Trace metals in New Zealand Greenshell™ mussel larvae and the effect of chelating agents on metal concentrations and larval survival

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Abstract

In many places, the natural concentrations of heavy metals in seawater are low, but with the activity of humans, such as the depositing of industrial wastewater and solid waste into the environment, increasingly soil, water, air, plants and animals are being contaminated with heavy metals. The toxicity of heavy metals commonly impacts the survival of crustacean and bivalve larvae in hatchery culture, and this has led to the widespread use of the chelating agent ethylenediaminetetraacetic acid (EDTA) to decrease this toxicity.

The research presented in this thesis improves our understanding about the known benefit of applying chelating agents to shellfish hatchery seawater, particularly at the earliest stages of development. Significant improvements in survival, which were associated with lower concentrations of several toxic heavy metals, were observed with EDTA treatment over the first 2 days of *P. canaliculus* larval development. The concentration and spatial arrangement of heavy metals in larvae was consistent with reduced bioavailability of several metals, especially copper and zinc.

Following the first study, *P. canaliculus* larvae were experimentally raised to 22 days 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 beyond the first 2 days. 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 reared without EDTA.

Since EDTA has a very poor biodegradability leading to potential persistent environmental effects, alternative methods to prevent heavy metal toxicity to shellfish larvae are needed. Ethylenediaminedisuccinic acid (EDDS) is a biodegradable potential alternative to EDTA for this application and was tested as a treatment of the seawater used for rearing aquaculture Greenshell™ mussel (*Perna canaliculus*) larval embryos in this study. Mussel embryos reared with EDTA or EDDS had significantly better survival than without. Similar improvements to those with EDTA were observed in the survival of *P. canaliculus* embryos over the first 2 days of development when reared with EDDS added to the seawater, resulting in significantly lower concentrations of zinc and higher concentrations of calcium observed in the embryos when compared to the control treatment which had no chelating agent added.

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Nature of contribution by PhD candidate	Conceiving the research ideas, planned and conducted experiment, analysed results, writing the manuscript
Extent of contribution by PhD candidate (%)	90%

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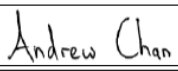
Andrew Chan		21/01/2020
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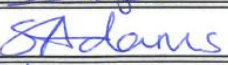
Serean Adams		2/3/20.
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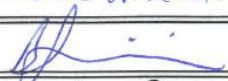
Bridget Finnie		02/03/2020
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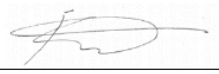
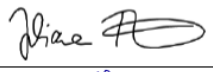
Shinji Kihara		14 Sep 2020
Juliane Reinhardt		13 Sep 2020
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1. Introduction

The experiments presented in this thesis (Chapters 2-4) are the first to investigate how the concentrations of heavy metals in *P. canaliculus* larvae are affected by treatment of seawater with chelating agents. Earlier studies investigated the effect of chelating agents on concentrations of heavy metals in other species of aquaculture shellfish, such as common mussels (*Mytilus* spp.) (George & Coombs, 1977), and shrimp (*Penaeus monodon*) (Lawrence et al., 1981). In particular, these studies saw clear effects of the chelating agent on zinc regulation and uptake (Nugegoda & Rainbow, 1988). As a result the addition of chelating agents to seawater used to rear larval shellfish became a standard protocol in many aquaculture hatcheries throughout the world to improve production by reducing the toxicity of heavy metals (Helm, 2004). Shortly after the establishment of a significant commercial green-lipped mussel hatchery near Nelson in 2015, it was quickly ascertained that the addition of EDTA greatly improved the production of mussel spat at the facility. It was found that early larval *P. canaliculus* benefitted in a similar manner to other bivalves with significant improvements in larval survival and a reduction of deformities and disrupted development. On many occasions *P. canaliculus* embryos fertilised in the hatchery would not survive at all without the addition of EDTA.

The question was first posed as to what role EDTA provides in this improvement for *P. canaliculus* by Gale et al. (2016), published a year prior to the start of the research for this PhD. Globally the mechanism of action of EDTA leading to benefits for shellfish has had little or no investigation, and it was believed that *P. canaliculus* would provide an excellent case study for investigating the mechanism of action of EDTA and its benefits for shellfish larval rearing in general. In the study by Gale et al. (2016), fertilised *P. canaliculus* eggs were reared for 2 days with and without 25 µM EDTA and the levels of oxidative stress in the developing embryos were measured. Significantly more oxidative stress was found in embryos grown without EDTA than with EDTA. The most common triggers of oxidative stress in living cells are raised levels of heavy metals, in particular iron and copper (Guerin et al., 2001). When these metals are present in the free ionic form ($Fe^{2+/3+}$, Cu^{2+}), they are inducers of reactive oxygen species (ROS) formation in living cells via the Fenton and Haber Weiss reactions (Guerin et al., 2001). However, it remained unknown whether the concentrations of heavy metals including iron and copper were affected, highlighting an important missing piece of information in the

understanding of the mechanism of action of EDTA for the benefit of developing shellfish embryos.

1.1 Effect of heavy metals on shellfish

Heavy metals (defined here as metals with a greater atomic weight than calcium) occur naturally in seawater at low levels, although in some locations these levels may be elevated naturally. For example, comparatively high levels of cadmium compared to the global seawater average are found in the seawater of Foveaux Strait and Tasman Bay (Nielsen, 1975). Heavy metal concentrations may also be elevated as a result of human activities, including mine drainage, industrial wastewater, storm water runoff, or contamination from antifouling paints used on boat hulls (Jarup, 2003; Brown & Peake, 2006; Fu & Wang, 2011; Wicke et al., 2012). In many places, the natural levels of heavy metals in seawater are low, but with the activity of humans, such as the depositing of industrial wastewater and solid waste into the environment, increasingly soil, water, air, plants and animals are being contaminated with heavy metals (Cheng, 2003). Typically the most toxic form of heavy metals is the free ionic (unbound) state, however, there are some exceptions (e.g., arsenic and mercury) which when bound with organic material, prove to be considerably more toxic (Leermakers et al., 2005; Y. Wang et al., 2015).

Heavy metals are not biodegradable and tend to accumulate in organisms, frequently causing toxic and/or carcinogenic effects. For example, traces of free metal cations such as $Fe^{2+/3+}$ and $Cu^{+/2+}$ are inducers of reactive oxygen species (ROS) formation in living cells via the Fenton and Haber-Weiss reactions, shown below (Guerin et al., 2001; Gale et al., 2016).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot \text{ (Fenton Reaction)} \quad (1.1)$$

$$Fe^{3+} + O_2^{-}\cdot \rightarrow Fe^{2+} + O_2 \quad (1.2)$$

$$\text{Net Reaction: } H_2O_2 + O_2^{-}\cdot \rightarrow O_2 + OH^- + OH\cdot \text{ (Haber-Weiss Reaction)} \quad (1.3)$$

The presence of unbound heavy metal ions, whether in the environment or an organism's diet, can interfere with shell and bone formation (i.e., through interfering with bio-mineralisation) in living organisms (Stromgren, 1982; Sunila & Lindstrom, 1985; Yap et al., 2002; Lopes-Lima et al., 2012; Goto & Sasaki, 2014).

Highly controlled bio-mineralisation of calcite and aragonite (polymorphs of $CaCO_3$) is achieved by molluscs, through the use of shell proteins which direct the formation of $CaCO_3$ (Marin et al., 2008). Unbound heavy metal ions are believed to cause problems for bio-

mineralisation through incorporation into the shell of molluscs as they are constructed. However, the process by which molluscs form their shells is poorly understood, and hence the nature by which the heavy metals are incorporated is uncertain (Lasseter et al., 2016).

Unbound heavy metal ions are also thought to affect the function of carbonic anhydrase, one of the key enzymes involved in the formation of molluscan shells (Zhang & Zhang, 2006). Carbonic anhydrase is a metalloenzyme that usually features a Zn^{2+} ion in the active site and catalyses the reaction of carbon dioxide and water to form bicarbonate and protons (or the reverse). This family of enzymes is common among animals, not only for the formation of shell, but also for removing carbon dioxide from tissues and maintaining pH balance in the body. In molluscs, this enzyme is crucial for conversion of CO_2 to HCO_3^- which can then dissociate to form CO_3^{2-}:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \quad (1.4)$$

$$HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \quad (1.5)$$

If the localised concentration of CO_3^{2-} is increased, then the rate of $CaCO_3$ mineralisation will increase due to super-saturation:

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3\ (s) \quad (1.6)$$

Cd^{2+}, Co^{2+}, and Ag^+ ions competitively inhibit carbonic anhydrase, by competing for the Zn^{2+} binding site (Vitale et al., 1999; Soyut et al., 2008). In contrast, Cu^{2+} can non-competitively inhibit carbonic anhydrase. High activities of carbonic anhydrase were observed prior to shell formation during the development of blue mussel (*Mytilus edulis*) larvae (Medakovic, 2000). Therefore, if heavy metal ion concentrations are high enough, they can indirectly affect the bio-mineralisation process in mussel larvae, through inhibiting the function of carbonic anhydrase.

Different heavy metals are known to have various toxic effects at different concentrations depending on the species of shellfish (Martin et al., 1981; Ramachandran et al., 1997; His et al., 1999; Rainbow, 2002). For example, the order of toxicity in terms of mortality is Hg>Ag>Cu>Ni for larvae of the eastern oyster (*Crassostrea virginica*) (Calabrese et al., 1977) compared to Hg>Cu>Ag>Ni for larvae of the hard clam (*Mercenaria mercenaria*) (Calabrese & Nelson, 1974) and embryonic development of *M. mercenaria* is much more sensitive to increased levels of Pb than *C. virginica*. These metals affect the growth (i.e., causing abnormalities, and slowed growth rate) and the survival of the larvae.

A number of studies have examined the toxicity effects of heavy metals in bivalve sperm, eggs, and larvae. During the larval development of the Pacific oyster (*Crassostrea gigas*), increasing the concentrations of zinc in the seawater from 125 to 500 µg L^{-1} resulted in decreased growth, and increased occurrence of abnormalities and mortality rates (Brereton et al., 1973). Increased levels of mercury (5.7 µg L^{-1}), silver (16 µg L^{-1}), and copper (10 µg L^{-1}) in seawater interferes with embryonic development of larvae of both *C. gigas* and the Mediterranean mussel (*Mytilus galloprovincialis*) (Glickstein, 1978; Coglianese & Martin, 1981; Beiras & His, 1994, 1995b). In contrast, concentrations of zinc and cadmium up to 100 µg L^{-1} have no apparent effect on the embryonic development of *C. gigas* (Watling, 1982). Finally, delayed larval settlement of *C. gigas* occurs in the presence of zinc at concentrations of 125 µg L^{-1} and above (Boyden et al., 1975).

In addition to problems for oysters, respiration in the sperm and eggs of the blue mussel (*Mytilus edulis*) is inhibited by increased levels of copper and zinc in seawater (Akberali et al., 1985). This is suspected to occur through inhibition of respiration in the mitochondria, as zinc inhibition has been found to inhibit mitochondrial respiration in other tissues of adult *M. edulis*, such as the mantle and digestive gland (Akberali & Earnshaw, 1982). Copper interferes with the development from unshelled trochophore to shelled veliger larvae of *M. edulis* at concentrations of 20 µg L^{-1} and above (Beaumont et al., 1987). Chances of survival and appearance of developmental abnormalities in *M. edulis* embryos are affected in the presence of elevated copper concentrations (8 µg L^{-1}) but this effect varies among localised populations of this mussel, indicating that there may be a genetic influence on copper toxicity within this species (Hoare, Beaumont, et al., 1995). However, copper present at 8 µg L^{-1} concentration has no significant effects on survival or shell growth once *M. edulis* veliger larvae are fully developed and as they transition to post-larval stages (Hoare, Davenport, et al., 1995).

Veliger larval development in terms of shape and pattern of cellular cleavage of the eastern mudsnail (*Ilyanassa obsoleta*), is affected by the presence of heavy metals including Hg, Cu, Zn, Cd, Pb and Cr at concentrations of at least 20 µg L^{-1} (Conrad, 1988). Cell division and growth of fully developed *I. obsoleta* larvae is retarded at concentrations of metal too low to cause mortality, through interference with polar body formation during cytokinesis (Conrad, 1988).

The results from these various studies of bivalve sperm, eggs, embryos and larvae point to a number of heavy metals being responsible for toxic effects that vary by bivalve species, developmental stage, and the concentrations of the heavy metals. Further to the importance of

the concentrations of heavy metals, toxic effects are also dependent on amounts of dissolved organic carbon (DOC), salinity, temperature and pH of the seawater. For example, the toxicity of Ni, Cu, Zn and Cd for bay mussel (*Mytilus trossulus*) larvae is reduced with increased amounts of dissolved organic carbon (DOC) in seawater (Nadella et al., 2009; Deruytter et al., 2015). This is thought to be due to the complexation of the unbound heavy metal ions with the DOC's, resulting in their decreased bioavailability.

Furthermore, the toxicity of Ni, Cu, Zn and Cd for mollusc larval development increases with changes in salinity and temperature (i.e., both increases and decreases), although it is not fully understood why this is the case (MacInnes & Calabrese, 1979; Coglianese, 1982; Robert & His, 1985; McLusky et al., 1986; Deruytter et al., 2015). Theoretically, an increase in cation concentration associated with elevated salinity should increase competition with heavy metal ions and decrease their toxicity. It seems likely that a change in larval physiology rather than a change in speciation or ion competition in the solution is causing the observed differences in toxicity of heavy metal ions at different salinities. These changes could include changes in the transcellular Na^+ gradient, and/or changes in the haemocyanin concentration and osmolarity of the haemolymph (Deruytter et al., 2015). Additionally, higher temperatures generally result in an increased solubility of metal ions, and a greater movement of solutes across cell membranes (Cairns et al., 1975), however, it was observed that the toxicity (i.e., the rate of abnormalities and survival) caused by copper for embryos and early larvae of *C. virginica* is higher at a seawater temperature of 20 °C than 30 °C (MacInnes & Calabrese, 1979).

As ocean acidification occurs as a result of climate change the bioavailability of metals in naturally occurring seawater can be expected to increase (Millero et al., 2009). For example, a study over a range of seawater pH consistent with current and anticipated changes due to climate change found significant decreases in the larval development success of *Perna perna* which was associated with increased bioavailability of heavy metal ions at reduced pH (Szalaj et al., 2017).

Collectively, these results suggest the mechanism of toxicity of heavy metal ions in shellfish is complex and dependent on a large variety of factors other than their concentration, and that for experimental purposes, it is important to monitor or control factors such as DOC's, salinity, pH, temperature and other factors where possible. Generally, the stages of shellfish development that are most vulnerable to increased levels of heavy metal ions are the embryonic and larval development stages (Calabrese et al., 1977; Ringwood, 1990). Furthermore, the

sensitivity of individual shellfish species to heavy metals can differ markedly, however, the mechanisms by which metals induce toxic effects could be expected to have similarities.

1.2 Concentrations of heavy metals found in seawater

Heavy metals in seawater vary in concentration with location and they exist in many different chemical states, including coordination with different chemical species (Sohrin & Bruland, 2011). Typically, seawater closer to urban areas, such as in marinas and harbours, has a higher concentration of heavy metals due to anthropogenic activities. For example, seawater sampled from the North Sea between the coast of the Netherlands and the coast of England, showed that concentrations of dissolved metals (Cu, Cd, Ni, Zn and Fe) was high near the Netherlands coast, decreasing in the seaward direction, then increasing to the highest measured levels upon reaching the coast of England (Nolting, 1986). A similar phenomenon has been observed at many other locations (Table 1.1. Concentrations of heavy metals in seawater from various locations.Table 1.1).

Table 1.1. Concentrations of heavy metals in seawater from various locations.

