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The value of EDTA treatment of hatchery water to rear Greenshell™ mussel (*Perna canaliculus*) larvae

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Abstract
The chelating agent ethylenediaminetetraacetic acid (EDTA) is used throughout the world to improve the yield of early stage D-larvae during bivalve hatchery production. Adding EDTA (12 μM) to seawater significantly increases the survival of Greenshell™ mussel (*Perna canaliculus*) larvae during their first 48 h of development. However, whether there are benefits of continuing to use EDTA beyond this first stage of larval development remain unknown and were tested in this study. After rearing for 48 h in the presence of EDTA, *P. canaliculus* larvae were experimentally raised to 22-day post-fertilisation in seawater with and without 12 μM EDTA. The survival, shell length growth, algal ingestion rate, swimming activity and potential toxic metal accumulation by the larvae were compared over this period. There were minimal benefits from continuing addition of EDTA. However, significant changes in metal concentrations within the larvae were observed. Zinc, cadmium and mercury were detected at significantly lower concentrations in 22-day-old larvae reared with EDTA versus those without EDTA. Collectively, the results indicate that the use of EDTA is critical only during the first 48 h of larval development, during which time larval shell formation is initiated and appears highly vulnerable to interference by heavy metal ions.

Keywords Greenshell™ mussel · EDTA · Larval rearing · Fitness · Metals

Introduction

Ethylenediaminetetraacetic acid (EDTA) is commonly used worldwide as a chelating agent in a variety of industrial, medical, cosmetic and laboratory applications (Oviedo and Rodriguez

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2003). The ability of EDTA to readily complex with metals in solution has an important role in these applications.

EDTA is widely used for the treatment of seawater for rearing the larvae of bivalves in aquaculture hatcheries because of its ability to significantly improve D-larval yield and overall production efficiency (Gale et al. 2016; Lawrence et al. 1981; Rainbow et al. 1980; Utting and Helm 1985). It is believed that EDTA mitigates heavy metal toxicity for shellfish larvae by means of its strong metal binding properties, reducing their overall bioavailability (Nicula et al. 2011).

Some heavy metals can prove to be toxic to bivalves in a variety of ways if present in sufficient concentration (Rainbow 2002). For example, certain heavy metals at trace levels in seawater can interfere with shell formation (Lasseter et al. 2016), affect protein function (Rainbow 2002) or cause oxidative stress (Gale et al. 2016), particularly for the more vulnerable larval stages. Several heavy metals, such as iron, copper and zinc, are biologically essential, and hence, organisms have the physio-chemical mechanisms to exert some control over the uptake, concentration and distribution of these metals in their tissues (Wang et al. 2018). However, other metals of no known biological function in the larvae, such as cadmium, mercury and lead, where they are bioavailable, are often able to substitute essential metal ions, potentially leading to malfunction of key biological processes (Wang et al. 2009; Wang et al. 2018).

Hatchery production of early juvenile bivalves is of major importance for aquaculture grow-out operations; otherwise, juveniles are sourced from the wild, which can be highly unreliable (Adams et al. 2009; Alfaro et al. 2010; Helm et al. 2004). For example, a substantial proportion of New Zealand's Greenshell™ mussel (*Perna canaliculus* Gmelin 1791) aquaculture industry relies on wild juveniles collected from Ninety Mile Beach in the upper North Island of New Zealand. However, the timing of supply is unpredictable, the quality of juveniles highly variable, and their long-term performance frequently unsatisfactory (Alfaro et al. 2010; Jeffs et al. 2018). Efficient hatchery production is thus important for Greenshell™ mussels, as it also is for ensuring a sustainable future for aquaculture globally.

Currently, the addition of EDTA to hatchery water during embryo incubation stages of some bivalve species is considered essential to ensure successful completion of early larval development in which shell formation is initiated (Helm et al. 2004). Current best practice for *P. canaliculus* larval rearing uses seawater aged for at least 24 h with 12 μM EDTA in the first 48 h initial incubation of mussel embryos to substantially improve the yield of D-stage veliger larvae (S. Adams, personnal communications; Gale et al. 2016). However, it remains unclear whether the continuation of EDTA dosing of larval culture water beyond the initial embryo incubation would continue to provide benefits for the later stages of larval development.

Therefore, the aim of the present study was to investigate whether dosing of seawater with 12 μM EDTA could also be beneficial for larval development subsequent to the first 48 h of larval rearing of Greenshell™ mussels.

Materials and methods

Gamete collection and incubation

Adult Greenshell™ mussels harvested from near-shore long line farms in the Marlborough Sounds, New Zealand, were transferred to the Cawthron Aquaculture Park where they were

maintained under ambient temperature and food conditions for 4 months before being used as broodstock for larval production in May–June 2018. Mussels were placed into spawning trays, and thermal cycling was used to induce mussels to release gametes (Helm et al. 2004; Adams et al. 2009). Spawning mussels were separated into individual containers once gamete release was first observed and the sex of each spawning mussel could be determined.

