skretting) and diced mussels (*Mytilus edulis*).

EB (99.1% pure; Sigma Aldrich, St. Louis) was dissolved in HPLC grade ethanol (Fisher Scientific, St. Louis) and an aliquot of the stock solution was mixed with a known quantity of dry silica sand to produce a nominal concentration of 400 µg EB/kg dw (sand). The sand was mixed in a rotary mixer for 24 h to ensure that it was well homogenized and to allow for the evaporation of ethanol. An equal amount of sand was mixed for 24 h and used as a blank control. Finally, a solvent control was prepared by mixing ethanol with sand in the same manner.

A 30-day, serial replacement bioassay was conducted using a single nominal concentration of 400 µg EB/kg dw (sand). This concentration is similar to levels of EB measured in marine sediments near a salmon aquaculture operation (Telfer et al. 2006). In preliminary studies, EB concentrations in sand were found to be stable for at least 10 days (data not shown).

During exposures, worms were held individually in solvent cleaned 250-mL beakers containing ~ 400 g of wet sand and covered with mesh to minimize escape of worms. Before the start of the bioassay, worms were removed from the holding tanks, rinsed with seawater to remove excess sand and mucus, blotted dry with paper towel, weighed, and then randomly assigned to beakers ($n = 50$/treatment). The beakers were placed in a flow-through water bath with a depth greater than the height of the beakers. Temperature and oxygen in the water baths were monitored daily during the exposure and general observations of the worms' behavior (i.e., burrowed or not burrowed) were made at the same time. Dead worms were removed, weighed, and stored at − 80 °C. Every second day, worms were offered a salmon feed pellet, and any uneaten material from the previous feeding was removed. Every 10 days, the sand in the beakers was replaced with freshly prepared sand, and all worms were weighed before being returned to the beaker. At the end of the experiment, worms were blotted dry, weighed, and euthanized using liquid nitrogen. Sand from five randomly selected beakers for each treatment was collected at $T = 0$, $T = 10$, 20, and 30 days and was immediately frozen at − 80 °C until used for chemical analysis. Samples were taken at the beginning and end of each 10-day period to assess initial and final concentrations of EB in the beakers for each batch of sand used.

Chemical Analysis

All samples of sand were analyzed for EB concentrations using a modified version of the method outlined by Van de Riet et al. (2001). Just before EB extraction, samples were thawed to room temperature. A subsample (5.0 g) was transferred to a 50-mL centrifuge tube, along with 15 mL of acetonitrile (ACN, Fisher Scientific, Fairlawn, NJ) and an internal standard of ivermectin (IVR). The mixture was shaken on a wrist-action shaker for 30 min and then centrifuged at 3000 rpm (1075 g) for 10 min. The supernatant was collected and transferred to a 125-mL Erlenmeyer flask. The sample was then re-extracted in the same manner using ACN. The combined supernatant was brought to 100 mL with the addition of 70 mL of distilled water and 100 µL triethylamine. This supernatant was than cleaned on a preconditioned Varian C-18 BondElut SPE cartridge. The supernatant was passed through the SPE cartridge followed by 6 mL of a 70:30 deionized Milli-Q H_20/ACN 0.1% triethylamine solution. The SPE cartridge was dried under vacuum for 2–3 min. The bound components were eluted from the SPE cartridge with 12 mL of ACN drawn through the cartridge at a rate of 5 mL/min into a 15-mL centrifuge tube. This elute was reduced to ~ 1.5 mL under nitrogen and then pipetted into a 5-mL borosilicate glass culture tube. The centrifuge tube was rinsed with ACN, and the rinsate was

added as well to the culture tube. The extract was evaporated to dryness under nitrogen at 50 °C.