Metal	**Location**	**Concentration ($\mu g\ L^{-1}$)**	**Reference**
Copper	Pacific Ocean ~7 km off Otago coast	0.136	(Croot & Hunter, 1998)
	Pacific Ocean ~30 km off Otago coast	0.0438	(Croot & Hunter, 1998)
	Tasman Sea - marine	0.06	(Batley 1995[a])
	Ireland - harbour	2.2 - 18.4	(Clancy *et al*. 1987[b])
	Sweden - marina	0.69 - 3.83	(Ohrn 1995[b])
	Greece - harbour	0.45 - 20.70	(Dassenakis *et al*. 1996[b])

	France - harbour	1.0 – 10.0	(Alliot & Frenet-Piron 1990[b])
	North Sea – England to Netherlands	0.235 – 0.864	(Nolting, 1986)
	Coast of New South Wales, Australia	0.025 – 0.036	(Apte et al., 1998)
	a – as reported in (Macleod & Eriksen, 2009) b – as reported in (Hall & Anderson, 1999)		
Zinc	North Atlantic Ocean	0.065 – 0.124	(Yeats 1988[c])
	NE Pacific Ocean	0.23 – 0.40	(Yeats 1988[c])
	SW Pacific Ocean	<0.04	(Batley 1995[c])
	North Sea[d]	0.196 - 4.710	(Nolting, 1986)
	c - as reported in (WHO, 2001) d - between England and Netherlands		
Iron	Pacific Ocean – surface ~7 km off Otago coast	0.236	(Croot & Hunter, 1998)
	Pacific Ocean – surface ~30 km off Otago coast	0.0575	(Croot & Hunter, 1998)
	North Sea[d]	0.614 – 5.580	(Nolting, 1986)
Cadmium	Pacific Ocean – surface ~7 km off Otago coast	0.00127	(Croot & Hunter, 1998)
	Pacific Ocean – surface ~30 km off Otago coast	0.00246	(Croot & Hunter, 1998)
	North Sea[d]	0.0157 – 0.153	(Nolting, 1986)

	Coast of New South Wales, Australia	0.0015 – 0.005	(Apte et al., 1998)

1.3 Concentrations of heavy metals found in shellfish

Mussels, as well as other shellfish, have been used as bio-accumulators for detecting the presence of heavy metals in both freshwater and seawater for over 40 years (Nielsen & Nathan, 1975; Phillips, 1976; Boening, 1999; Huang et al., 2007). This is because the shellfish often accumulate these metals, leading to higher concentrations in the flesh and shells than are present in their aquatic or sediment environment. This phenomenon has led to a quantification of the ability of individual species to bioaccumulate metals, known as the metal bioconcentration factor (BCF) (Neely, 1979). The BCF is the concentration of a chemical within an animal divided by the concentration found externally (ANZECC/ARMCANZ, 2000). This factor allows us to estimate the relative extent of bioaccumulation of heavy metals in individual species, and to compare this bioaccumulation for different heavy metals.

An attempt to establish a baseline level for heavy metal concentrations in molluscs around New Zealand found the concentration of heavy metals was highly variable depending on the mollusc species (Nielsen & Nathan, 1975). For example, a study of the concentrations of copper observed in several mollusc species sampled from a variety of locations around New Zealand found they varied by as much as two orders of magnitude (Nielsen & Nathan, 1975) (Table 1.2).

Table 1.2. Concentrations of copper in the flesh of various New Zealand molluscs (Nielsen & Nathan, 1975)[a], (Peake et al., 2006)[b], (Peake et al., 2010)[c]

Species	**Range of concentration ($\mu g\ g^{-1}$)**
Pacific oyster[a] *Crassostrea glomerata*	4.4 - 380
Bluff oyster[a] *Ostrea chilensis*	1.0 - 41.0
Green-lipped mussel[a] *Perna canaliculus*	0.2 - 28.0
Mediterranean mussel[a] *Mytilus galloprovincialis*	1.7 - 18.0
Toheroa[a] *Paphies ventricosa*	1.1 - 2.0
Pipi[a] *Paphies australis*	0.7 - 1.3
Tuatua[a] *Paphies subtriangulata*	1.4 - 8.7
NZ ribbed mussel[a] *Aulacomya maoriana*	3.3 - 19
NZ cockle[b] *Austrovenus stutchburyi*	3.0 – 60.0
Common NZ scallop[c] *Pecten novaezelandiae*	6.0 – 9.0

Zinc and copper are biologically essential metals in shellfish, with zinc commonly coordinated by proteins such as carbonic anhydrase (Lindskog, 1997) and copper coordinated with the oxygen transport protein hemocyanin (Van Holde et al., 1992). Hence, their measured concentrations tend to be higher than other metals (Table 1.3).

Table 1.3. Concentrations of various metals previously recorded in the flesh of adult *P. canaliculus.* (Chandurvelan et al., 2015)

Metal	**Range of concentration ($\mu g\ g^{-1}$)**
Arsenic	5 - 28
Cadmium	0.5 – 1.7
Copper	2 - 18
Lead	0.1 – 1.5
Nickel	0.1 – 11.8
Zinc	45- 148

Decreasing the bioavailability of the heavy metals in seawater should result in decreases in the bioaccumulation of the heavy metals for shellfish, and mitigation of their toxicity. The main method currently used to decrease heavy metal bioavailability is chelation. Natural levels of metals in some locations can cause problems for shellfish production, leading to the addition of chelating agents to tank seawater, particularly at the most vulnerable early stages of shellfish development as a means to arrest heavy metal toxicity. The most widely used chelating agent worldwide for any application is ethylenediaminetetraacetic acid (EDTA), and from an examination of the available literature it appears to be the only chelating agent that has been used for shellfish production that consistently provides significant benefits.

1.4 Use of EDTA in global shellfish production

Ethylenediaminetetraacetic acid (EDTA) forms octahedral complexes with metal ions through its N and O^- entities (Lopezalcala et al., 1984) (Figure 1.1).

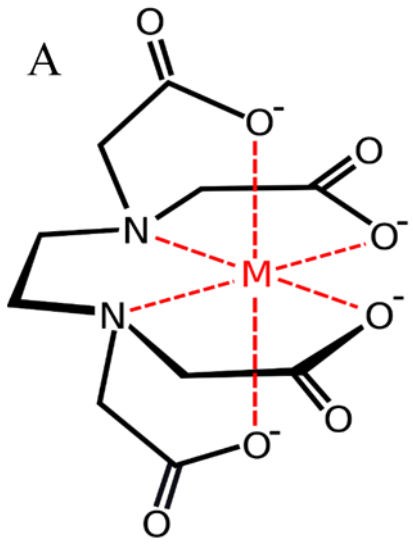
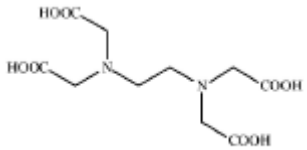
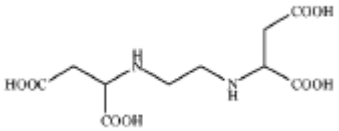
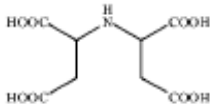
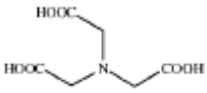


Figure 1.1 EDTA in complex with a metal ion.

It has long been known that EDTA can be added to certain environments to chelate with heavy metal ions and that this can benefit organisms that otherwise when exposed to the metals would experience toxic effects (B. Sun et al., 2001). The pK_a values of EDTA are such that at the pH of seawater, ~8.0, EDTA will be present in predominately the mono-protonated state, $EDTA^{3-}$ (Dawson et al., 2002) (Table 1.4).

Table 1.4. Structures and pK_a values of EDTA and related chelators (Dawson et al., 2002)

Abbreviation	**Full name**	**Structure**	**pK_a values**
EDTA	ethylenediaminetetraacetic acid		$pKa_1 = 2.0$ $pKa_2 = 2.7$ $pKa_3 = 6.7$ $pKa_4 = 10.3$
EDDS	ethylenediaminedisuccinic acid		$pKa_1 = 2.4$ $pKa_2 = 3.9$ $pKa_3 = 6.8$ $pKa_4 = 9.8$
IDSA	iminodisuccinic acid		$pKa_1 = 3$ $pKa_2 = 3.8$ $pKa_3 = 4.8$ $pKa_4 = 10.12$
NTA	nitrilotriacetic acid		$pKa_1 = 1.9$ $pKa_2 = 2.5$ $pKa_3 = 9.73$

EDTA is widely used in industry, medicine (Vaara, 1992), cosmetics, and laboratory applications for its chelation properties, low cost and low toxicity (Oviedo & Rodriguez, 2003) (Figure 1.2).

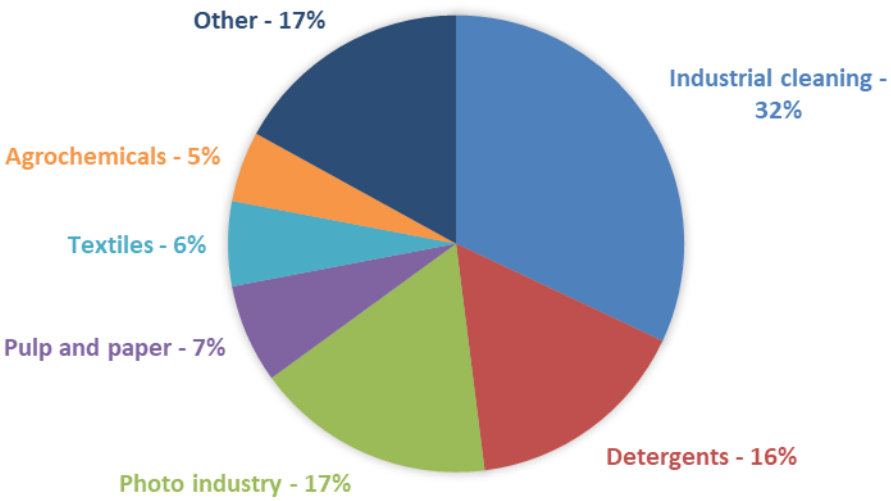


Figure 1.2. Range of uses of EDTA. Adapted from (Nowack & VanBriesen, 2005).

With such widespread use of EDTA, there are concerns about whether it has the potential to be a persistent organic pollutant because the degradation of EDTA is slow, and occurs mainly abiotically in the presence of sunlight (Bucheli-Witschel & Egli, 2001). EDTA is nontoxic for mammals and it has been used for decades in cosmetics and pharmaceuticals with no adverse effects on humans, but its strong ability to complex metals, along with its persistence in the environment, can result in changes in natural metal speciation when present in the environment (Pinto et al., 2014). For example, the increased presence of chelating agents in water increases the solubilisation of metals from sediments and soils (Nowack & VanBriesen, 2005), which in turn will increase the potential for heavy metals to exert their toxic effects.

There is potential to use microorganisms in wastewater and sewage treatment plants to degrade EDTA (Kaluza et al., 1998) as this has been found to work at a laboratory scale (Tucker et al., 1999). Alternatively, there has been growing interest in advanced oxidation processes, however, this approach has not been scaled up to practical application at a waste management scale, due to several technical and economic reasons (Sillanpää et al., 2011). If EDTA was replaced with readily biodegradable alternatives, these waste treatment processes would not be required. The long-term effects of using EDTA worldwide, with it being a persistent organic pollutant, provides an incentive to look for more biodegradable alternatives for its current

applications. A search for alternatives could include the use of EDTA treatment of seawater in shellfish hatcheries, to ensure that the used seawater subsequently discharged into the environment as waste has minimal environmental impact (Oviedo & Rodriguez, 2003; Sillanpää, 2005). Alternatives to EDTA used in many industrial applications may be suitable for seawater application, however, their effectiveness remains to be determined.

Where heavy metal ions in seawater cause problems for the breeding of shellfish and production of their juveniles in shellfish hatcheries, EDTA is commonly used to reduce the bioavailability of the metals, and thereby their toxicity to larvae (Lawrence et al., 1981; Utting & Helm, 1985; Nicula et al., 2011; Gale et al., 2016). For example, the bioavailability of copper in seawater is related to the concentration of the free Cu^{2+} ions rather than the total copper concentration (Sunda & Guillard, 1976; Zamuda & Sunda, 1982; Florence et al., 1992). Due to its charge at a pH of ~8.0, $EDTA^{3-}$ is highly soluble in seawater and since EDTA chelates cations, its addition to seawater reduces the bioavailability of the metal ions, which prevents the toxic effects these metal ions would otherwise cause to shellfish larvae.

EDTA addition at concentrations of up to 20 µM has been shown to reduce toxicity and abnormalities in oyster embryos, larvae and adults (*Crassostrea gigas, Crassostrea virginica*), mussel embryos, larvae and adults (*M. edulis*) shrimp embryos and larvae (*Penaeus stylirostris*), and adult barnacles (*Semibalanus balanoides*) caused by copper, zinc, cadmium, and manganese (George & Coombs, 1977; Rainbow et al., 1980; Castille & Lawrence, 1981; Knezovich et al., 1981; Lawrence et al., 1981; Hung, 1982; Nugegoda & Rainbow, 1988), and it is thought that EDTA plays a similar role for New Zealand green-lipped mussel (*Perna canaliculus*) embryos (Gale et al., 2016).

Disodium EDTA is added to seawater at 4 mg L^{-1} (12 µM) to greatly improve larval production in the commercial green-lipped mussel (*P. canaliculus*) hatchery in Nelson, New Zealand, usually to 100% survival yield of 2-day-old *P. canaliculus* D-larvae from fertilised eggs. Previously the concentration used was lower (3 µM), however, after years of trials it was determined that the currently used concentration works best to consistently provide better survival outcomes (Dr Rodney Roberts, SPATnz Ltd, pers. comm.). An experiment with a further increased concentration of EDTA added to the seawater (25 µM), resulted in 78.6% survival of D-larvae at 2 days old (Gale et al., 2016), suggesting there may be drawbacks from using higher concentrations of EDTA. However, at the higher concentration the survival of larvae was still significantly improved compared to only 1.2% survival of larvae without EDTA, and there was less oxidative stress observed in larvae grown with EDTA (Gale et al.,

2016). To ensure this research is industrially relevant, where survival yield is the most important benefit of adding EDTA for hatchery production, concentrations of EDTA of 3 µM and 12 µM were used in this current research project.

In order for a more acceptable alternative chelating agent to have potential for use in a shellfish hatchery it needs to be; 1) biodegradable 2) able to chelate *toxic* metals and benefit the growth of shellfish through decreasing heavy metal toxicity with the same efficiency or better as EDTA, 3) cost effective.

1.5 Potential alternatives to EDTA

Methods for reducing the effect of unbound heavy metal ions in water include processes such as chelation, chemical precipitation, membrane filtration, electrochemistry, and adsorption to surfaces (Fu & Wang, 2011).

1.5.1 Chelating agents

The best alternative chelating agents for potential commercial application in reducing the bioavailability of heavy metal ions in seawater currently include ethylenediaminedisuccinic acid (EDDS), iminodisuccinic acid (IDSA), and nitrilotriacetic acid (NTA). All of these agents have been found to successfully complex with metal ions to varying degrees (Tandy et al., 2004; Pinto et al., 2014), and are already used in selected industrial applications with the aim of reducing the environmental impact of EDTA (Pinto et al., 2014).

The common feature of the molecular structure of these chelating agents is that they have one or more nitrogen centres surrounded with branches of carboxylic acid groups. Each nitrogen centre has a lone pair of electrons and at the pH of seawater the carboxylic acid groups have a negative charge allowing the whole molecule to coordinate around a single positively charged metal ion. Different metals have different sizes and different charge densities which results in a variation of efficiency for this coordination with the chelating compound and hence the variation in the coordination efficiency of these ligands for different metal ions. The chelating abilities of these ligands are also dependent on pH and their pK_a values.

At the pH of seawater, around 8.0, the abilities of these ligands for chelation with metal ions are different, and some of these chelating agents, such as EDDS, are better at binding with certain metals than EDTA (Tandy et al., 2004). Furthermore, some chelating agents are more

biodegradable than others through naturally occurring microbial degradation (Bucheli-Witschel & Egli, 2001).