Ten spawning male mussels were rinsed with filtered seawater (FSW; 1 μm filtered, UV sterilised at 100 mJ cm^{-2} s^{-1} and passed through activated carbon), placed anterior end up in separate 50 ml plastic bottles and left to spawn without the addition of seawater. Concentrated sperm spawned from each male dripped into the container and was collected every 30 min over a period of 2 h and was stored at 5 °C until the samples from each male were pooled ready to be used in experiments. Sperm concentration was determined using a Neubauer haemocytometer.

Spawning female mussels were rinsed with FSW and placed in individual containers with 500 ml FSW at 9 °C. Concentrated suspensions of released eggs were then collected every 30 min and stored at 5 °C. Eggs were checked under the microscope for morphological normality and to ensure an absence of polar body (i.e. remained unfertilised). A pool of unfertilized eggs with 16 females contributing was produced. Then, the eggs were fertilized with sperm at a ratio of 500: 1 (sperm: egg). The embryos were then split evenly into six aliquots of approximately 10 million embryos, and each aliquot was added to one of six 170 L cylindro-conical incubation tanks. At 24 h prior to the addition of the embryos, each incubation tank was filled with FSW at 16–17 °C and pH of 8.4 which was dosed with analytical grade ethylenediaminetetraacetic acid disodium salt (EDTA) (Fisher Scientific UK, CAS: 6381-92-6) at a concentration of 12 μM and continuously aerated from the base of the tank (Ragg et al. 2019). For the first 48 h, the embryos were incubated without replacement of seawater.

Larval system and rearing procedure

Following a 48 h static incubation period, during which time the embryos progressed to D-stage veligers, each tank was drained, and the swimming D-stage larvae were collected on a 40 μm nylon screen and counted, and the yield of viable D-stage larvae determined as a percentage of initial egg population. The larvae were pooled and distributed among 12 replicate 2.5 L purpose-built acrylic larval rearing tanks (six control and six EDTA treated) at approximately 1 million D-stage larvae per tank and reared in flow-through according to standard procedure previously detailed in Ragg et al. (2010).

The rearing system consisted of 12 larval tanks supplied by gravity-fed seawater from two 100 L header tanks via a manifold. Six individual tanks were the control treatment and were supplied continuously with FSW adjusted to 18 °C at 80 ml min^{-1}. The remaining six tanks were the EDTA treatment and were supplied continuously by means of a submersible pump at 80 ml min^{-1} from a 1000-L reservoir tank that was filled with FSW and dosed with 12 μM EDTA 24–48 h prior to use.

Every 2 days, each larval rearing tank was completely drained through a screen with a mesh screen (size 43–175 μm) that was previously determined to retain > 90% of live larvae, whilst separating detritus and empty shells. Once drained, each vessel was also cleaned with hot freshwater to reduce the development of biofilm on the tank surface.

A plurispecific diet of cultured microalgae (*Chaetoceros calcitrans* forma *pumilum*, *Tisochrysis lutea*, *Chaetoceros muelleri*) was added to the supply tanks and delivered to the

larval rearing tanks to reach a steady density in the water being supplied to the larval rearing tanks of 5 to 30 cells μl^{-1}.

The larval rearing phase was completed after 22-day post-fertilisation (PF), when most of the larvae reached competency for settlement as made apparent by the presence of an eyespot.

Sampling procedure and assessments

Larval fitness, developmental rates and survival were compared with metal content throughout the experiment. While it is recognised that uptake of metals from seawater is influenced by the bioavailability of their free ions in solution (Florence et al. 1992), it remains technically challenging to obtain information about trace metal speciation in biological systems. Therefore, in this study, we were limited to the determination of total metal concentration only in larval mussels.

Larval performance assessment

Larval survival, shell length and algal ingestion rates were assessed throughout the rearing period. D-stage yields were evaluated on day 2 post-fertilisation (D2), by taking three 0.2 ml aliquots and counting the number of larvae that had formed a complete D-shaped shell within each incubation tank. On D4, D6, D8, D10, D14, D17, D20 and D22, larval survival was estimated in each tank, by counting the number of live larvae in three 0.2 ml aliquots sampled from the surface water from each larval tank. To minimise the effects of differences in stocking density caused by different mortality rates, an appropriate number of larvae were removed on D10, and tanks were re-standardized so that all tanks matched the larval numbers of the tank with the lowest resident population.

Shell lengths of 30 larvae per tank were measured after fixation with 10% buffered formalin, on D4, D6, D8, D10, D14, D17, D20 and D22, using the cellSens image analysis software (inverted microscope CX41 × 40 magnification, DP74 camera; Olympus).

Percent larval swimming activity was evaluated from each replicate tank on D6, D10, D14, D17 and D20 by counting the number of resting larvae versus the total number of larvae 1 min after disturbance by swirling in a culture dish containing a 1.0 ml sample. Normal swimming behaviour consisted of arcing trajectories and excluded spinning larvae (abnormal) and resting larvae.