The concentrated residue was dissolved in 1.5 mL of anhydrous ACN, mixed and placed on ice. After 15 min, 200 µL of 50% methylimidazole (Sigma Aldrich; ≥ 99% purity, Muskegon, MI) in anhydrous ACN solution was added and then mixed briefly on a vortex mixer. After 15 min, 200 µL of 50% trifluoroacetic anhydride (Sigma Aldrich, 99% purity) in anhydrous ACN solution was added, and samples were again mixed on a vortex mixer. After a final 15-min interval, 600 µL of anhydrous ACN was added to each sample and vortexed for a final time. Using a 1.5-mL syringe, a 1-mL subsample was placed into autosampler vials. The derivatized extracts were analyzed for EB, its desmethyl metabolite (DM), and IVR by high-performance liquid chromatography (HPLC) on an Agilent 1100 liquid chromatograph: G1379A Degasser, G1311A Quatanary pump, G1313A autosampler, and G1321A fluorescence detector (FLD), using methods modified from Tauber et al. (2006). A 100-µL sample was injected onto an ES Industries Exsil ODS B 5µ 100 Å, 25-cm × 3.2-mm column. The column temperature was set at 35 °C. The mobile phases were a 98% ACN: 2% Millipore water (A) and 100% methanol (B), with the following gradient program: time 0–10 min: 100% A; time 10–11 min progression from 100% A to 100% B: time 11–19 min: 100% B; time 19–20 min: progression from 100% B to 100% A; mobile phase flows were maintained at 1 mL/min. The fluorescence excitation wavelength was 365 nm, and the emission wavelength was 470 nm. Each run of samples contained a calibration series (25, 100, and 150 ng on column for each EB, DM, and IVR), with solvent blanks being run every 5 samples and an extraction blank every 20 samples. All samples were run from low to high concentrations, and a tip rinse vial was used between every sample. This method was validated before processing of samples from the 30-day exposure.

Data and Statistical Analysis

Because worms were housed individually during the bioassay, it was possible to assess individual changes in growth. Absolute changes in worm mass were determined by weighing worms before (T_1) and after (T_2) each 10-day period and calculating the difference in the two measures. Specific growth rates (SGR) of worms were determined as follows:

$$\%\ \mathrm{SGR}\ (\mathrm{g/day}) = (\ln(\mathrm{Mass}_{T2}) - \ln(\mathrm{Mass}_{T1}))/(T_2 - T_1) \times 100$$

(Schreck and Moyle 1990; T in days). Normality of the data was assessed using the Kolmogorov–Smirnov test and homogeneity of variance was tested using F max and/or Levene's test; no transformations were needed. Concentrations of EB in sand on days 0, 10, 20, and 30 were compared using one-way ANOVA ($\alpha = 0.05$) and Tukey HSD post hoc analysis. Any changes in EB concentrations within each of the 10-day intervals were compared using t tests that compared initial ($T = 0$) and final ($T = 10$) EB values. Additionally, SGR's from the 10-day intervals were compared across and between the three exposure concentrations using repeated measures ANOVA. Growth was also compared among treatments, within time, using a one-way ANOVA and a Tukey's HSD post hoc analysis. Behavioral data were expressed as a percentage of burrowed animals to total animal in each treatment and these effects were assessed qualitatively. Graphs were prepared using Excel. All normality, ANOVA, and Tukey analyses were performed using Minitab® 16 statistical software, and α was set at 0.05 for all tests. Unless otherwise stated, all data are shown as means ± 1 SE.

Results

Initial concentrations of EB in the sand on days 0, 10, and 20 averaged 291 ± 10.5, 213 ± 40.4, and 198 ± 21.8 µg/kg wet wt, respectively (Table 1), indicating that measured concentrations were between 49 and 73% of nominal. There was a significant decrease between the initial and final EB concentrations in both the first and second 10-day intervals ($T = 0$–$T = 10$; $T = 10$–$T = 20$; $p < 0.005$ and 0.012, respectively) but not in the final 10 days ($T = 20$–$T = 30$; $p = 0.11$). At the end of the first two 10-day periods, EB concentrations were on average 46% lower than initial concentrations (Table 1; Fig. 1). EB concentrations averaged 170 ± 15.8 µg/kg wet wt across all treatment beakers and for all sampling times. The desmethyl metabolite of EB was not detected in any of the samples analyzed nor was EB detected in any of the control samples.

Table 1 Predicted (nominal) and mean measured concentrations of EB in sand samples (N = 5/treatment/date) collected during a 30-day bioassay

Time interval (days)	Days 0–10, initial	Days 0–10, final	Days 10–20, initial	Days 10–20, final	Days 20–30, initial	Days 20–30, final
Batch (time prepared)	0	0	10	10	20	20
Concentration (µg/kg) ± SE	291.64 ± 10.5	105.93 ± 7.16	213.10 ± 40.46	101.12 ± 13.78	198.82 ± 21.83	83.03 ± 21.83
IVR recovery (%) ± SE	92.075 ± 11.34	93.13 ± 8.92	95.35 ± 9.15	89.84 ± 14.60	80.14 ± 8.98	83.03 ± 6.97