EDDS

Ethylenediaminedisuccinic acid (EDDS) is a readily biodegradable structural isomer of EDTA and it has been increasingly replacing EDTA in soil washing and phytoextraction of heavy metals as well as many other applications (Tandy et al., 2006; Leštan et al., 2008; Fine et al., 2014). The [S,S] isomer was shown to be the only fully and practically degradable isomer of EDDS when evaluated using several biodegradability tests (Schowanek et al., 1997). EDDS is produced naturally by a number of microorganisms such as *Amycolatopsis japonicum* (Nishikiori et al., 1984; Goodfellow et al., 1997). The typical method for industrial production is a one step process involving the addition of NaOH to 2-aminosuccinic acid in the presence of 1,2 dibromoethane (Figure 1.3) (Neal & Rose, 1968).

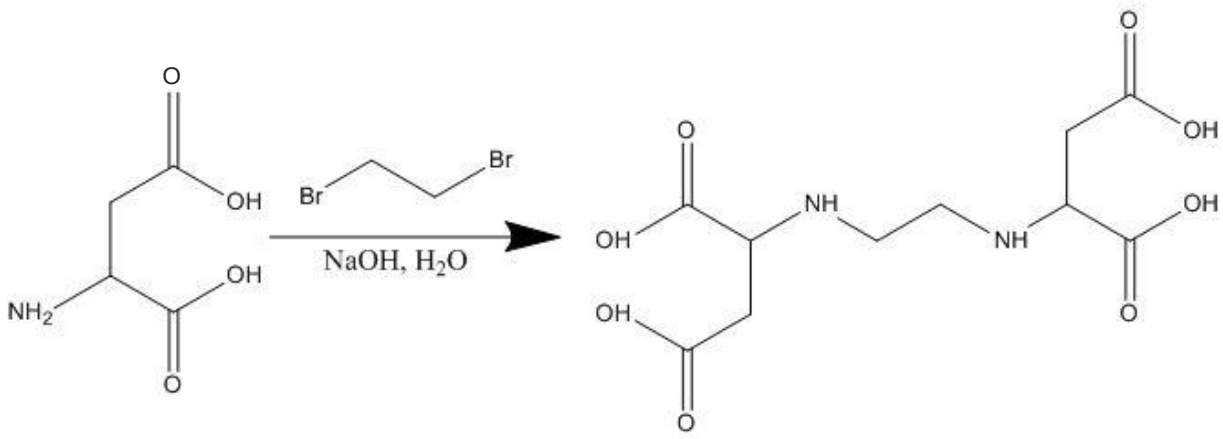


Figure 1.3. Reaction scheme for production of [S,S] EDDS. (Neal & Rose, 1968)

During the process of making paper, EDTA is used to improve the quality of the end-product by inhibiting the generation of radicals at the hydrogen peroxide bleaching stage by chelation with Mn, Fe and Cu ions. However, there tends to be high quantities of Ca in the process which the EDTA also binds with. This has parallels with the use of EDTA in seawater in hatcheries, where it is added to alleviate the effects of toxic transition metal ions, but it can also interact with useful ones such as Ca^{2+}. Simulated modelling has been used effectively to elucidate the comparative efficiency of EDTA and EDDS for the paper making process (Jones and Williams (2001). The modelling revealed that at a pH of 8, EDDS was far superior to all other chelating agents as it chelated with 100% of the transition metal ions and avoided complexing with Ca^{2+}

more than EDTA. This finding suggests EDDS would have good potential for use in shellfish hatchery application, because it could reduce the concentration of free and unbound toxic metal ions such as Cu^{2+}, while leaving more Ca^{2+} unbound and available for $CaCO_3$ shell formation by the organisms.

EDDS is better at removing Cu^{2+} and Zn^{2+} ions from soil solution than EDTA at a pH of 7, but less effective for lead ions, due to the different complexation efficiencies (Tandy et al., 2004). The stability (based on equilibrium constant logK, Figure 1.4) of EDDS-Cu complexes at a pH of 8 are as good as EDTA-Cu complexes (Orama et al., 2002). The higher the stability constant is, the more likely the metal ions are to be bound by the chelating agent. If Cu^{2+} proves to be the main free metal ion causing problems for development of *P. canaliculus* for developing D-larvae then EDDS would be a good potential alternative to EDTA in shellfish hatcheries.

The formation of a metal-chelator complex is an equilibrium in solution. A conditional formation constant for ML being formed by reaction between a metal, M, and a ligand, L, is defined in the following equation:

$$K_{ML} = \frac{[ML]}{([L]total-[ML])([M]total-[ML])} \quad (1.7)$$

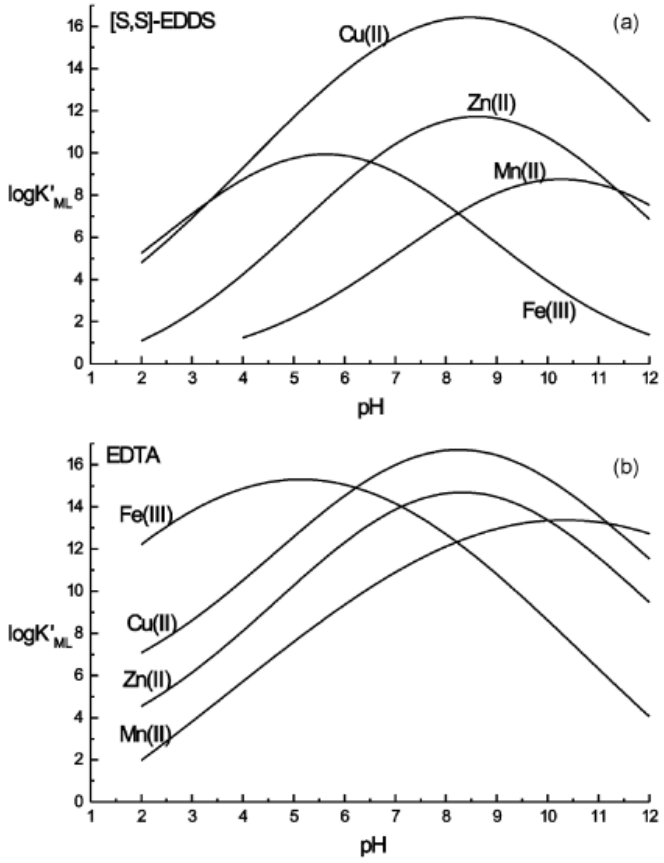


Figure 1.4. Conditional formation constants for metal-chelator complexes versus pH for EDDS (a) and EDTA (b) Source: (Orama et al., 2002).

Analytical grade EDDS is commercially available in a pure form from Sigma Aldrich in solution (concentration 35% w/w) for $184 per 100 mL. However, it is also commercially available in larger quantities for industrial use under the trademark Enviomet^{TM} by Innospec Inc. at a lower cost.

IDSA

Another chelating agent with increasing interest in its applications is iminodisuccinic acid (IDSA) (Zhao et al., 2010) which has been commercialised since 1998 (Kolodynska, 2011a). It contains one nitrogen instead of two and this decreased nitrogen content has been proposed to make IDSA more biodegradable than EDDS (Pinto et al., 2014). The synthesis of IDSA is also generally considered to be environmentally friendly with no generation of off-gases or effluents (Kolodynska, 2011a).

IDSA has been shown to be a competitive alternative to EDTA in terms of its chelating ability for the heavy metals Cu, Zn, Mn, and Fe in solution (Hyvonen et al., 2003). Furthermore, IDSA has been used in solution to chelate metal ions and improve adsorption rates onto chitosan (Kolodynska, 2012). However, IDSA is not as efficient as EDDS or EDTA for chelation of heavy metals in solution, because IDSA does not form as stable metal complexes (Kolodynska, 2012).

Production of IDSA is very straightforward through a two-step process required for manufacture (Figure 1.5), and production is likely to increase in the future with concomitant increase in availability and decrease in cost (Kolodynska, 2011a).

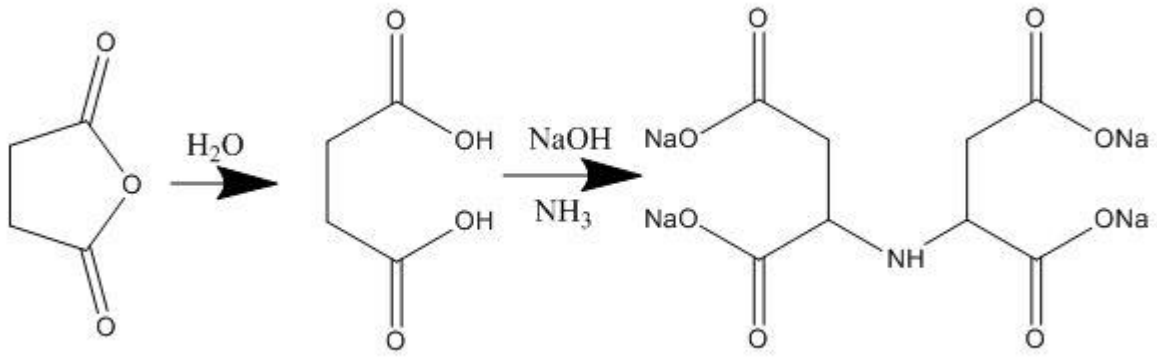


Figure 1.5. Reaction scheme for production of IDSA from succinic anhydride

IDSA is currently commercially available under the name of Baypure CX 100 from Lanxess Aktiengesellschaft at a cost of NZ $16 per kg (Pers. Comm., Bruce Holmes, Lanxess Aktiengesellschaft).

NTA

Nitrilotriacetic acid (NTA) is the oldest of the synthetic chelating agents mentioned here, first produced in the 19th century (Figure 1.6).

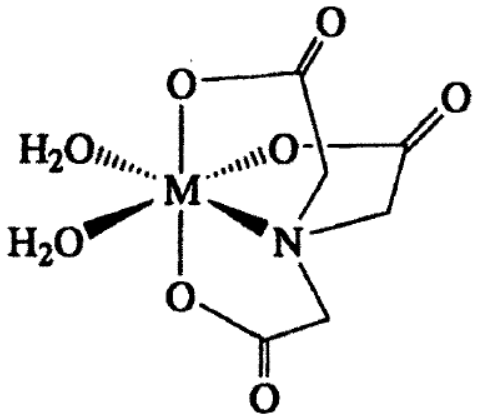


Figure 1.6. NTA in complex with a metal ion (M).

Inspired by NTA, EDTA was developed in the 1920s and has effectively replaced NTA for applications since then, because EDTA has much better chelating effectiveness. NTA is more biodegradable than EDTA (Kolodynska, 2011a). However, specific and environmentally uncommon conditions are required for effective biodegradation to occur, and in colder waters such as those of New Zealand there is potential for NTA to carry and increase solubilisation of toxic metals such as Cd, Cu, and Zn (Thom, 1971). In addition to this concern there are possible adverse health effects for mammals exposed to NTA (Ebina et al., 1986).

NTA is the best of the chelating agents mentioned here at extracting Zn^{2+} ions from solution at a pH of 7, and second only to EDDS and EDTA for extracting Cu (Tandy et al., 2004).

Analytical grade NTA is supplied commercially by Sigma Aldrich at a cost of NZ $236 per kg.

MGDA

Methylglycine, N,N, diacetic acid (MGDA) is another biodegradable chelating agent capable of forming complexes with Cu^{2+}, Ni^{+} and Pb^{2+} ions to reduce bioavailability in aqueous solutions (Jachula et al., 2011, 2012). It is being investigated for its potential to complex heavy metal ions so that anionic exchangers can be used as adsorbents, in the hope that this will provide a novel method of heavy metal wastewater treatment (Jachula et al., 2016). However, MGDA is a very good chelator of Ca^{2+} (Bretti et al., 2017), and therefore could worsen the quality of the seawater for larval culture if used in shellfish hatcheries.

MGDA is supplied as Dissolvine M-40 which is 40% MGDA in solution, by Akzo Nobel N.V. for an unspecified cost.

PAA

Polymerisation of aspartic acid forms polyaspartic acid (PAA), which can be controlled in molecular length (Roweton et al., 1997). Being a naturally occurring amino acid derived compound means that PAA is highly biodegradable. PAA can be completely biodegraded by microorganisms present in water treatment plants (Freeman et al., 1996).

PAA can form complexes with Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+} ions in solution, which may then readily adsorb to chitosan or polystyrene anion exchangers, as the metal complexes with PAA have an overall negative charge (Low et al., 1996; Kolodynska et al., 2008; Kolodynska, 2011b). Cross-linked PAA hydrogels are effective removing agents for lead ions through both an ion exchange mechanism and a chelation mechanism (Bo Sun et al., 2005).

Analytical grade PAA is supplied by Sigma-Aldrich Corporation for NZ $183 per 100 mg.

1.5.2 Adsorption

Chelation is not the only method for reducing the concentration of bioavailable heavy metal ions in solution. For example, heavy metal ions can adsorb to clay, hydroxides, and organic matter, including agricultural waste products (Table 1.5) (Bailey et al., 1999; Babel & Kurniawan, 2003; Bradl, 2004; Paulino et al., 2006; Demirbas, 2008). The organic matter component includes various forms of vegetation including coconut fibres, bark, tree wood, and even chrysalides of silkworms (Paulino et al., 2006). These methods remove metals from solution and decrease their overall concentration in solution. However, metals that remain in solution and are not adsorbed to the surfaces of these materials will not have changed in their bioavailable proportion, as they would with chelating agents.

Table 1.5. List of adsorption materials and their properties for reducing bioavailable heavy metal ion concentrations in solution.

Material	**Heavy metals adsorbed from solution**	**Comments**	**References**
Clay	Cr, Zn, Cd, Hg, Pb	High surface area -ve charged surface Low cost - abundant	(Cadena et al., 1990; Pradas et al., 1994; Bailey et al., 1999; Celis et al., 2000).
Coconut Fibre	Cr, Fe, Ni, Cu, Zn, As, Hg, Pb	Low cost - waste product of coconut husk processing	(Noguera et al., 1998; Igwe et al., 2008; Gopalakrishnan & Jeyadoss, 2011; Shukla & Shukla, 2013)
Tree Bark	Cr, Ni, Cu, Zn, Cd, Hg, Pb	Low cost – forestry waste product	(Deshkar et al., 1990; Vazquez et al., 1994; AlAsheh & Duvnjak, 1997; Gloaguen & Morvan, 1997; Gaballah & Kilbertus, 1998; Vazquez et al., 2002)

$CaCO_3$ Shells	Cu, Zn, Cd	Waste product from fisheries and aquaculture	(Kohler et al., 2007; Thind, 2013)
Zeolites	Cr, Co, Ni, Cu, Zn, Cd, Hg Pb	Low cost - naturally occurring aluminosilicate minerals	(Zamzow et al., 1990; Malliou et al., 1994; Erdem et al., 2004)
Chitosan	Cr, Co, Ni, Cu, Zn, Cd, Hg	De-acetylated chitin - a highly abundant naturally occurring biopolymer, waste product of fisheries and aquaculture Chemical modifications can be used to enhance adsorption	(Rhazi et al., 2002; Guibal, 2004; Rinaudo, 2006; Gerente et al., 2007; Ngah et al., 2011)

1.5.3 Summary

In all forms of its use, the increasingly agreed aim for industrial and agricultural applications is to replace EDTA, for the benefit of the environment. Aquaculture is one of the fastest growing food production industries and should be seriously considering alternatives to EDTA for use in shellfish hatcheries. Based on the review of available information the most promising candidate for replacing EDTA for use in shellfish hatcheries is EDDS. This inspired a test of its effectiveness in the work of this thesis (Chapter 4).

1.6 The life cycle of New Zealand green-lipped mussels

The New Zealand green-lipped mussel (*Perna canaliculus*) is an endemic bivalve species which is found in shallow coastal waters around much of the country (Jeffs et al., 1999). This species is an important traditional seafood or kaimoana for Māori for whom it is known as kutai or kuku. This mussel species is also known by its trademark name of Greenshell™ when grown in commercial aquaculture in New Zealand.