Larval food ingestion rate was assessed from each replicate tank on D4, D6, D10, D14, D17, D20 and D22 by measuring the difference in the micro-algal cell counts between the water supplied into the larval tanks versus the water draining from the tanks on each assessment day. Micro-algal cell counts were made using a Coulter Counter (Beckman Coulter Multisizer4 particle analyser, Beckman Coulter Inc., California; 2.5–20 μm). To ensure the dilution of micro-algal density was consistent among tanks, the seawater inflow rates were carefully confirmed volumetrically for each tank. Estimates of micro-algal cell depletion were standardised by the larval population in each tank to provide a measure of individual larval ingestion rate.

Larval sampling for metal content

Following spawning, three replicate samples of 2 million unfertilised eggs were collected from the pooled eggs to be used in this study, rinsed with cold freshwater on a 15 μm screen, and

transferred into a cryovial. Cryovials were then plunged into liquid nitrogen and stored at − 80 °C. All samples were then freeze-dried and stored at − 80 °C for later metal analysis.

The same process was repeated on D2 when samples of 210,000 to 280,000 larvae were collected from each tank. On D10, a sample of between 200,000 and 300,000 larvae was sampled from each tank, and on D22, a sample of between 200,000 and 300,000 larvae were collected from each tank.

Metal analysis

Freeze dried mussel eggs and larvae were all microwave acid digested in pre-weighed 100 ml Teflon tubes to which the sample of mussel larvae were added and re-weighed. An aliquot of 1 ml of H_2O_2 and 3 ml of concentrated HNO_3 (69%) was added to each tube, which was sealed with a screw-on cap, before 70 min of digestion in a microwave digestion system (Milestone Srl, Sorisole, Italy), at a maximum temperature of 180 °C. Then, 36 ml of type 1 deionised water was added to all tubes before they were weighed with solution. The solutions were then analysed with inductively coupled plasma mass spectrometry (ICP-MS) (7700x, Agilent, Santa Clara, CA), and the concentrations of Cr, Fe, Co, Ni, Cu, Zn, As, Cd, Hg and Pb were back-calculated for the dry mass of the original freeze-dried samples. The ICP-MS was operated in He mode to reduce polyatomic interferences. Calibration standards were prepared in a matrix-matched solution from 1000 ppm single element standards (Peak Performance, CPI International, Santa Rosa, CA) and a blank matrix-matched solution containing no single element standards. An online internal standard (20 ppb Y & Tb) was used to monitor and correct for instrument drift and matrix effects. Detection limits were 10^{-5} (Cr), 10^{-4} (Fe), 10^{-6} (Co), 10^{-5} (Ni), 10^{-4} (Cu), 10^{-4} (Zn), 10^{-5} (As), 10^{-5} (Cd), 10^{-5} (Hg) and 10^{-5} (Pb) μg g dry matter^{-1} (or ppm).

Statistical analyses

All percentage data were arcsine square root transformed to stabilise variances. The normality of transformed data was assessed using the Shapiro-Wilk test ($p > 0.05$) while homogeneity of variances was confirmed with the Brown-Forsythe test ($p > 0.05$). After fulfilment of these two conditions, larval performances at each sampling time were compared between the two treatments with a two-way repeated measure ANOVA, followed by a multiple pairwise comparison using Tukey's post hoc tests. All heavy metal concentration data that did not pass the Shapiro-Wilk test ($p < 0.05$) were log transformed to improve normality. Comparisons of heavy metal concentrations between eggs and D-larvae, and between treatments (control vs EDTA) at each stage of development were made using paired Student's t tests. Significant difference was accepted at $p \leq 0.05$. Statistical analyses were performed using the software Sigma-Plot 14.0 (Systat Software Inc.). Means are presented with standard errors (SEM) throughout the results.

Results

The mean yield of D-stage larvae on D2 for the six 170 L tanks was 89 ± 3% SEM. One replicate 2.5 L tank from the EDTA treatment overflowed on D3 and was subsequently excluded from all analyses.

Larval survival

There was a statistically significant interaction between treatment and day PF ($F_{1,7} = 9.711$, $p = 0.029$); therefore, the effect of EDTA was dependent on when survival was measured. On D4, there was a significant difference between control and EDTA treated larvae, with survival percentages of 93.7% ± 3.3% and 84.8% ± 4.6%, respectively ($t = 2.872$, $p = 0.019$). However, on all other assessment days, there was no significant difference between control and EDTA treated larvae ($p > 0.05$) Fig. 1.

Shell length

EDTA treatment of the seawater did not result in a significant difference in mean shell length of the cultured larvae ($F_{1,7} = 0.00173$: $p = 0.888$). In both treatments, larvae reached ≈ 250 μm mean size after 22 days (Fig. 2).