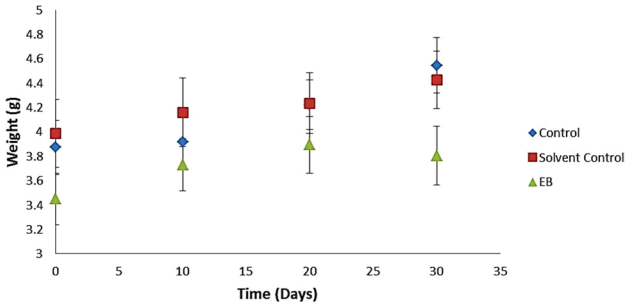

Fig. 1 Mean (± SE) total mass (g) of *N. virens* at four sampling periods during 30-day exposure to 171 µg/kg wet wt EB in sand, ethanol control or blank control

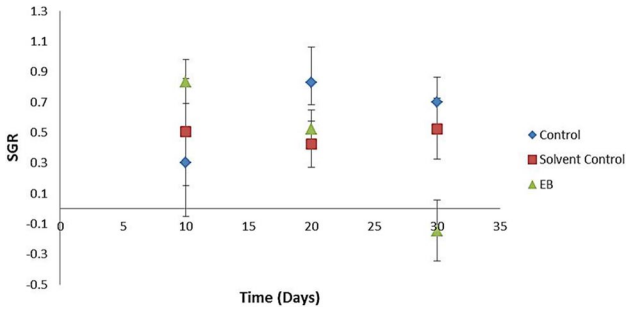

Fig. 2 Measured (mean ± SE) SGR for *N. virens* at 4 sampling periods during a 30-day exposure to either 171 µg/kg wet wt EB in sand, ethanol control or blank control. *Significant difference $\alpha = 0.05$

During the first 10 days, several worms escaped from their containers: three from the blank control, seven from the solvent control, and six from the EB exposure. Because it was impossible to tell which beaker the worms had escaped from, new worms and EB-treated, or control, sand were put into these beakers at day 10. The replacement worms were then held for a full 30 days with appropriate sand changes every 10 days. The number of worms in each treatment group started at 50 and only changed over time due to the death of some worms. One worm died in the solvent control group, and six died in each of the blank control and EB-treated groups.

Worms from the controls had mean total masses of 3.87 ± 0.22 g ($N = 50$) at $T = 0$, 3.91 ± 0.20 g ($N = 50$) at $T = 10$, 4.22 ± 0.20 ($N = 47$) at $T = 20$, and 4.55 ± 0.23 g ($N = 44$) at $T = 30$ (Fig. 1). Worms in the ethanol controls had higher average masses across all times of 3.99 ± 0.28 g ($N = 50$) at $T = 0$, 4.16 ± 0.28 g ($N = 50$) at $T = 10$, 4.23 ± 0.25 ($N = 49$) at $T = 20$, and 4.42 ± 0.24 g ($N = 49$) at $T = 30$ compared with worms in the blank controls (Fig. 1). Worms in the EB exposure had lower mean masses of 3.45 ± 0.21 g ($N = 50$) at $T = 0$, 3.73 ± 0.22 g ($N = 48$) at $T = 10$, 3.89 ± 0.23 ($N = 45$) at $T = 20$, and 3.81 ± 0.24 g ($N = 44$) at $T = 30$ compared with the other two treatments (Fig. 1). On average, worms in the blank control and solvent control beakers increased in mass by 0.61 ± 0.18 g ($N = 44$) and 0.48 ± 0.11 g ($N = 49$), respectively, over the 30 days. Worms in the EB exposure increased in mass by only an average of 0.07 ± 0.15 g ($N = 44$) over the same time. Despite these differences, total masses of the worms were not significantly different across treatments within each time period (ANOVA; $p = 0.118$). In fact, mass across all three exposures was not significantly different for any time period ($p > 0.05$).

Specific growth rates (Fig. 2) of the worms in the EB exposure decreased over time and were negative in the final 10 days of the experiment. This was in contrast to the control worms that either increased in SGR over time (control) or had SGRs that were similar after days 10, 20, and 30 (solvent control). This change in SGR in the EB exposure was found to be significantly different ($p = 0.001$) across the exposures following analysis via repeated measures ANOVA ($\alpha = 0.05$). Change in SGR also was significantly different in the final 10-day period of the EB exposure from the last 20-day periods (days 10–20 and 20–30, respectively) of both controls ($p = 0.025$ and $p = 0.001$ for the ethanol and blank controls, respectively).