The life cycle of the New Zealand green-lipped mussel commences when reproductively mature male and female mussels release their sperm and eggs into the water column followed by fertilisation (Jeffs et al., 1999). Unfertilised eggs appear brown and spherical in shape with a diameter of around 60 µm (Rusk et al., 2017). Immediately after fertilisation, cell division begins, and if conditions are suitable over the following 48 hours, the embryo develops into a healthy D-shaped veliger larva, around 100 µm in size (Redfearn et al., 1986; Rusk et al., 2017). At this point the larva has started the development of a pair of calcium carbonate shells, is able to swim using a velum rimmed with bands of cilia, and has developed a stomach and begun to filter feed on microalgae in the water column (Redfearn et al., 1986; Rusk et al., 2017). Problems with larval rearing at this stage could be due any combination of factors such as: broodstock, hygiene, pathogenic bacteria, toxic metabolites, chemical residues, hatchery design, hatchery components and toxic metals (Helm, 2004).

After 10 days post-fertilisation, further organogenesis has taken place and the larvae have developed a complete nervous system throughout their bodies (Rusk et al., 2017). Larvae at 13 days post-fertilisation have developed a series of muscles and are able to open and hold closed their shells. After 18 days the larvae have developed an eye spot, and often secrete a mucous thread to stick to a surface. At this stage the larvae have also developed a foot which they can extend out of the shell to hold onto substrate for the purpose of settling (Rusk et al., 2017).

Once a suitable substrate for settlement has been found, a larva begins metamorphosis into a juvenile or plantiger, but more commonly called a spat (Buchanan, 1999). This metamorphosis to a juvenile may be delayed several weeks until a suitable substrate is found (Buchanan, 1999). The life cycle is completed when *P. canaliculus* juveniles grow in size to reach reproductive maturity at a size of at least 27 mm in shell length, usually taking around 4-9 months in the wild (Alfaro et al., 2001).

The Greenshell™ mussel is New Zealand's primary aquaculture species earning over 250 million USD in export revenue per annum (Jeffs et al., 2018). For decades this industry has

been reliant on wild-caught spat that is washed up on Ninety Mile Beach in Northland, New Zealand (Alfaro et al., 2010; Jeffs et al., 2018). The dependence of the industry on the spat supply from the wild is concerning, as farmers have no control over wild spat supply timing, quantity or quality, and the location of the adult broodstock providing these spat remains unknown.

The hatchery rearing of mussel spat involves spawning adult mussels and raising the resulting larvae through to spat in captivity before transfer onto farms in coastal waters. The hatchery production of juvenile shellfish is increasingly replacing the harvesting of wild spat for use in seeding shellfish aquaculture operations worldwide. Hatchery production can greatly improve the reliability for producing juveniles of many species of aquaculture shellfish, whilst also allowing for the application of selective breeding to provide for long term farm stock improvements. A significant amount of ongoing research activity has enabled the emergence and continuing development of shellfish hatchery technology.

One important breakthrough was the addition of EDTA to seawater used for larval rearing of shellfish species in order to improve larval survival (Utting & Helm, 1985; Helm, 2004). It is now common practice in commercial shellfish hatcheries to use EDTA in larval rearing, especially for the initial embryo incubation stage for bivalves. The application of EDTA in this manner has provided similar larval benefits for larval production in the only commercial hatchery for *P. canaliculus* in New Zealand (Gale et al., 2016). By using EDTA, the yield of healthy D-larvae after 48 h post-fertilisation can be improved significantly in hatchery production (Gale et al., 2016). However, an understanding of exactly how EDTA provides this improvement in larval survival has not been concluded. For example, oxidative stress in *P. canaliculus* is reduced with the addition of EDTA to the incubation seawater during the first 48 h of development from a fertilised egg, which is thought to be because of a reduction in the bioavailability of Fe and Cu (Gale et al., 2016), however, the concentrations of the heavy metals and how the concentrations in *P. canaliculus* change with EDTA addition has not been investigated.

1.7 Research aims

The primary aim of this study was to better understand the mechanism of the effectiveness of EDTA for shellfish in aquaculture hatcheries. Prior to this study, understanding of the role of EDTA for rearing of aquaculture shellfish has been lacking. For example, questions remain about which heavy metals EDTA is detoxifying and how EDTA reduces their toxicity. The

secondary aim of this study was to test whether continuing the use of EDTA beyond the first two days post-fertilisation provides any benefits to *P. canaliculus* as they develop into spat that are ready to settle (22 days post-fertilisation). The third aim of this study was to investigate the potential for biodegradable alternatives to EDTA to be applied to aquaculture shellfish rearing, as it has been shown that EDTA is not readily biodegradable. Finally, being able to quantify any changes in metal content and distribution during the larval development of *P. canaliculus* using ICP-MS and synchrotron XFM, will provide valuable information for the hatchery production of shellfish larvae in general.

The thesis is structured in the following manner with the following overall aims;

Chapter 1

- To provide a general introduction to the research presented in the thesis by presenting an overview of the toxicity of heavy metals to shellfish, and the heavy metal chelating abilities of some commonly used chelating compounds, including EDTA, which is widely used in the hatchery production of shellfish species.
- To describe the larval biology of the green-lipped mussel as well as the effect of EDTA in the commercial hatchery production of this mussel.
- To present an outline of the overall aims of the research work presented in the thesis.

Chapter 2

Published as: McDougall et al., 2019. Examining the role of ethylenediaminetetraacetic acid (EDTA) in larval shellfish production in seawater contaminated with heavy metals. *Aquatic Toxicology*. doi:10.1016/j.aquatox.2019.105330

- To determine if the presence of EDTA at a concentration of 3 µM improves the survival of developing embryos of the *P. canaliculus* over the 2 day period post-fertilisation.
- To determine if the presence of EDTA affects the mean concentration of heavy metals found within developing embryos of *P. canaliculus* using ICP-MS, when compared to the mean concentration found in embryos raised in seawater without EDTA.
- To determine the spatial distribution, at 1 µm spatial resolution, of calcium and heavy metals within individual 2-day-old *P. canaliculus* D-larvae.
- To determine whether the spatial distribution of heavy metals within D-larvae is affected by the presence of EDTA during larval rearing compared to their distribution in D-larvae reared in the absence of EDTA.

Chapter 3

Published as: McDougall et al., 2020. The value of EDTA treatment of hatchery water to rear Greenshell™ mussel (*Perna canaliculus*) larvae. *Aquaculture International*. doi:10.1007/s10499-020-00543-y

- To measure the survival, growth, ingestion rate, and swimming activity of *P. canaliculus* through development from fertilised egg for 22 days to spat that are ready to settle both in the presence and absence of EDTA.
- To determine if there are any benefits to adding EDTA to seawater used for rearing *P. canaliculus* beyond the first 2 days of development from fertilised egg.
- To determine if the presence of EDTA in seawater used for rearing spat affects the mean concentration of heavy metals found within *P. canaliculus* using ICP-MS, as they develop throughout the full 22 day rearing period post-fertilisation.

Chapter 4

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- To measure the effectiveness of EDDS for improving *P. canaliculus* larval survival over 2 days of development from a fertilised egg and compare to the use of EDTA at equivalent concentrations.
- To measure how the mean concentrations of heavy metals detected in 2-day-old *P. canaliculus* embryos using ICP-MS are affected by the addition of EDDS, and how it compares to the EDTA used at equivalent concentrations.
- To determine if administering EDDS affects the spatial distribution and concentrations of heavy metals within individual 2-day-old *P. canaliculus* embryos compared mussel embryos raised with equivalent concentrations of EDTA.

Chapter 5

- To provide a general discussion of the research presented in the thesis by comparing and discussing the sets of results presented in each of the chapters of the thesis.

2. Examining the role of ethylenediaminetetraacetic acid (EDTA) in larval shellfish production in natural seawater

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2.1 Introduction

In commercial shellfish hatcheries, fertilised eggs (ova) are raised through their full larval development to form juveniles, which provide the seed supply that underpins much of the world's annual 20M t shellfish aquaculture production. For bivalves, such as mussels, in the first stage of their development the ova are fertilised with sperm and then develop into D-shaped veliger larvae over 48 h. The percentage yield of healthy D-veliger larvae raised from fertilised ova after 48 h is suspected to be adversely affected by the toxicity of certain metals in the incubation seawater, to which the larval shellfish are thought to be highly sensitive. This seawater is most often pumped into the shellfish hatchery from adjacent shallow water coastal environments, which are prone to anthropological sources of heavy metal pollutants.

Heavy metals (defined here as metals heavier than calcium in the periodic table) occur naturally in seawater at background levels that vary depending on location (Duffus, 2002). For example, high levels of cadmium are found naturally in the seawater and shellfish inhabiting Foveaux Strait and Tasman Bay in New Zealand (Nielsen & Nathan, 1975). Heavy metals are also often elevated above background levels as a result of human activities including mine drainage, industrial wastewater, storm water runoff, and ablation of antifouling paints used on boat hulls (Jarup, 2003; Brown & Peake, 2006; Fu & Wang, 2011; Wicke et al., 2012). In many places, the natural background levels of heavy metals are low, but they may still interfere with normal larval development during this sensitive developmental stage in shellfish. Nevertheless, several heavy metals are also biologically essential at trace levels for shellfish (Wintz et al., 2002).

Different heavy metals are known to have various toxic effects at different concentrations depending on the species and developmental stage of shellfish (Martin et al., 1981; Ramachandran et al., 1997; His et al., 1999; Rainbow, 2002). In particular, some heavy metals are known to affect the growth (i.e., abnormalities, slowed growth rate) (Brereton et al., 1973; Calabrese & Nelson, 1974; Calabrese et al., 1977), development (i.e., cell formation and cell

cleavage) (Glickstein, 1978; Coglianese & Martin, 1981; Beaumont et al., 1987; Conrad, 1988; Beiras & His, 1994, 1995a), health (respiration) (Akberali & Earnshaw, 1982; Akberali et al., 1985), settlement (Boyden et al., 1975), and the survival of shellfish larvae which are much more vulnerable to the toxic effects than adults (Ringwood, 1990). This is why the presence of heavy metals, even at low concentrations is thought to be a major problem for shellfish hatchery production.

A number of mechanisms by which heavy metals may prove toxic to molluscs have been described, mostly in adults, and often associated with shell formation. The presence of sufficient levels of heavy metals, whether in the environment or in the diet of a mollusc, disrupts shell formation by interfering with bio-mineralization (Stromgren, 1982; Sunila & Lindstrom, 1985; Yap et al., 2002; Lopes-Lima et al., 2012; Goto & Sasaki, 2014). The highly controlled bio-mineralization of calcite and aragonite (crystalline polymorphs of $CaCO_3$) are achieved by molluscs through the use of shell-formation proteins which direct their precise microstructural crystalline formations (Marin et al., 2008). When sufficiently bioavailable, heavier metal ions with smaller ionic radii than calcium will replace calcium ions, which in turn has the potential to affect the structural integrity of calcite within mollusc shells (Lorens & Bender, 1980). Furthermore, alternative heavy metal ions can replace zinc ions within important enzymes used for mineralization, such as carbonic anhydrase, and thereby disrupting their functionality (Lasseter et al., 2016).

Carbonic anhydrase is a key enzyme responsible for increasing the localized concentration of CO_3^{2-} at key locations in the bodies of molluscs where shell formation occurs (Lindskog, 1997; Zhang & Zhang, 2006; Marin et al., 2008). For example, high activities of carbonic anhydrase were observed immediately prior to shell formation during the development of blue mussel (*Mytilus edulis*) larvae (Medakovic, 2000). The function of carbonic anhydrase is affected by the presence of heavy metal ions. For example, Cd^{2+} and Co^{2+} ions competitively inhibit carbonic anhydrases, by competing for the Zn^{2+} binding site, and Cu^{2+} can non-competitively inhibit the enzyme (Vitale et al., 1999; Soyut et al., 2008). Metal ions such as Fe^{2+} and Cu^{2+} are known to induce and catalyse the production of reactive oxygen species (ROS) O^{2-}, H_2O_2, and OH, through the Fenton and Haber-Weiss reactions (Guerin et al., 2001). ROS cause oxidative stress, which has also been shown to impair the development of embryos of the green-lipped mussel, *Perna canaliculus* (Gale et al., 2016).

Assessing the ecological impact of pollutants in the aquatic environment is of great importance and heavy metal pollution remains of significant environmental importance. For example, 25%

of perceived ecological impacts in reviewed scientific literature were associated with metal pollution (Rochman et al., 2016). Nevertheless, there remains a lack of understanding about the ecological impact of heavy metal pollution in the aquatic environment.

Ethylenediaminetetraacetic acid (EDTA) is known to influence interactions between dissolved metals and aquatic organisms. Disodium EDTA is added to incubation water at 1 mg L^{-1} (3 µM) as standard practice for seawater "conditioning" that ensures successful larval rearing in many of the commercial shellfish hatcheries around the world. For example, for raising bivalve molluscs, such as mussels, the addition of this chemical to seawater used for larval rearing greatly increases the proportion of ova developing to D-larvae (Utting & Helm, 1985; Gale et al., 2016). However, despite the widespread use of EDTA in hatcheries, its mode of action and its possible interaction with metals remain undetermined. EDTA is thought to reduce the bioavailability of heavy metal ions (Rainbow et al., 1980), and thereby their toxicity to larvae, but there is a lack of evidence to confirm this modality (Lawrence et al., 1981).

The primary goal of this study was to test if the previously hypothesized benefit of adding EDTA is due to this chelating agent binding with and reducing the bioavailability of toxic metals. This in turn would reduce metal toxicity and increase successful larval shellfish development. To achieve this, the ICPMS and synchrotron XFM were used to determine differences in total metal concentrations and distributions (all metal species included) between green-lipped mussel D-larvae raised in seawater with and without added EDTA to incubation seawater.

2.2 Materials and Methods

2.2.1 Adult mussel collection and spawning

Adult *P. canaliculus* in reproductive condition were collected from mussel farms in the Marlborough Sounds near Nelson, New Zealand on 5 March 2018 and taken to the nearby SPATNZ Ltd shellfish hatchery located at Glenduan. At the hatchery the outer shells of the mussels were gently scrubbed clean of biofouling. The mussels were then placed in shallow plastic spawning trays and subjected to thermal cycling until spawning began. The spawning female mussels were removed from the spawning trays and placed in individual spawning containers (5 L bucket containing 25 °C seawater).

The ova spawned by each of 10 female mussels was sampled for later analysis of metal composition. A 1.5 mL aliquot of the ova suspended in seawater spawned from each of the mussels was sampled with a disposable pipette and put into a 1.7 mL micro centrifuge tube. The ova were allowed to settle before the seawater was decanted off. The tubes were then snap frozen in liquid nitrogen for transport to the laboratory and remained frozen until subsequent analysis. The remaining ova from each of the spawning containers were mixed together to create a pooled sample of ova from all the female mussels to ensure that embryos entering the tanks were of a similar composition at the outset of the experiment. In the laboratory, the ova samples were thawed then dried in a vacuum desiccator, and the dry weight of the ova was measured. The dried ova were acid digested in concentrated HNO_3 (67%) at 80 °C for 30 min and the heavy metal content was analysed with Inductively Coupled Plasma Mass Spectrometry (ICPMS).

2.2.2 Experimental design

Twelve conical larval culture tanks of filtered seawater (160 L) were prepared for this experiment. Within 24 h of tank preparation, three replicate samples of 10 mL of the seawater were obtained from the surface water of each of the tanks and subsequently analysed for heavy metal content using ICPMS. Then 160 mg of disodium EDTA (LabServ, Analytical Grade) was added to six randomly selected tanks to a concentration of 1 mg mL^{-1} (3 µM) to be consistent with commercial treatment of seawater at the hatchery facility at the time. The seawater was kept circulating with air bubbles introduced at the base of the conical tanks. After 24 h of circulation with EDTA, three replicate samples of 10 mL of the seawater were obtained from the surface water of each of the tanks and were subsequently analysed for heavy metal content using ICPMS using the same instrument that was used for analysis of acid digested mussel ova. The tanks were maintained at a temperature of 18 °C throughout the following larval experiment.