Swimming activity

There was a statistically significant interaction between treatment and day PF ($F_{1,4} = 16.810$, $p < 0.001$). The swimming activity of mussel larvae depended on what day it was measured (Fig. 3). On D10, larvae from the control treatment (78 ± 5% of sample) were significantly more active than larvae from the EDTA treatment (27% ± 20%, $p < 0.001$; Fig. 3). The opposite was observed on D14, when larvae from the EDTA treatment were significantly more active than larvae from the control treatment ($p < 0.001$; Fig. 3).

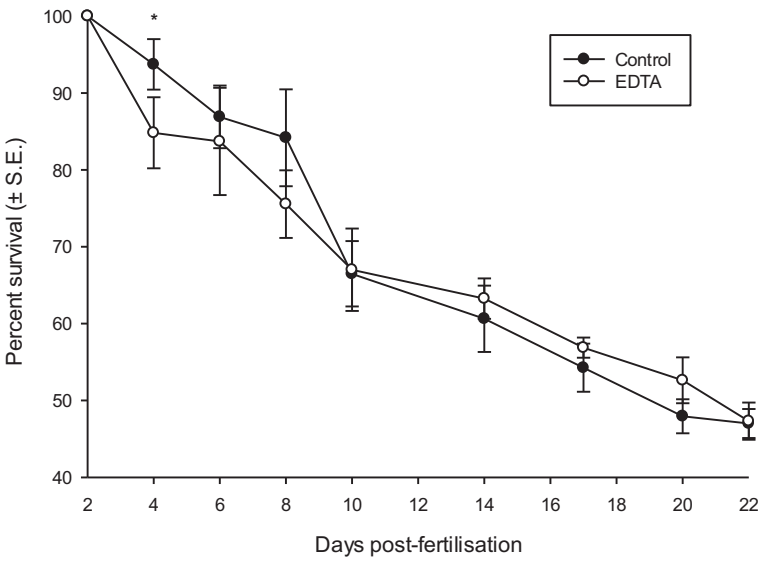

Fig. 1 Mean percent survival (± SEM, $n = 5/6$) of mussel larvae reared in EDTA-treated FSW (hollow marker) and control treatment without EDTA (solid markers) over 22 days of experimental culture. Net survival was based on the number of D-stage larvae estimated on D2. Results are adjusted to account for the larvae removed on D10 during biomass standardisation and tissue sampling for ICP-MS. Asterisk indicates a significant difference between the control treatment and the EDTA treatment ($p < 0.05$)

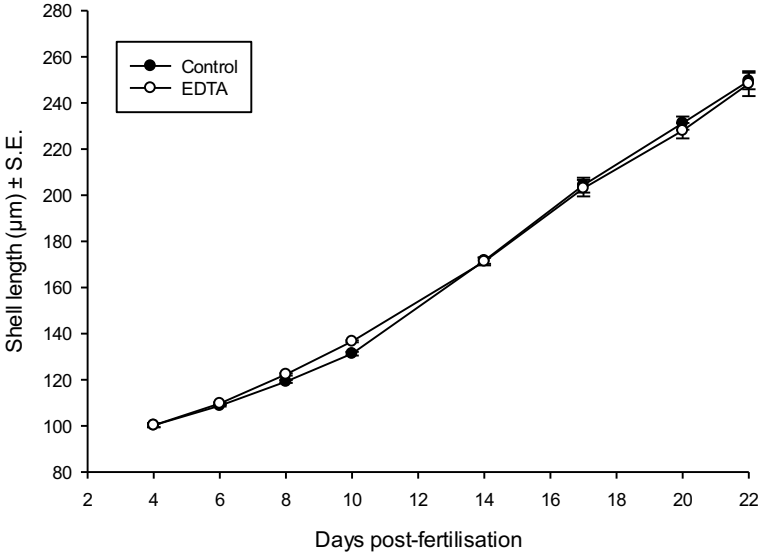

Fig. 2 Mean shell length of mussel larvae reared in EDTA treated seawater (hollow markers) and seawater without EDTA (solid markers) from D4 to D22 (± SEM, $n = 5/6$)

Feeding rate

Throughout the trial, feeding rates increased for both treatments, starting at ≈ 2000 cells/larva/day on D4 and finishing at ≈ 17,000 cells/larva/day at D22. Overall, there was no significant