There were some differences in the burrowing of worms among treatments (Fig. 3). In the control beakers, between 58 and 100% of the worms remained burrowed each day over the entire experiment. This behavior was a little more variable in the solvent control treatments, with 32–92% of worms burrowed in the first 10 days, 88–94% of worms burrowed in the next 10 days, and 78–97% of worms burrowed in the final 10 days. In contrast, > 50% of worms exposed to EB-treated sand were found emerged from their burrows over the first 20 days and few or none were burrowed in the final 10 days of the experiment. These (emerged) worms also did not burrow for the remainder of the bioassay.

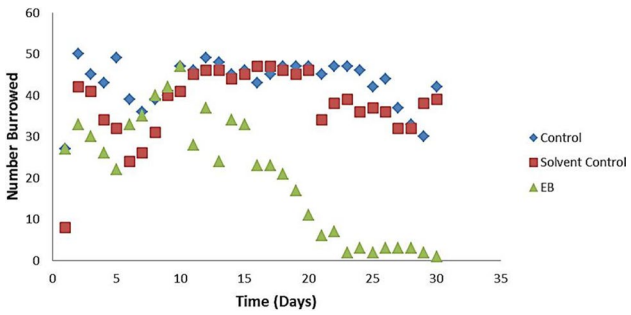

Fig. 3 Burrowing behavior of *N. virens* exposed to either 171 µg/kg wet wt EB in sand, ethanol control, or blank control during a 30-day bioassay

Discussion

This study examined the effects of an anti-sea lice treatment, EB, on a species of worm that is being considered for IMTA and is common in temperate marine environments. *N. virens* showed no treatment-related mortality; however, they had lower specific growth rates than the control worms and exhibited behavioral changes as manifested by reduced burrowing into the sand. Overall, these results suggest that EB could present a hazard to wild or cultured worms at cage sites where it is used to treat sea lice and may affect the worm's ability to act as an extractive and bioturbative species in IMTA.

The lack of burrowing that was observed in the latter part of the study suggests that wild or cultured worms may become more susceptible to predation if exposed to EB from cage culture operations. These behavioral effects were found for worms exposed to average EB concentrations that were less than half of that in sediments near a salmon aquaculture site (366 µg/kg dw; Telfer et al. 2006) but higher than those reported in the laboratory study of Mayor et al. (2008; 28 µg/kg dw). Reports of EB concentrations near aquaculture sites were not common when these trials were performed. In a more recent study, Tucca et al. (2016) collected sediment samples at sites 0–100 m away. The authors reported concentrations of EB in sediment ranging from 5.29 to 9.97 ng/g dw. The authors did not, however, state when the treatment occurred relative to the time of sampling. Regardless, field measurements are likely to be highly variable, due to site-specific differences in environmental conditions, such as sediment quality and water exchange and EB treatment requirements. The 366 µg/kg reported by Telfer et al. (2006) likely represents a worst-case scenario for field sampling, because these samples were collected immediately after treatment and from directly under the cages. However, given the intended purpose of *Nereis* in an IMTA system, sampling directly beneath the cages and immediately following EB treatment suggests that 366 µg/kg may better reflect potential clam worm exposures. In addition, EB is persistent in sediments and has been measured 4 (Haya et al. 2005) and 12 months (Telfer et al. 2006) after treatment, and its half-life has been estimated at 400 days in the lab and potentially years in the environment (Benskin et al. 2016). While the purpose of the current study was to determine the affect of EB on caged worms within the confines of an IMTA setting, the behavioral effects observed and the environmental persistence of EB indicate that longer-term assessments of EB's effects on other indigenous infaunal organisms is warranted.

In the current study, worms typically completed their burrows within the first few hours after being added to the control beakers. However, EB affected their burrowing behavior; fewer or no worms burrowed in the final 10 days of the study. In addition, the EB-exposed worms that burrowed made more irregular burrows, with numerous branches and openings on the top of the sand (McBriarty personal observation). Also in the final 10 days of the experiment, worms would either thrash violently before settling on the surface of the sand or continually circle the beaker (McBriarty personal observation). In contrast, burrowing and other behaviors remained normal for *N. virens* in the controls; the presence of EB resulted in abnormal behaviour.

Marked behavioral changes in *N. virens* also were described by Olla et al. (1984). In their study, low concentrations of oil in sand resulted in total or partial emergence of *N. virens* from burrows, whereas mortality only occurred at the highest test concentrations. They warned against using only mortality, because it was a less sensitive indicator and recommended that changes in behavior be used to assess sublethal effects of sediment contaminants. Results of the current study suggest that burrowing behavior is a more sensitive and ecologically relevant endpoint. In the environment, these worms would be more susceptible to predation at the surface of the sediments.