Having inoculated six of the tanks with 3 µM EDTA and six without, mussel ova from the pooled suspension were equally distributed into twelve 1 L beakers containing 500 mL of the seawater from each tank, respectively. Four 10 µL aliquots of the resulting 500 mL solutions were observed under a microscope and the number of ova present in each beaker was estimated (ranging from 25 – 50 ova per 10 µL). Sperm were then added equally to each beaker such that 2-7 sperm were visible (from count estimates via microscopy) per ova to ensure complete fertilisation, and to avoid polyspermy. This was checked by observing four 10 µL aliquots under a microscope. The 500 mL of seawater containing fertilised ova in each beaker was then

poured into its respective 160 L larval rearing tank. This established estimated embryo densities ranging from 1,600,000 to 2,200,000 per 160 L tank.

The fertilised embryos were allowed to develop over a period of 40 h over which time they would normally progress to D-veliger larvae. After 40 h, each of the 12 tanks were drained through a 40 µm nylon mesh sieve. Retained larvae were sucked off the surface of the sieve with a Pasteur pipette and placed into 50 mL centrifuge tubes containing 40 mL of the respective seawater from each tank for later analyses. The tubes were refrigerated at 4 °C until analyses.

After 40 h of larval development, three 10 µL aliquots of seawater from each tank were observed under an optical light microscope, and a count was made to estimate a mean percentage yield of larvae for comparison with counts of ova for control tanks and tanks with EDTA at the outset of the experiment. Larval yield is defined as the percentage of embryos that develop into D-veliger larvae with no shell deformities and a straight D-hinge.

All seawater sourced for this study was pumped from an intake located 200 m off the coast from the hatchery and stored in high density polyethylene holding tanks until required. This seawater was then filtered through a 5 µm mesh bag filter before being supplied to fill the twelve 160 L tanks.

2.2.3 Analytical chemistry

Seawater samples were diluted 20× in 2% HNO_3 solution prior to analysis. Ova and larvae samples were digested in 2 mL tubes with 1 mL of 67% HNO_3 added dropwise and the tubes were immersed for 45 min in a boiling water bath. The resulting solutions were poured into 15 mL sterile polypropylene tubes. Adsorption of metals to the walls of these tubes is expected to be minimized because of the acidification step (Batley & Gardner, 1977). The original sample tube was then rinsed four times with deionized water (1 mL) and the washings poured into the 15 mL tube. The solutions were then diluted to 11 mL with deionized water prior to analysis. The solutions were quantitatively analysed for chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) concentrations on an Agilent 7700x ICP-MS operated in helium mode to reduce polyatomic interferences. Calibration standards were prepared in a matrix-matched solution from 1000 ppm single element standards (Peak Performance, CPI International) as well as a blank matrix-matched solution containing no single element standards. An online internal standard (20 ppb Yttrium & Terbium) was used to monitor and correct for instrument drift and

matrix effects. All results are reported in ng mL^{-1} (ppb) for seawater samples or µg g^{-1} (ppm) for ova and larvae samples. The EDTA (LabServ, Analytical Grade) used at SPATNZ was also analysed with ICPMS for heavy metal content.

2.2.4 Synchrotron X-ray Fluorescence Microscopy (XFM)

Silicon nitride (SiN) windows with a 2 mm^2 aperture size were prepared by the Melbourne Centre for Nanofabrication for mounting larvae samples. A simple method was used for the preparation of samples ready for examination under XFM given that previous research has found that minimizing the preparation steps of biological samples for analysis of metal content is vital to ensure that the metals are observed in as close to their native state as possible (New et al., 2018). Using a Pasteur pipette ~ 0.5 mL of concentrated larvae were extracted from the bottom of the refrigerated larval samples tubes (50 mL) and transferred to 1 mL of deionized water in an Eppendorf tube to dilute the salt concentration. The Eppendorf tube was then shaken and 0.5 µL of the resulting suspension was extracted with an auto pipette. The 0.5 µL droplet was carefully placed in the centre of a SiN window. The window was dried by placing it on a clean Kimwipe tissue on a hot plate set to 40 °C. The dried droplet was observed under a binocular optical light microscope to ensure sufficient numbers of isolated larvae were present for each sample. This method was repeated for each sample from the twelve tanks.

The XFM beamline at the Australian Synchrotron was used to focus a monochromatic beam of 15.8 keV x-rays into a microprobe of order 2 µm high by 4 µm wide, enabling analysis for elements ranging from P to Se via K-shell excitation up to Hg and Pb via L-shell excitation (Paterson et al., 2011). The specimens mounted on the SiN windows were oriented at 45° to the incident beam. For each measurement, the specimen was scanned continuously through the focus and x-ray fluorescence events were collected using a Vortex – EM-90 detector positioned 90° with respect to the incident X-ray beam and interpreted using a XiA FalconX pulse processor. Specimen position encoder events were merged with fluorescence photon data to form a single list-mode event stream which was written to disk for later analyses using GEOPIXE software (C. G. Ryan et al., 2005). A 375 µm Kapton film filter was placed between the Vortex detector and the specimen to selectively attenuate the low energy fluorescence peaks, particularly the strong calcium X-ray fluorescence that originated from the comparatively high calcium content of the larval shell. In this case the use of the Kapton filter prevented the reporting of quantitative elemental concentrations as is typical with XFM, however, it is possible to compare elemental content on a relative scale due to the careful control of the measurement conditions. The possibility of artefacts resulting from X-ray

induced damage was assessed through inspection of large-area scans taken before and after fine detailed scanning. Minimal differences between the before and after scans were taken to indicate that there was no significant elemental redistribution resulting from the measurement procedure.

2.2.5 Statistical analyses

All data were tested for normality and homogeneity of variances prior to analyses and if noncompliant (i.e., failed either Shapiro–Wilk's or Bartlett's tests) were log transformed and rechecked for compliance. Nested ANOVA was used to compare the individual heavy metal concentrations in seawater between the two sets of six tanks designated to be treated with EDTA and without treatment. Seawater concentrations of metals for the two sets of tanks were compared both prior to and following the addition of EDTA. Two-tailed t-tests were used to compare heavy metal concentrations in larvae raised in seawater with and without EDTA added. Likewise, a two-tailed t-test was used to compare the abundance of healthy D-larvae between tanks with and without EDTA added. For cadmium in larvae the data remained non-compliant after log transformation, so a Welch's two tailed t-test was used. All statistical testing was assessed for significance at $\alpha = 0.05$.

2.3 Results

2.3.1 Larval yields

The mean yield of D-larvae in the six tanks where the seawater was treated with EDTA was 15-fold higher than for those tanks without EDTA ($t = 11.95$, $P<0.05$), i.e., 39.1 ± 5.0 % versus 2.7 ± 1.1 %.

2.3.2 Larval metal content

Larvae cultured in seawater without EDTA had 3× the concentration of chromium, 5× the concentration of copper, 2× the concentration of zinc, 20× the concentration of cadmium and 11× the concentration of lead that was found in the larvae cultured in seawater with EDTA (Figure 2.1) ($p < 0.05$). In contrast, larvae cultured with EDTA contained 3× the cobalt concentration, and double the arsenic and mercury concentrations, of that found in the larvae cultured in seawater without EDTA (Figure 2.1) ($p < 0.05$).

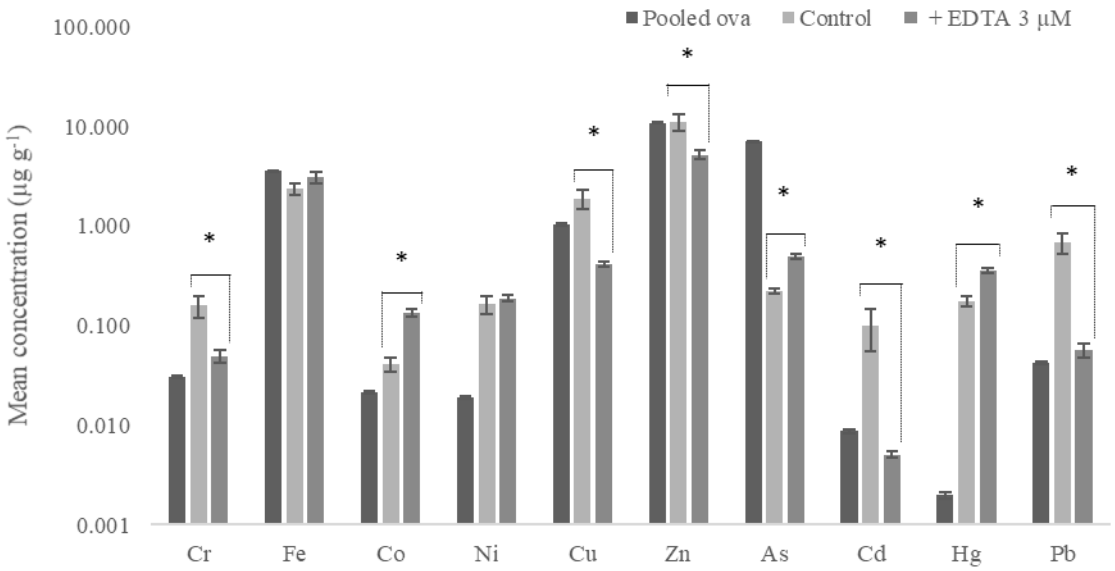

Figure 2.1. Mean concentrations (± S.E.) of heavy metals in a pooled sample of *P. canaliculus* ova prior to being fertilised and used in the experiment, control larvae (reared in seawater without EDTA) and larvae reared in seawater with EDTA added. Note: concentrations are on a log scale. Significant differences in the mean concentrations between larvae from control tanks and larvae from EDTA-treated tanks for Cr, Co, Cu, Zn, As, Cd, Hg and Pb ($p < 0.05$), but no significant difference for Fe and Ni ($p > 0.05$).

2.3.3 Seawater metal content

There was no significant difference in the mean concentration of any of the seven heavy metals detected in the seawater used for larval rearing between the six control tanks and the six tanks which subsequently had EDTA added ($p > 0.05$, Figure 2.2). Copper was the most abundant heavy metal detected in the seawater with an overall mean concentration of 10.5 ± 0.5 ppb. Cadmium, tin, mercury and lead concentrations in seawater were below the detection limits of the ICPMS, i.e., < 0.001 ppb.

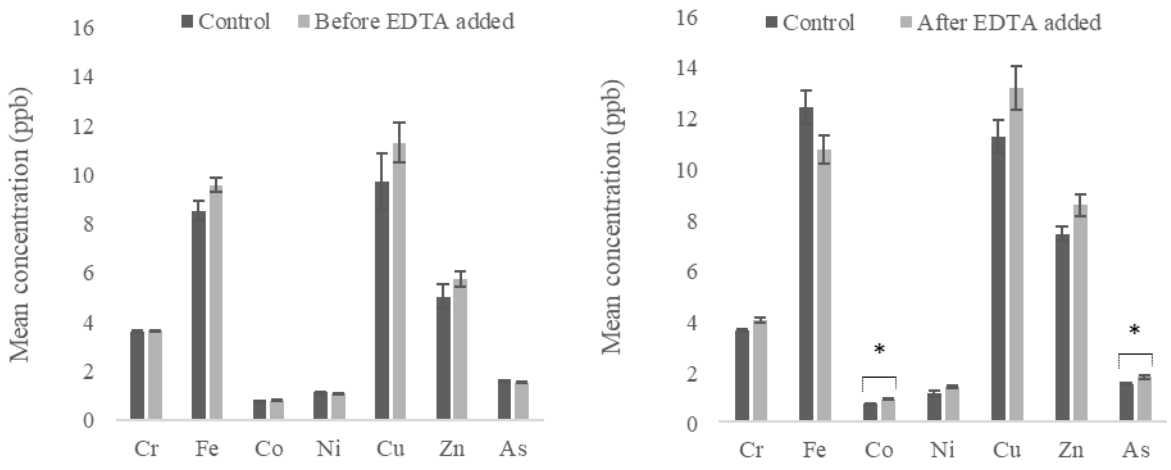

Figure 2.2. Mean concentrations (± S.E.) of seven heavy metals present in seawater sampled from 12 experimental larval mussel rearing tanks, before (left) and after (right) six were treated with EDTA. Nested ANOVA confirmed there were no significant differences between the means for all heavy metals for the two sets of six tanks prior to experimental treatment ($p > 0.05$) but found significant differences (*) for Co and As ($p < 0.05$) after EDTA treatment.

For the seven measured heavy metals in the seawater following the addition of EDTA, only the mean concentrations of cobalt and arsenic were significantly different (Figure 2.2). Both cobalt and arsenic showed small increases in concentration following the addition of EDTA, i.e., 18% and 16%, respectively. Both cobalt and arsenic were below detection limits in the EDTA powder itself. Copper was the most abundant heavy metal in the seawater after EDTA addition with an overall mean concentration of 12.2 ± 0.7 ppb. Copper, iron and zinc concentrations increased over time from before EDTA was added to after EDTA was added.

The EDTA powder contained 0.038 ± 0.003 µg g^{-1} of chromium, 1.531 ± 0.150 µg g^{-1} of iron, 0.184 ± 0.010 µg g^{-1} of copper, 1.934 ± 0.076 µg g^{-1} of zinc, and 0.061 ± 0.005 µg g^{-1} of cadmium. All other heavy metals in the EDTA powder were below ICPMS detection limits.

2.3.4 XFM

The XFM scans clearly showed calcium concentrations are highest at the hinge and the margins of the larval shells, reflecting the locations of increased projected thickness of the shells and of shell formation (Figure 2.3). No visually obvious differences in calcium deposition were observed between larvae raised in seawater with and without the addition of EDTA.

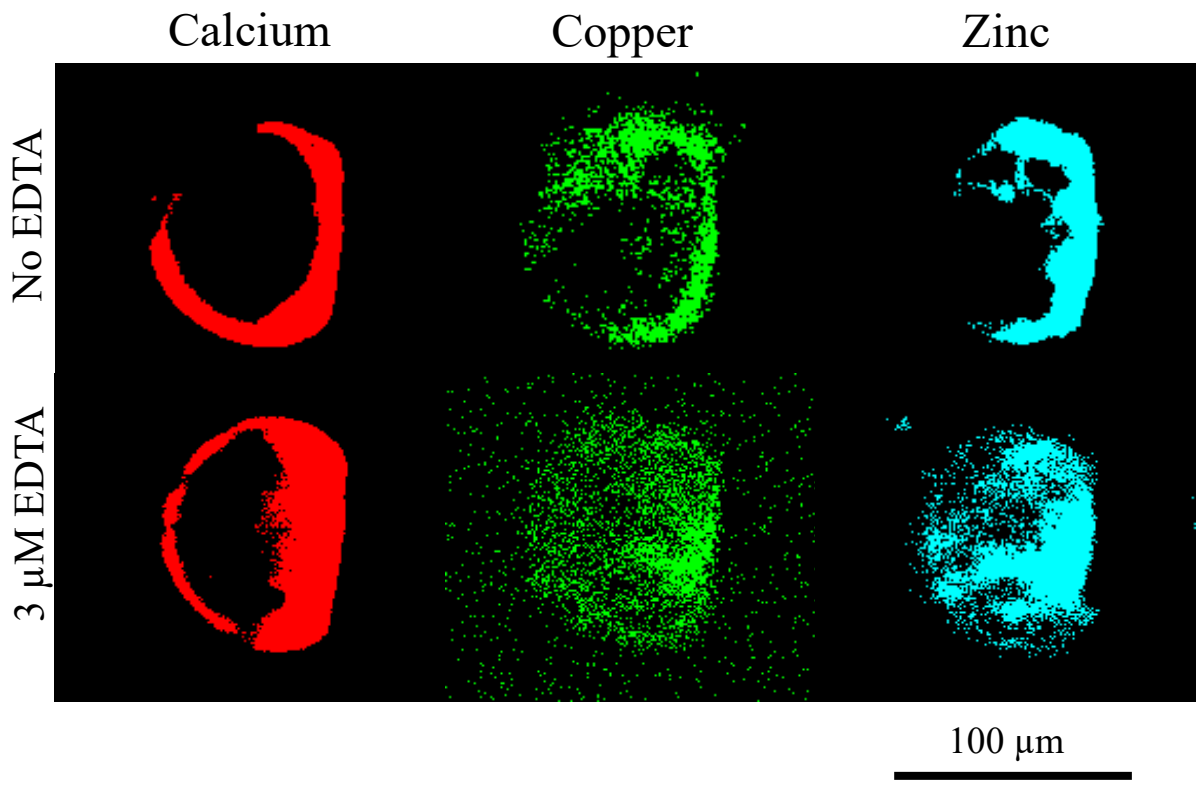

Figure 2.3. Distributions of calcium, copper, and zinc within 2-day-old *P. canaliculus* larvae raised with and without EDTA added to incubation seawater. Pixels with the highest counts for the respective metal (10% of all counts) are displayed to visualize the distribution of metal concentrations without undue influence from measurement background.