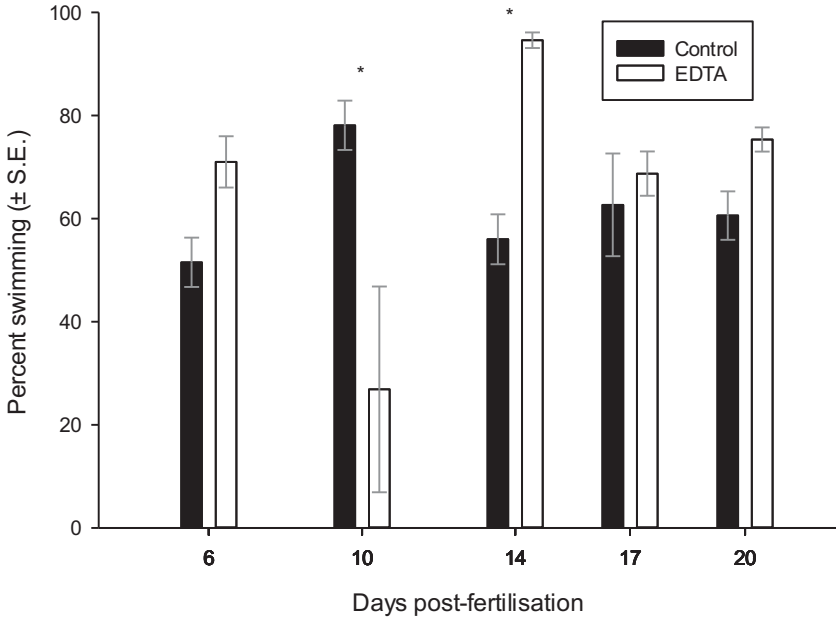

Fig. 3 Mean percentage of swimming larvae reared in EDTA-treated (white) and EDTA-free seawater (black) from D6 to D20 (± SEM, $n = 5/6$). Asterisks indicate significant differences between treatments ($p < 0.001$)

difference in the mean feeding rates between the control and EDTA treatment ($F_{1,7} = 0.701$, $p = 0.496$). However, feeding rate was significantly affected by the use of 12 μM EDTA at D20 (Tukey $q = 3.120$, $p = 0.033$). No significant differences in feeding rates were observed on any other assessment day ($p > 0.05$, Fig. 4). There was not a significant interaction between treatment and day PF ($p = 0.200$).

Metal content

In eggs (D0) and D-stage larvae (D2)

The most abundant metals in mussel eggs prior to fertilisation were iron (Fe) and zinc (Zn), with mean concentrations of 19.9 ± 4.8 μg g dry matter^{-1} and 19.6 ± 0.5 μg g^{-1}, respectively (Fig. 5). After fertilization and 48 h of incubation in FSW treated with 12 μM EDTA, mean concentrations of total chromium (Cr), cobalt (Co), nickel (Ni), cadmium (Cd) and mercury (Hg) were significantly increased in the resulting D-stage larvae ($p < 0.05$; Fig. 5). In particular, the concentration of total Cd in D-stage larvae increased from 0.009 ± 0.001 to 6.668 ± 2.895 μg g^{-1} ($t = 7.394$, $p = 0.002$). Similarly, total concentration of Hg in D-stage larvae was significantly increased from below detection limits in the eggs, to 1.00 ± 0.04 μg g^{-1} in the D-stage larvae ($t = 92.6$, $p < 0.001$: Fig. 5).

In contrast, the levels of Zn and arsenic (As) in the D-stage larvae were significantly lower than the eggs (Fig. 5, $p < 0.001$). Iron (Fe) and copper (Cu) concentrations were not affected by the use of EDTA during the 48 h incubation of mussel embryos (Fig. 5), while lead (Pb) remained below detectable levels in both mussel eggs and D-stage larvae (i.e. < 0.001 μg g^{-1}).

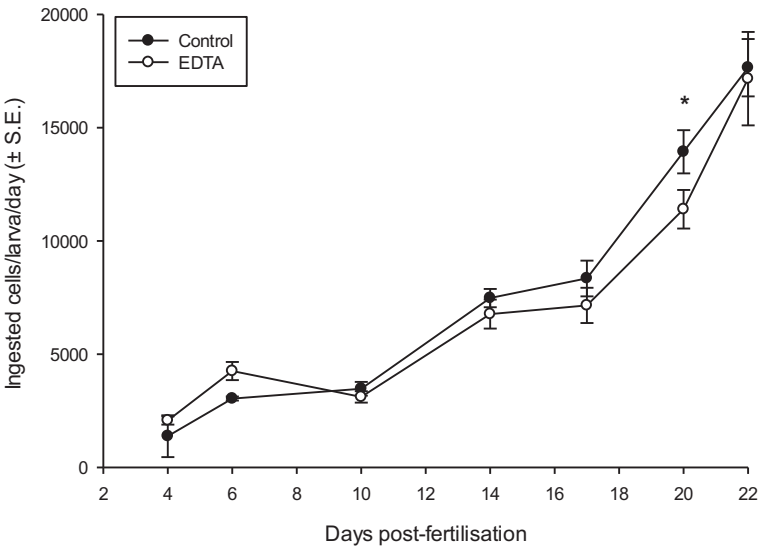

Fig. 4 Mean feeding rate of mussel larvae reared in control seawater (black) and EDTA-treated seawater (white) from D2 to D22, expressed as number of algal cells ingested per larva per day (± SEM, $n = 5/6$). Asterisk indicates a significant difference between the control treatment and the EDTA treatment on D20 ($p = 0.033$)