EB concentrations in the sand were consistently lower after 10 days than the initial concentrations, and these losses were higher than those observed in preliminary trials using sand only (no worms; data not shown). This is likely because the ventilation and burrowing activities of the worms increased the breakdown of EB in the sand. Using the ventilation rate for *N. virens* reported by Miron et al. (1994) of 86 mL water/g worm per h, the largest worm in the 30-day bioassay would have moved ~ 21 L of water through its burrow during a 24-h period. Previous studies have shown that *N. virens* and other burrowing invertebrates change the chemical nature of the sediments through vertical and lateral mixing as well as the exchange of interstitial pore water in marine sediments with overlying water (Piot et al. 2008; Papaspyrou et al. 2006; Kristensen 2000). Another possibility for the decline in EB concentrations in the sand over time is the bioaccumulation of this chemical by the worms because of its low water solubility (Haya et al. 2005). Van Geest et al. (2014) showed that *N. virens* held under the same conditions as described here accumulated measurable levels of another persistent anti-louse pesticide (AlphaMax®, active ingredient deltamethrin). No chemical analyses were performed on worm tissues in the current study. Interestingly, during the final 10 days of the bioassay, there was a buildup of presumably anoxic sand (layer of black sand; G. McBriarty, personal observation), and no significant decrease in EB concentrations in the sand was observed when most of the worms were not forming burrows. Similarly, Kuo et al. (2010) showed that EB concentrations in sediment remained stable over a 10-day period in studies with the surface-dwelling amphipod *Eohaustorius estuaries*.

EB did not cause any treatment-related mortality of the worms during this bioassay, and these results are consistent

with those of Mayor et al. (2008) who reported an LC_{50} of 1368 µg/kg wet wt for *Hediste diversicolor*, a polychaete similar to *Nereis.* Their estimate was based on nominal concentrations in sediment. This may result in an overestimation of the LC_{50}, because our data indicated that the concentration of EB decreased over time.

Exposure of *N. virens* to EB in sand affected their SGR but only after exposures of 20 days or more. This coincides with when the treated worms stopped burrowing but appeared to continue to eat. Because no effect on SGR was noted in control worms, this indicates a direct effect of EB on these individuals. Similar declines in growth of *N. virens* also have been observed when the worms were exposed to AlphaMax®, an anti-sea lice pesticide that contains deltamethrin (Van Geest et al. 2014). Because growth of worms was affected at the single concentration tested in the current study, exposures to broader range of EB concentrations are recommended to determine concentrations at which no growth-related effects occur.

The significant impact of EB on the growth and behavior of *N. virens* indicates that use of EB medicated food may affect its commercial viability as a co-extractive species in IMTA. If *N. virens* are not growing then they are not providing an extractive service, and, as such, it would not be possible to recoup the economic investment required to incorporate *Nereis* into IMTA practices. In addition, if the worms are unable to burrow they are not providing a bioturbative service that would reduce impacts of aquaculture wastes on the environment. Exposure conditions used in the current study may differ from those in the field, because sediments near cage sites will contain organic matter that could change uptake of EB into the worms. While conditions in the current study mimicked those of previous bioassays, it is possible that effects of EB would be reduced if exposures occurred in sediments rather than sand. For example, Van Geest et al. (2014) exposed *N. virens* for 7 days to AlphaMax® (active ingredient deltamethrin) in either sediment or sand and found that the worms emerged in both treatments but at lower concentrations in the sand exposures; however, effects on growth and its accumulation into the worm were greater in sediments than sand at similar concentrations of deltamethrin. As such, further study of EB's effects on worms in sediments is warranted.

Conclusions

While EB was not acutely lethal to *N. virens* under these experimental conditions the sublethal, behavioral implications of EB exposure may limit the effective use of *Nereis* as a commercially valuable co-extractive species. A number of effects of EB on *N. virens* were observed. *N. virens* exposed to EB over 30 days showed a loss of weight as well as a significant drop in SGR compared with controls. Environmentally significant behavioral responses also were noted. Exposed worms either emerged from their burrows or failed to reestablish burrows after > 15 days exposure to 171 µg EB/kg wet wt. Local conditions at aquaculture sites will affect the concentrations of EB to which *N. virens* are exposed. However, for IMTA purposes, the location that maximizes the likelihood that worms can consume wastes from the salmon aquaculture site also maximizes the opportunity to be exposed to food-borne therapeutants. Because only one treatment concentration was used herein, additional studies are suggested to determine a threshold concentration and a no-effect concentration of EB for behavioral responses of *N. virens*.

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