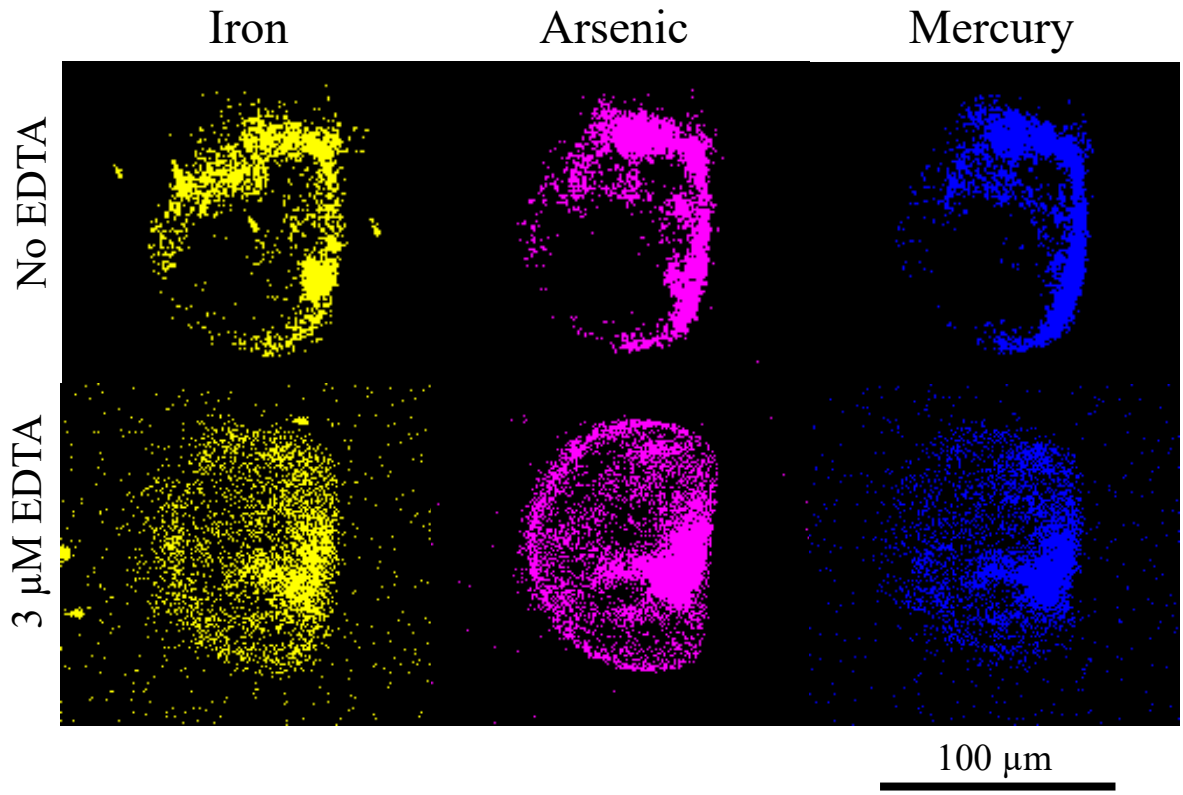

Figure 2.4. Distributions of iron, arsenic, and mercury within 2-day-old *P. canaliculus* larvae raised with and without EDTA present in the incubation seawater. Pixels with the highest counts for the respective metal (10% of all counts) are displayed to visualize the distribution of metal concentrations without undue influence from measurement background.

In larvae cultured without EDTA, heavy metals were often observed at comparatively higher concentrations at locations of shell formation around the margins of the larval shell (Figure 2.3, Figure 2.4). In larvae cultured with EDTA, heavy metals were often observed at comparatively higher concentrations near the visceral mass, and to a lesser extent in areas of high calcium content and shell formation around the margins of the larval shell (Figure 2.3, Figure 2.4).

2.4 Discussion

The aim of this research was to determine how the addition of EDTA to larval incubation seawater provides a higher yield of healthy mussel larvae, by investigating how EDTA influences the concentration and distribution of heavy metals in the larvae. The yield of mussel D-larvae was considerably improved with EDTA addition to seawater in this experiment (i.e., 15 fold increase), which is consistent with previous findings documenting the benefits of the addition of EDTA to larval shellfish production (Utting & Helm, 1985; Gale et al., 2016). Larvae incubated without EDTA present in the seawater for 40 h contained more chromium, copper, zinc, cadmium and lead than larvae incubated with EDTA, despite both treatments having the same concentration of these heavy metals in the seawater at the outset of the experiment. This suggests the beneficial effect of adding EDTA for mussel larvae is the reduction in bioavailability and bioaccumulation, and correspondingly reduced toxicity of these naturally occurring heavy metals in the larvae.

Some heavy metals were present at concerning concentrations in the ova at the outset of this experiment (Figure 2.1). The adult mussels were obtained from the Marlborough Sounds, and will have bio-accumulated these metals from the seawater there. As the ova were pooled together, all tanks had the same concentrations of heavy metals in the ova at fertilisation, therefore any effect of these metals on the subsequent development of embryos should have been consistent for both the control and the EDTA treated tanks.

Copper (Cu^{2+}) present in seawater at a concentration of 5 ppb or higher (i.e., less than half than in this study), interferes with embryonic development of Asian green mussel (*Perna viridis*), Pacific oyster (*Crassostrea gigas*) and blue mussels (*Mytilus edulis* and *M. trossulus*) larvae (Coglianese & Martin, 1981; Martin et al., 1981; Hoare, Beaumont, et al., 1995; Mai et al., 2012). For example, in the congener mussel *P. viridis* the EC50 for copper for the first 48 h of larval development is 11.7 ppb which was similar to the concentration of copper in the larval incubation seawater in the current study (Figure 2.2) (Purbonegoro and Hindarti, 2019). Similarly, a copper concentration of 10 ppb resulted in a marked decrease in the viability of larvae of Pacific blue mussel (*M. trossulus*) incubated over their first 48 h compared to those in lower copper concentrations (Fitzpatrick et al., 2008). One mechanism for copper toxicity for developing *C. gigas* was via DNA damage (Mai et al., 2012). In addition, copper is known to non-competitively inhibit carbonic anhydrase (Soyut et al., 2008). As seawater copper concentrations were detected here at 9.7-13.2 ppb (Figure 2.2), and significant decreases in copper concentrations within larvae were found when EDTA was added to the incubation

seawater (Figure 2.1), EDTA is very likely to have a role in reducing the toxicity of copper for these mussel larvae.

Copper and iron are known to catalyse production of reactive oxygen species, which can cause oxidative stress, and in previous work the oxidative stress in *P. canaliculus* larvae decreased with the addition of 3 µM EDTA (Gale et al., 2016). However, there was no significant decrease in iron concentration within 2-day-old larvae with the addition of EDTA (Figure 2.1), suggesting that copper is the problematic heavy metal for oxidative stress for *P. canaliculus* larvae, and that iron concentrations are tolerable. The increase in concentration of iron and copper over time from before EDTA addition to after EDTA addition could be a result of the iron and copper from the air at the hatchery accumulating in the water (Figure 2.2). Further experiments beyond the scope of this thesis are required to confirm this. Air conditioning fans, vents and wiring are made of steel and copper, and airborne particles can contaminate very dilute seawater samples (Noble et al., 2020).

Hexavalent chromium (Cr^{6+}) caused 50% abnormal development for *C. gigas* and *M. edulis* embryos at a seawater concentration of 4538 ppb (Martin et al., 1981), which is three orders of magnitude higher than the concentrations in the seawater in this experiment (3-5 ppb) (Figure 2.2). This suggests that the effect of EDTA on chromium levels within mussel larvae (Figure 2.1) is not a major benefit of its addition.

Concentrations of zinc (Zn^{2+}) in seawater up to levels of 100 ppb, have no apparent effect on the larval development of *C. gigas* (Martin et al., 1981). Zinc concentrations of only 5 – 9 ppb in the seawater were observed in this experiment (Figure 2.2), yet EDTA still significantly reduced the accumulation of zinc by *P. canaliculus* larvae (Figure 2.1). Zinc is an essential element, required for the function of carbonic anhydrase for example (Medakovic, 2000), so reduction of its bioavailability may not be beneficial. Results from this study suggest that adding EDTA could be altering the distribution of zinc (Figure 2.3), as well as reducing overall zinc concentrations within shellfish larvae.

The sample preparation method for investigating mussel larvae with synchrotron X-ray Fluorescence Microscopy (XFM) proved to be very effective, and images are likely to be very good representations of actual elemental distributions within the larvae, because of minimal exchange of fluid during sample preparation (New et al., 2018). Furthermore, this study demonstrates that sample preparation methods commonly used for microscopy, such as alcohol dehydration, are not always necessary for analysis of mussel larvae with XFM.

Healthy D-larvae observed in XFM elemental maps had well-formed D-shaped shells with high concentrations of calcium, providing great reliability for location of the larvae for investigating heavy metal distributions (Figure 2.3). Interestingly, zinc was concentrated around the margins of shells in larvae developed without EDTA, but in larvae raised in seawater with EDTA, zinc was more evenly distributed throughout the larva (Figure 2.3). Zinc coordinates with the enzyme carbonic anhydrase, which is an important enzyme involved in bio-mineralisation of the $CaCO_3$ shell (Miyamoto et al., 1996; Medakovic, 2000; Zhang & Zhang, 2006). This could explain the observed localisation of zinc in areas of high shell formation.

Zinc is a vital metal for several biological processes including shell formation, however, it is known to become toxic for mussels when present at sufficiently high concentration (≥ 100 ppb) (Nadella et al., 2009). If zinc concentrations are too high, it may adversely affect bio-mineralisation processes by replacing calcium in the crystalline formation of shell material (Lasseter et al., 2016). Zinc has a smaller radius than calcium (0.74 Å and 0.99 Å, respectively) (Miyaji et al., 2005), and readily can be incorporated into calcite, and increased levels of zinc can skew the proportion of calcite in shellfish shells, decreasing the proportion of aragonite (Miyaji et al., 2005; Lasseter et al., 2016). This change in chemical composition of the shell is likely to affect its structural integrity and therefore potentially the survival of larval shellfish. Incorporation of zinc into larval shell material is another potential explanation for the distributions observed with XFM (Figure 2.3).

Cadmium and lead were undetectable in the seawater, yet they were measurable in the larvae and there was a significant decrease in their accumulation in larvae with EDTA present (Figure 2.1). As cadmium and lead are seldom present at sufficient concentration in seawater naturally, animals have not developed specialist transporters for them (W. X. Wang et al., 2018). Therefore, the mechanism of uptake of these metals is believed to occur inadvertently via pathways intended for essential metals such as iron, copper, zinc, and calcium (W. X. Wang et al., 2018). However, the minimum reported concentrations of cadmium and lead that cause adverse effects for developing shellfish larvae are 100 ppb and 40 ppb, respectively (Nadella et al., 2009; Q. Wang et al., 2009; Nadella et al., 2013), much higher than concentrations in seawater in this study (detection limits 0.004 ppb for cadmium and 0.008 for lead). For example, in the congener mussel *P. viridis* the EC50 for cadmium for the first 48 h of larval development was 1.97 ppm which is well above the undetectable concentration of cadmium in the larval incubation seawater in the current study (Figure 2.2) (Purbonegoro and Hindarti, 2019). Cadmium and lead are known to replace metals in the metal binding site of various

important proteins, affecting the protein's function (Vitale et al., 1999; Bertin & Averbeck, 2006; Flora et al., 2012). Lead is also known to cause oxidative stress, and problems for the nervous system at internal concentrations of 200 ppb (Flora et al., 2012). The results of this study suggest that EDTA helps to decrease the inadvertent transport of cadmium and lead into shellfish larvae. In contrast, an increase in arsenic and mercury concentration was observed within the larvae when EDTA was present in the seawater (Figure 2.1). Mercury is known to be especially toxic for shellfish larvae, by affecting embryogenesis, survival, growth, and metamorphosis (Glickstein, 1978; Beiras & His, 1994, 1995a; Q. Wang et al., 2009), at seawater concentrations as low as 0.01 ppb, however, mercury was undetectable in the seawater here (<0.001 ppb). EDTA can increase the uptake of arsenic into biological tissue (Rahman et al., 2008), and arsenic is known to cause abnormalities in *C. gigas* larvae at seawater concentrations of 100 ppb (Moreira et al., 2018), which is two orders of magnitude higher than arsenic seawater concentrations detected here (Figure 2.2). Thus, even though enhancement of the accumulation of arsenic and mercury in *P. canaliculus* larvae (Figure 2.1) by the presence of EDTA may be of concern, the low levels observed here are unlikely to be problematic.

Cobalt is a biologically essential heavy metal as a component of vitamin B12 in particular, a key vitamin for DNA synthesis, and one often present in high amounts in shellfish (Watanabe, 2007). The accumulation of B12 occurs via the filter feeding of microorganisms in the seawater (Watanabe et al., 2001). Interestingly, minimum cobalt concentrations that cause toxic effects in larval shellfish have not been reported in the literature, with the majority of current research focussed on the other more commonly toxic heavy metals. EDTA significantly increased the concentration of cobalt detected in mussel larvae (Figure 2.1). This suggests that a benefit of adding EDTA may be by increasing the cobalt bioavailability, which could improve vitamin B12 production in bacteria, and increase the supply of the vitamin when the larvae filter feed.

There is growing evidence that EDTA is an antimicrobial and antibiofilm agent (Finnegan & Percival, 2015; Percival et al., 2015). It is possible that these effects are a contributing factor towards the improved survival in larval rearing of *P. canaliculus* by affecting the microbiome in the rearing tanks. In a previous study on *C. gigas* larval rearing, it was shown that the microbiome is primarily affected by temporal changes and that reduced microbial levels in the seawater did not provide significant improvements in the survival of larvae (Laroche et al., 2018). That study was with oysters (*C. gigas*) which may not be affected by changes in microbial levels, whereas green-lipped mussels (*P. canaliculus*) could be much more sensitive to changes in the microbiome. Further research could address this issue, carrying out a study

of the microbiome at the *P. canaliculus* hatchery and the effect of EDTA on the microbiome there.

2.5 Conclusion

This study suggests that the chelation of low concentrations of heavy metal pollutants in seawater is the likely basis of the effectiveness of EDTA which is used globally in hatcheries for conditioning seawater to ensure success in the commercial rearing of shellfish larvae. In this study, EDTA greatly improved the survival of mussel D-larvae and reduced the concentrations of chromium, copper, zinc, cadmium, and lead that are otherwise accumulated by the larvae from ambient seawater concentrations. In particular, microscopic XFM imaging indicates that mineralisation that is fundamental to shell formation in D-larvae is disrupted by the accumulation of zinc. Despite large improvement in larval yields with the use of EDTA, healthy D-larvae yields can remain lower than desired in commercial production, and this could potentially be the result of increased bioavailability of non-essential metals, arsenic and mercury, resulting from conditioning with EDTA. This suggests that the development of more effective methods for managing bioavailable heavy metal pollutants are required for improving large scale commercial production of *P. canaliculus* D-larvae.