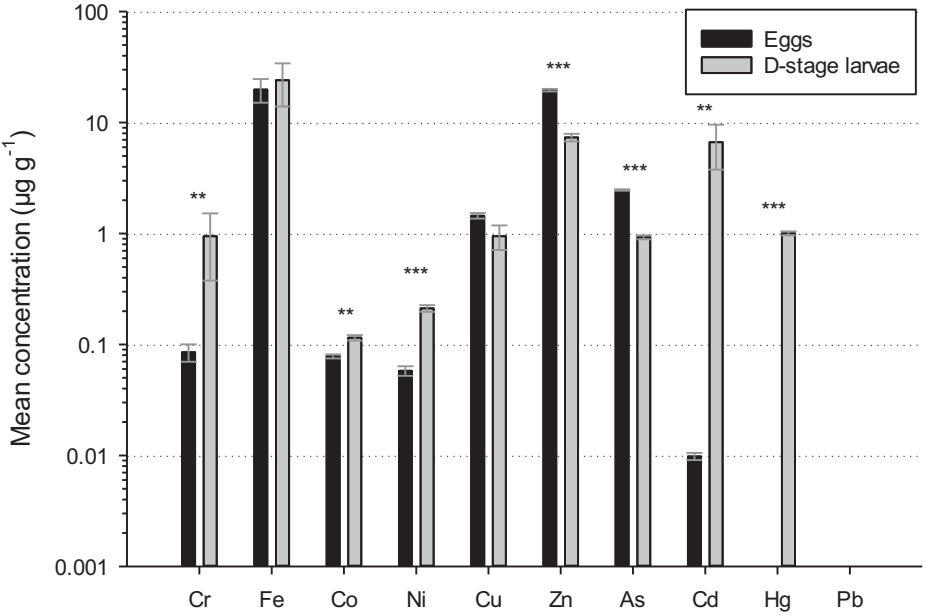

Fig. 5 Mean concentrations of chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) in unfertilized mussel eggs (black) and subsequent D-stage larvae (grey) following 48 h incubation in 12-µM EDTA-treated seawater. Values expressed in µg g^{-1} dry matter (or ppm) ± SEM ($n = 5/6$. Note: concentrations are on a log scale. Asterisks denote a statistically significant difference between eggs and D-stage larvae (Student's t test *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

In 10-day old larvae (D10)

In 10-day-old larvae reared with EDTA treatment, the mean concentration of cadmium was significantly lower than the control treatment, i.e. 0.0036 ± 0.0001 µg g^{-1} versus 0.0105 ± 0.0007 µg g^{-1} ($t = 11.707$, $p = 0.002$, Fig. 6). In contrast, the mean concentration of nickel was lower in the control treatment, i.e. 0.27 ± 0.02 µg g^{-1} versus 0.17 ± 0.04 µg g^{-1} ($p = 0.03$, Fig.6). Concentrations of the other assayed metals in the larvae from the control treatment were not different from those from EDTA-treatment ($p > 0.05$, Fig. 6).

In 22-day old larvae (D22)

Concentrations of Zn, Cd and Hg were significantly lower in the 22-day-old larvae reared with EDTA treatment compared with the control treatment ($p < 0.05$). The greatest difference was observed for Cd which had a concentration three times higher in the control treatment than the EDTA treatment, whereas the concentrations of both Zn and Hg were less than double in the control treatment.

In contrast, mean concentrations of Ni and As were significantly higher at D22 in larvae reared with EDTA treatment compared with the control treatment ($p < 0.05$). The greater difference was observed for As which had 30% higher concentration in the EDTA treatment than the control, whereas the concentration of Ni was 20% higher in the EDTA treatment.