3. The value of EDTA treatment of hatchery water to rear Greenshell™ mussel larvae beyond 2 days post-fertilisation

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3.1 Introduction

Currently, the addition of EDTA to hatchery water during embryo incubation stages of some shellfish species is considered essential to ensure successful completion of early larval development in which shell formation is initiated (Helm, 2004). The current best practice for larval rearing of *P. canaliculus* (Greenshell™ mussel) uses EDTA for 48 h during the initial incubation of embryos to substantially improve the subsequent yield of D-stage veliger larvae. However, it remained unclear whether the continuation of EDTA dosing of rearing water beyond the initial embryo incubation would continue to provide benefits for the later stages of larval development.

Therefore, the aim of the research presented in this chapter 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. Larval fitness, developmental rates, survival and the metal content of larvae were measured throughout the experiment and compared between the two treatments of the rearing seawater, i.e., with and without EDTA. Uptake of metals from seawater is highly dependent on the bioavailability of their free ions in solution (Florence et al., 1992). However, it is difficult to obtain information about trace metal speciation in biological systems. Therefore, this study was limited to the determination of total metal concentration in the larval mussels.

3.2 Materials and Methods

3.2.1 Gamete collection and incubation

Adult *P. canaliculus* 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 four 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 spawn (Helm, 2004). Spawning mussels were separated into individual containers once gamete release was first observed and the corresponding gender of each spawning mussel could be determined.

Ten spawning male mussels were rinsed with filtered seawater (FSW, i.e., 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 mussel dropped 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 with 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.

3.2.2 Larval system and rearing procedure

Following a 48 h larval 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, counted, and the yield of viable D-stage larvae were determined as a percentage of initial egg population. Larval yield is defined as the percentage of embryos that develop into D-veliger larvae with no shell deformities and a straight D-hinge. The larvae were pooled together and then 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 according to standard procedures 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 with FSW adjusted to 18 °C continuously 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 1,000 L reservoir tank that 24 h prior was filled with FSW and dosed with 12 µM EDTA.

Every two days, each larval rearing tank was drained through a screen with a mesh size (43–175 µm) previously determined to retain > 95% 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 diet consisting of a mix of axenically cultured microalgae (*Chaetoceros calcitrans* forma *pumilum*, *Tisochrysis lutea*, *Chaetoceros muelleri*) was added to the header 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 days post-fertilisation (PF), when most of the larvae reached competency for settlement as made apparent by the presence of an eyespot.

3.2.3 Larval performance assessment

Larval survival, shell length, and food ingestion 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 ensure that higher mortalities did not improve the growth of the remaining survivors, an appropriate number of larvae were removed on D10 so that all tanks matched the larval numbers of the tank with the lowest number.

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 CellSens image analysis software (inverted microscope CX41 40× magnification, DP74 camera; Olympus).

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

Larval food ingestion was assessed 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 drained 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 was standardised by the larval population in each tank to provide a measure of individual larval ingestion rate.

3.2.4 Metal content in tissue and water

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 analysis of metal content.

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.

3.2.5 Analytical chemistry

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 was added and re-weighed. An aliquot of 1 ml of H_2O_2 and 3 ml of concentrated HNO_3 (69%) were added to each tube, which was sealed with a screw-on cap, before 70 minutes 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 chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) were back-calculated for the dry mass of the original freeze-dried samples. The ICP-MS was operated in helium 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) as well as a blank matrix-matched solution containing no single element standards. An online internal standard of 20 ppb yttrium (Y) and terbium (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), 10^{-5} (Pb) µg g^{-1} (or ppm).

3.2.6 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 measures 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 versus EDTA) at each stage of development were made using paired Student 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 throughout the results.

3.3 Results

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

3.3.1 Larval survival

There was a statistically significant interaction between treatment and days PF ($p = 0.029$), therefore the effect of EDTA was dependent on when survival was measured (Figure 3.1). 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 (Figure 3.1, $p = 0.019$). However, on all other assessment days, there was no significant difference between control and EDTA treated larvae (Figure 3.1, $p > 0.05$).

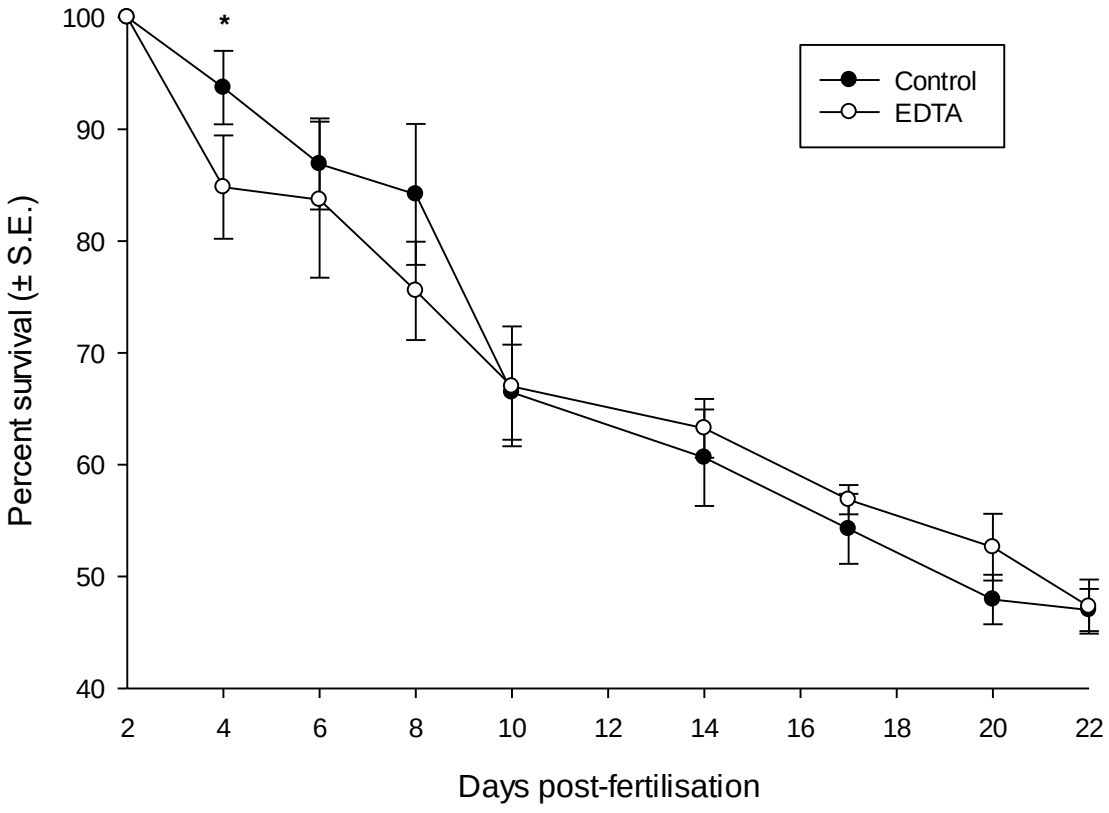

Figure 3.1. Mean percent survival (± SE) of mussel larvae reared in EDTA-treated FSW (hollow marker) and control treatment without EDTA (solid markers) over 22 days of experimental culture. Net survival was based on the number of D-stage larvae estimated on D2. Results are adjusted to account for the larvae removed on D10 during larval biomass standardisation and tissue sampling for ICPMS. Asterisk indicates a significant difference between the control treatment and the EDTA treatment ($p < 0.05$).

3.3.2 Shell length

EDTA treatment of the seawater did not result in a significant difference in mean shell length of the cultured larvae (ANOVA F = 0.00173: p = 0.888). In both treatments, larvae reached ≈ 250 µm mean size after 22 days (Figure 3.2).

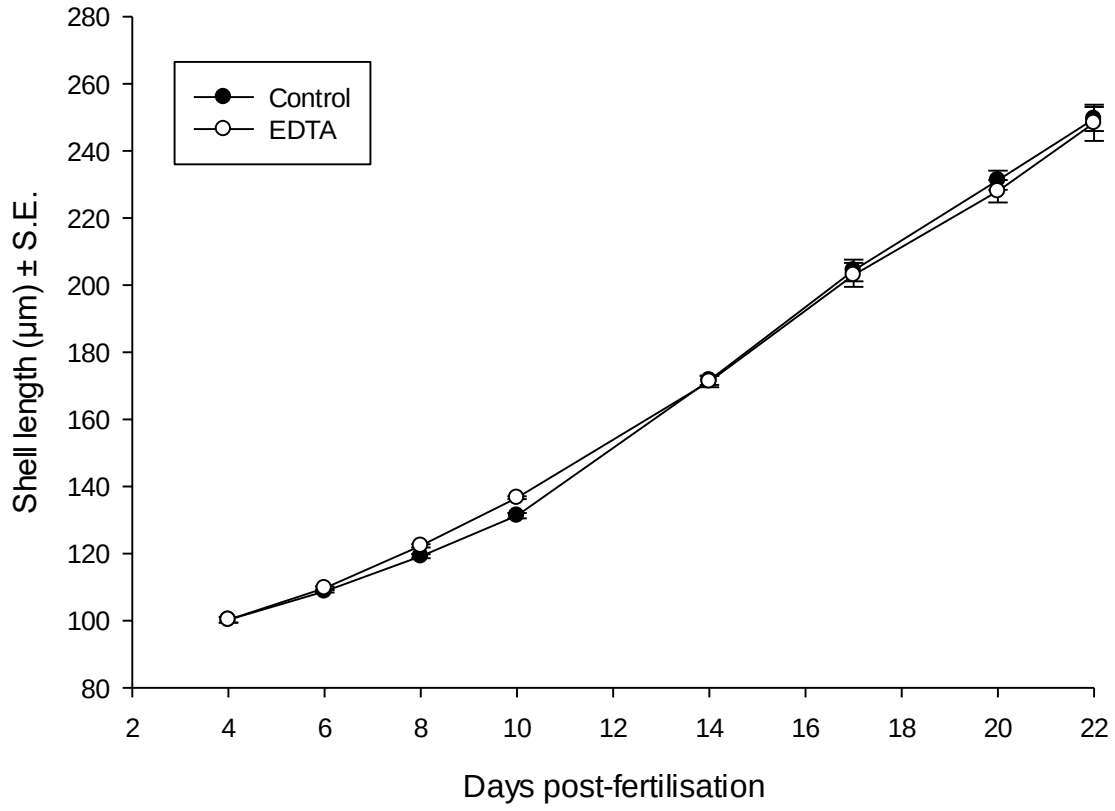

Figure 3.2. Mean shell length of mussel larvae reared in EDTA treated seawater (solid markers) and seawater without EDTA (hollow markers) from D4 to D22 (± SE).

3.3.3 Swimming activity

There was a statistically significant interaction between treatment and days PF ($p < 0.001$). The swimming activity of mussel larvae depended on what day it was measured (Figure 3.3). On D10, larvae from the control treatment (78% ± 5%) were significantly more active than larvae from the EDTA treatment (27% ± 20%, $p < 0.001$; Figure 3.3). The opposite was observed on D14, when larvae from the EDTA treatment were significantly more active than larvae from the control treatment ($p < 0.001$; Figure 3.3).

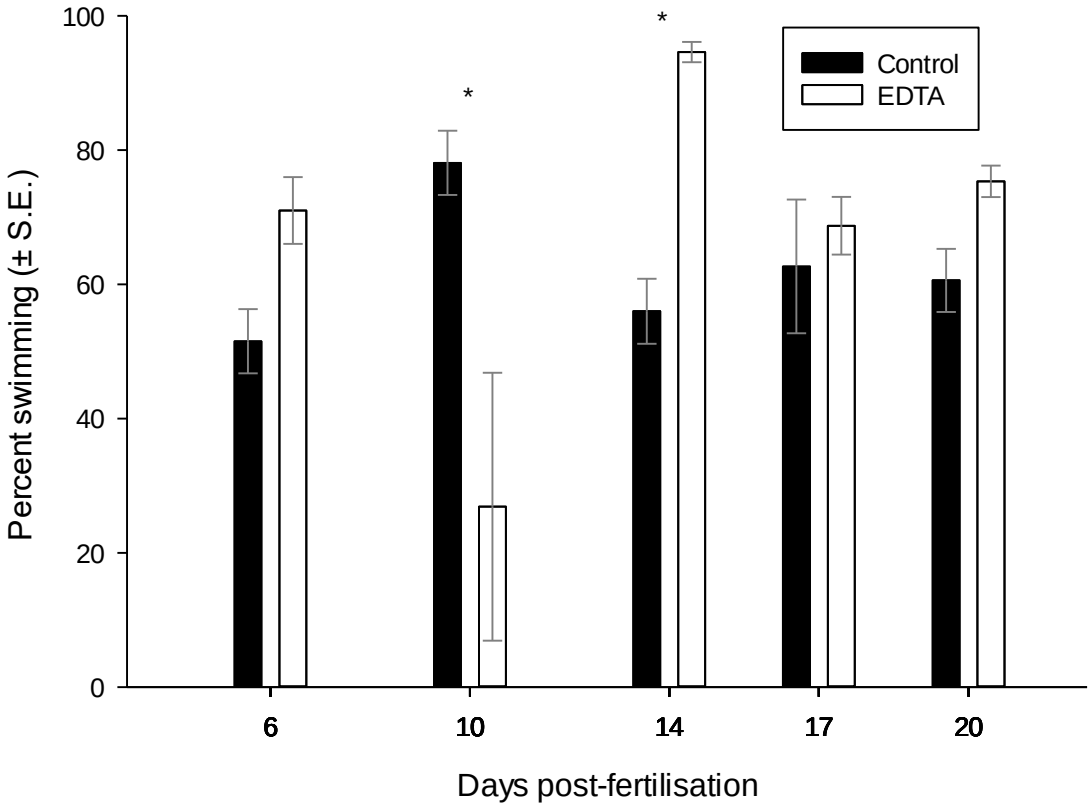

Figure 3.3. Mean percentage of swimming larvae reared in EDTA treated seawater (black) and without (white) from D6 to D20 (±SE). Asterisks indicate significant differences between the control treatment and the EDTA treatment ($p < 0.001$).

3.3.4 Feeding rate

Throughout the trial, feeding rates increased for both treatments, starting at ≈ 2,000 cells/larva/day on D4 and finishing at ≈ 17,000 cells/larva/day at D22. Overall, there was no significant difference in the mean feeding rates between the control and EDTA treatment (Figure 3.4, $F = 0.508$, $p = 0.496$). However, feeding rate was significantly affected by the use of 12 µM EDTA at D20 (Figure 3.4, Tukey $q = 3.120$, $p = 0.033$). No significant differences in feeding rates were observed on any other assessment day ($p > 0.05$, Figure 3.4). There was not a significant interaction between treatment and days PF ($p = 0.200$).