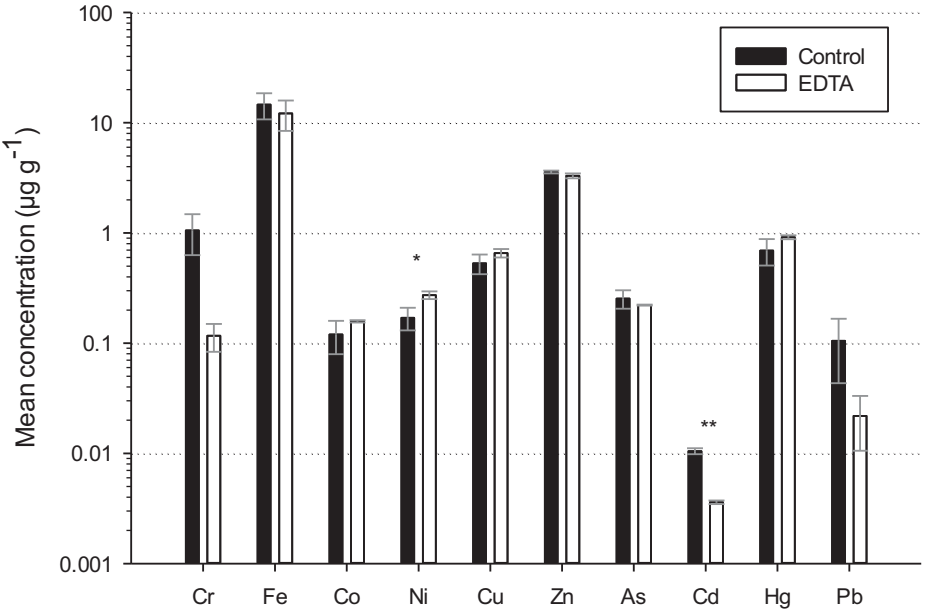

Fig. 6 Mean concentrations of chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) measured in 10-day-old larvae reared in the control treatment (black) and EDTA-treated FSW (white), expressed in µg g^{-1} dry matter (or ppm) ± SEM ($n = 5/6$). Note: concentrations are on a log scale. Asterisks denote a statistical difference compared with the corresponding control treatment (Student's t test * $p < 0.05$; ** $p < 0.01$)

Discussion

The highly effective use of the chemical EDTA is poorly understood in larval bivalve rearing, and it is not known at what point of development it is required. Greenshell™ mussel (*P. canaliculus*) provides an opportunity to further understand the role of EDTA usage in shellfish larval rearing. As for many other bivalve species, the yield of healthy D-stage Greenshell™ mussel larvae is greatly improved for the first 48 h of larval development (from fertilised eggs to prodissoconch I D-larvae) with the presence of 12 µM EDTA in the seawater (Gale et al. 2016; Helm et al. 2004; Utting and Helm 1985; Adams et al., unpublished data; McDougall et al. 2019). The current study investigated whether extending the use of 12 µM EDTA could be beneficial for subsequent larval developmental stages by experimentally rearing larval mussels with and without EDTA while comparing survival, swimming and feeding activity and shell length over the 22 days of larval development.

Results from all of these measures show that there is no major advantage to extending the use of EDTA. This is consistent with previous findings that the most vulnerable stage of shellfish larval development to metal toxicity is the first 48 h of embryogenesis, observed through higher mortality rates at that stage compared with the later stages of development (Beiras and His 1994; Connor 1972; Wang et al. 2009). For example, one- to three-day-old larvae of the European flat oyster (*Ostrea edulis*), common shrimp (*Crangon crangon*), European shore crab (*Carcinus maenas*) and common lobster (*Homarus gammarus*) all were shown to be considerably more sensitive to Cu, Zn and Hg than their respective adults, ranging from 14 to 1000 times more sensitive (Connor 1972).

The first 48 h of larval development is when the mineralisation of the shell is initiated with the deposition of mostly amorphous calcium carbonate, rather than crystalline calcite or aragonite (Medakovic 2000; Weiss et al. 2002). It is possible that larvae at this stage of development have less physio-chemical control over the acquisition of Ca for the formation of amorphous calcium carbonate shell, with an inability to control alternative metal ion incorporation resulting in toxicity effects due to substitution with non-essential metals. However, after the first 48 h, shell formation involves the deposition of crystalline calcite or aragonite, and greater selectivity of metal species may be possible.

During shellfish embryogenesis, developmental processes are vulnerable to toxicity from heavy metal ions commonly resulting in abnormalities (Coglianese and Martin 1981; Glickstein 1978), most likely caused by toxic effects such as oxidative stress and binding with vital enzymes (Gale et al. 2016; Lasseter et al. 2016; Rainbow 2002). It is likely that these toxic effects are most influential during the first 48 h of larval development.

Zn is an essential element, required by larvae as a coordination ion for a variety of proteins, including carbonic anhydrase, which is essential for shell mineralisation (Miyamoto et al. 1996). However, Zn is known to be toxic for bivalve larvae at sufficient concentrations (Brereton et al. 1973). The threshold level of these concentrations is dependent on the species and life stage of bivalve larvae (Rainbow 2002). For example, during the larval development of the Pacific oyster (*Crassostrea gigas*), increasing the concentrations of Zn in the seawater from 125 to 500 μg L^{-1} decreased rate of growth, increased occurrence of abnormalities and increased mortality rates (Brereton et al. 1973). In contrast, concentrations of Zn up to 100 μg L^{-1} have no apparent effect on the embryonic development of *C. gigas* (Watling 1982). In the present study, the growing larvae consistently accumulated less than 10 μg g dry $matter^{-1}$, implying that Zn may in fact be limiting in this system. Further investigation to determine the levels of bioavailable Zn species would therefore be valuable, when considering the benefits and impacts of EDTA enrichment.

Overall, survival of control larvae was not significantly different to the EDTA treated larvae (Fig. 1). Both treatments had roughly 50% survival at the end of the 22-day experiment, which is considered to be typical of a healthy high-density larval rearing system (Ragg et al. 2010). This suggests that any reduction in the bioavailability of any metal by EDTA had no significant biological benefit to larvae, beyond the first 48 h of development. However, if this experiment was repeated at a time where higher metal concentrations in the water is observed (e.g. following a severe rain event), one may expect EDTA to have some biological benefits to larval development beyond 48 h. It is possible that the reduced bioavailability of certain metals is not beneficial, due to their essential requirement, and this counteracts the benefit of reducing toxic metal (e.g. Cd and Hg) bioavailability.

Cadmium concentrations were high in D-stage larvae (6 ± 3 μg g^{-1}, Fig. 5,), and decreased considerably over time, resulting in much lower concentrations in 10-day-old larvae. This decrease was greater for larvae grown with EDTA treatment than the control (Fig. 6). It could be possible that EDTA binds with Cd and moves into the developing embryo during the first 48 h with it, providing protection against its toxic effects. It is known that EDTA can provide this benefit for mammals and is used medically to provide a treatment for people with Cd poisoning (Andersen 1984). As the larvae grow over the 22-day period, the Cd is most likely diluted by the increase in biomass (Fig.6 and Fig.7). Furthermore, this reduction of Cd concentration over time is improved with the presence of EDTA in the seawater throughout larval development.

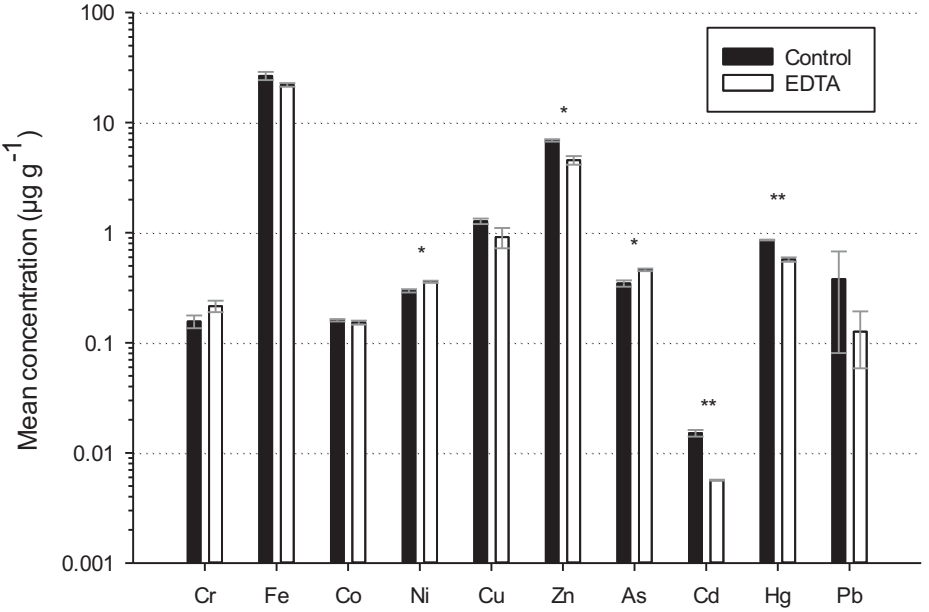

Fig. 7 Mean concentrations of chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) measured in 22-day-old larvae reared in the control treatment (black) and EDTA-treated FSW (white), expressed in µg g^{-1} dry matter (or ppm) ± SEM ($n = 5/6$). Note: concentrations are on a log scale. Asterisks denote a statistical difference compared with the corresponding control (Student's t test $*p < 0.05$; $**p < 0.01$)

Nickel and As are typically anthropogenic, non-essential elements for bivalve larvae, and arsenic is known to be highly toxic and bioavailable for developing larvae (Moreira et al. 2018; Zamble 2015). In this study, EDTA treated larvae had significantly more Ni and As than control larvae on D22 (Fig.7). This corresponds with previous results where arsenic concentration is increased for *P. canaliculus* D-stage larvae (up to 2-day PF) (McDougall et al. 2019). Therefore, another cost of using EDTA treatment of seawater for larval development is the potential to increase arsenic bioavailability. This possibility warrants further investigation and alternatives to EDTA for reducing the toxicity of metals while not increasing the bioavailability of As should also be considered.

Conclusion

The significant benefits for protecting larval mussels from heavy metal toxicity using EDTA during the first 48 h after fertilization do not continue in the later stages of larval development. Consequently, the effectiveness of using EDTA in the later stages of larval rearing of other various shellfish species reared around the world should be reviewed as it may also prove unnecessary. Eliminating EDTA use from the later stages of larval rearing would reduce production costs and reduce environmental concerns, given the non-biodegradability of EDTA. Alternatives to EDTA for reducing the toxicity of metals to shellfish should be considered such as other chelating agents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval "All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors."

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