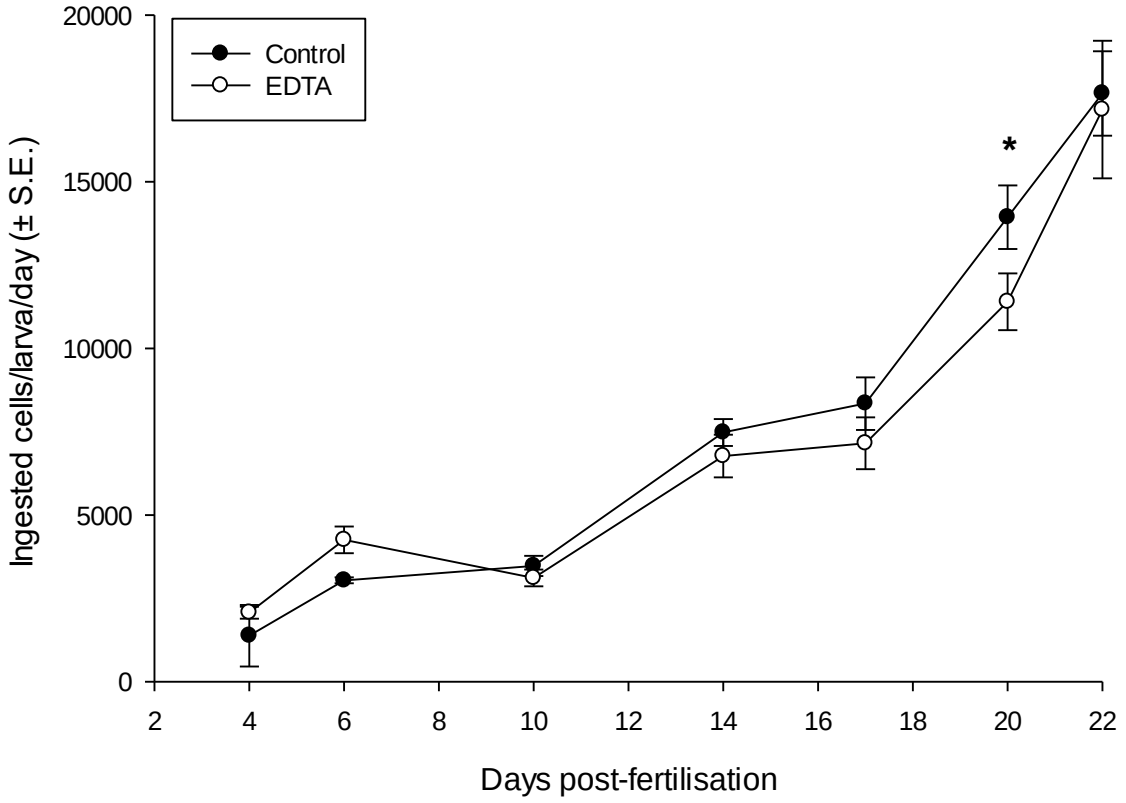

Figure 3.4. Mean feeding rate of mussel larvae reared in control seawater (black) and EDTA-treated seawater (white) from D2 to D22, expressed as number of algal cells ingested per larva per day (±SE). Asterisk indicates a significant difference between the control treatment and the EDTA treatment on D20 ($p = 0.033$).

3.3.5 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^{-1} and 19.6 ± 0.5 μg g^{-1}, respectively (Figure 3.5). 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 ($p < 0.05$; Figure 3.5). In particular, the concentration of total cadmium (Cd) in D-stage larvae increased from 0.009 ± 0.001 μg g^{-1} to 6.668 ± 2.895 μg g^{-1} ($t = 7.394$, $p = 0.002$, Figure 3.5). Similarly, total concentration of mercury (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 ($t = 92.6$, $p < 0.001$, Figure 3.5).

In contrast, the levels of zinc (Zn) and arsenic (As) in the D-stage larvae were significantly lower than the eggs ($p < 0.001$, Figure 3.5). Iron (Fe) and copper (Cu) concentrations were not affected by the use of EDTA during the 48 h incubation of mussel embryos (Figure 3.5), while lead (Pb) remained below detectable levels in both mussel eggs and D-stage larvae (i.e., < 0.001 µg g^{-1}).

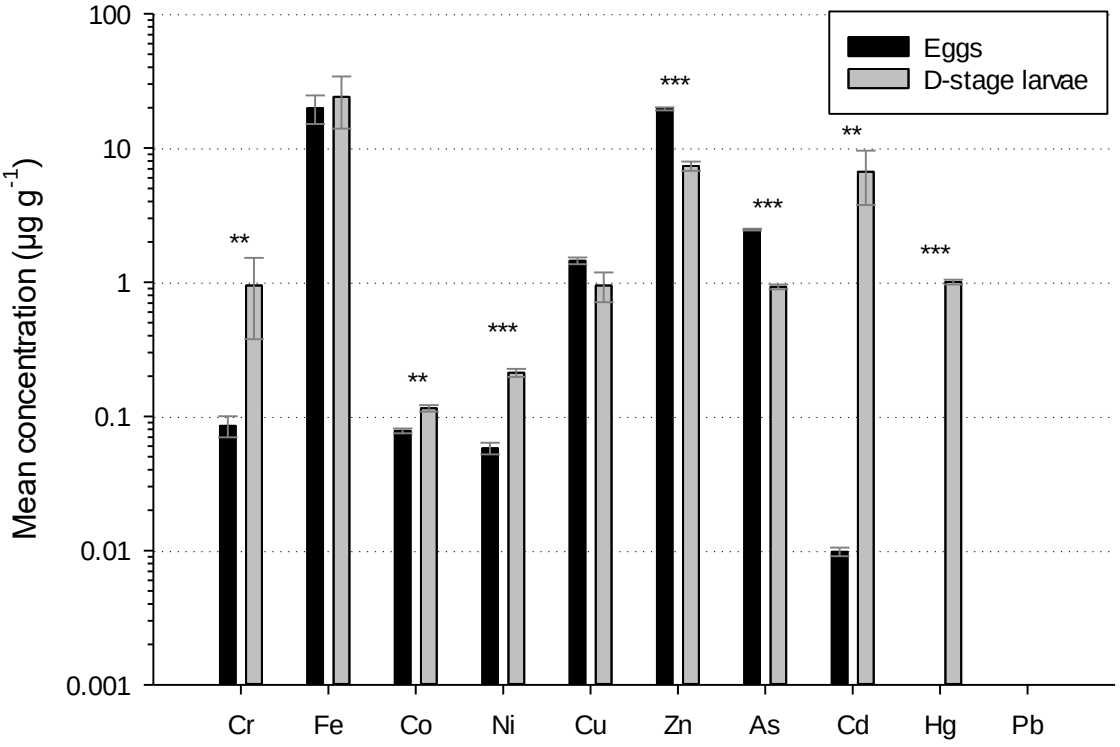

Figure 3.5. Mean concentrations of chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) in unfertilized mussel eggs (black) and subsequent D-stage larvae (grey) following 48 h incubation in 12 µM EDTA-treated seawater. Values expressed in µg g^{-1} dry matter (or ppm) ± SE with the concentrations presented 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} ($t = 11.707$, $p = 0.002$, Figure 3.6). 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} ($p = 0.03$, Figure 3.6). Concentrations of the other assayed metals in the larvae from the control treatment were not different from those from EDTA-treatment ($p > 0.05$, Figure 3.6).

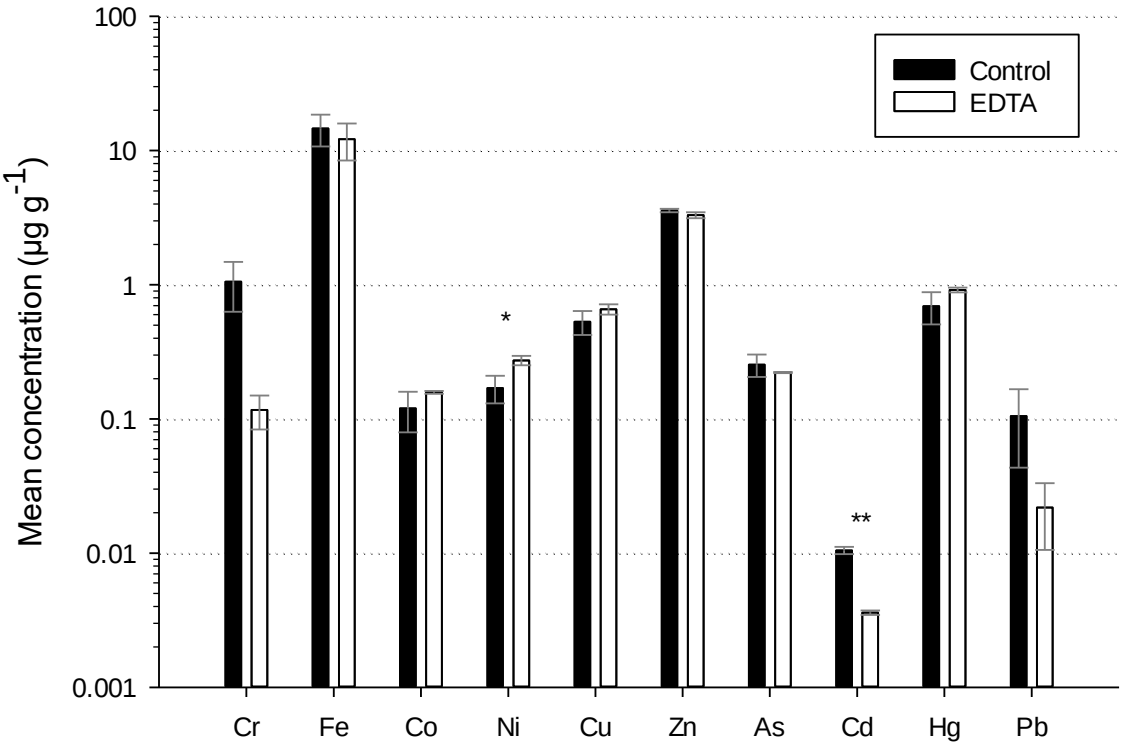

Figure 3.6. Mean concentrations of chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) measured in 10-day-old larvae reared in the control treatment (black) and EDTA-treated FSW (white), expressed in µg g^{-1} dry matter (or ppm) ± SE with concentrations presented on a log scale. Asterisks denote a statistical difference compared with the corresponding control treatment (Student's t-test *: $p < 0.05$; **: $p < 0.01$).

In 22-day-old larvae (D22)

Concentrations of zinc, cadmium, and mercury were significantly lower in the 22-day-old larvae reared with EDTA treatment compared to the control treatment ($p < 0.05$, Figure 3.7). The greatest difference was observed for cadmium which had a concentration three times higher in the control treatment than the EDTA treatment, whereas the concentrations of both zinc and mercury were less than double in the control treatment (Figure 3.7).

In contrast, mean concentrations of nickel and arsenic were significantly higher at D22 in larvae reared with EDTA treatment compared to the control treatment ($p < 0.05$, Figure 3.7). The greater difference was observed for arsenic which had 30% higher concentration in the EDTA treatment than the control, whereas the concentration of nickel was 20% higher in the EDTA treatment (Figure 3.7).

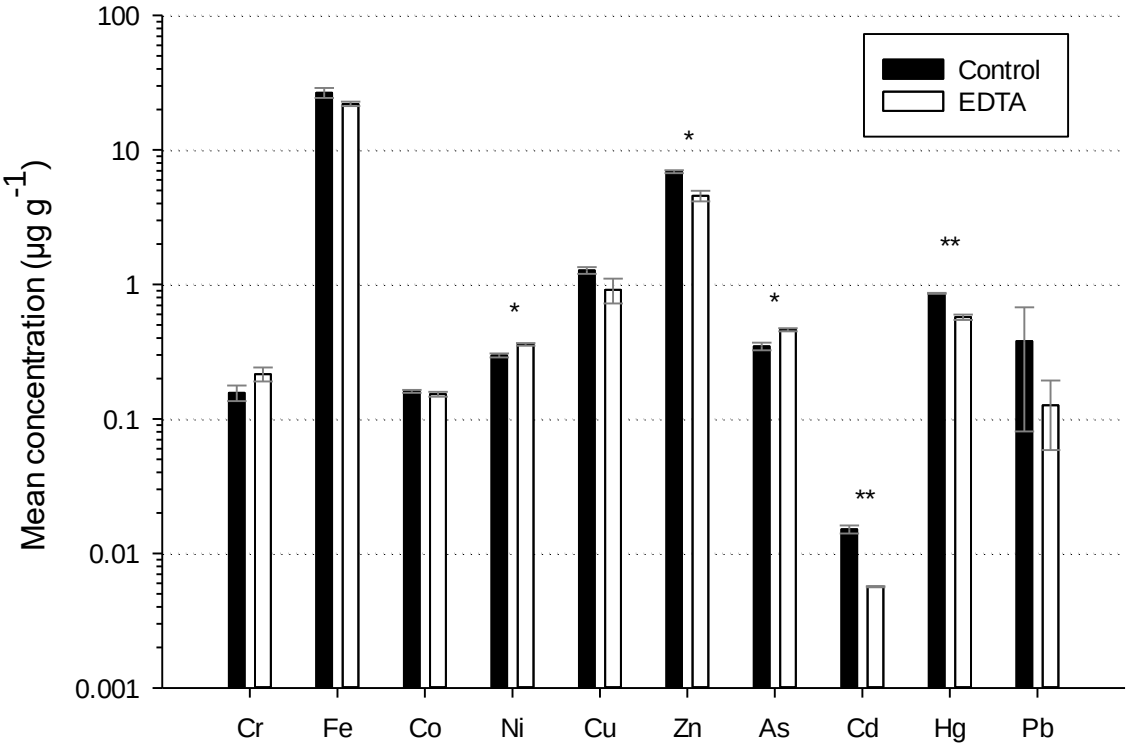

Figure 3.7. Mean concentrations of chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) measured in 22-day-old larvae reared in the control treatment (black) and EDTA-treated FSW (white), expressed in µg g^{-1} dry matter (or ppm) ± SE with concentrations presented on a log scale. Asterisks denote a statistical difference compared with the corresponding control (Student's t-test *: $p < 0.05$; **: $p < 0.01$)

3.4 Discussion

The highly effective use of the chemical EDTA is poorly understood in larval shellfish rearing and it is not known at what point of development it is required. Green-lipped or Greenshell™ mussels (*P. canaliculus*) provide an opportunity to further understand the role of EDTA usage in shellfish larval rearing. As for many other shellfish species, the yield of healthy D-stage green-lipped 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 EDTA in the seawater (Utting & Helm, 1985; Helm, 2004; Gale et al., 2016). 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 during this period of larval development compared to later stages of development (Connor, 1972; Beiras & His, 1994; Q. 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 European lobster (*Homarus gammarus*) all were shown to be considerably more sensitive to copper, zinc and mercury 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 calcium 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.

Some heavy metals were present at concerning concentrations in the ova at the outset of this experiment (Figure 3.5). The adult mussels were obtained from the Marlborough Sounds, and will have bio-accumulated these metals from the seawater there. As the ova were pooled

together, all tanks had the same concentrations of heavy metals in the ova at fertilisation, therefore any effect of these metals on the subsequent development of embryos should have been consistent for both the control and the EDTA treated tanks. Comparisons of concentrations within larvae with concentrations in the ova 48 h earlier provide insight as to where the heavy metals originated. Chromium, cobalt, nickel, cadmium and lead concentrations were significantly higher in the 2 day old larvae than in the ova, suggesting these metals were accumulated from the seawater over the first two days of development. In contrast, concentrations of zinc and arsenic were significantly lower in the 2 day old larvae than in the ova. This may be due to these metals leaching out of the ova as they developed or could be due to the increase in mass of the larvae, diluting the concentrations of the metals that remained from the ova.

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

Zinc is an essential element, required by larvae as a coordination ion for a variety of proteins, including carbonic anhydrase, which is vital for shell mineralisation (Miyamoto et al., 1996). However, zinc 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 zinc in the seawater from 125 μg L^{-1} 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 zinc up to 100 μg L^{-1} have no apparent effect on the embryonic development of *C. gigas* (Watling, 1982).

Overall, survival of control larvae was not significantly different to the EDTA treated larvae (Figure 3.1). Both treatments had roughly 50% survival at the end of the 22 day experiment. 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. 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 the cadmium and mercury bioavailability.

Cadmium concentrations were high in D-stage larvae (6 ± 3 µg g^{-1}, Figure 3.5), and decreased considerably over time resulting in much lower concentrations in 10-day-old larvae (Figure 3.6). This decrease was greater for larvae grown with EDTA treatment than the control (Figure 3.6). It could be possible that EDTA binds with cadmium 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 cadmium poisoning (Andersen, 1984). As the larvae grow over the 22 day period, the cadmium is most likely diluted by the increase in biomass (Figure 3.7). Furthermore, this reduction of cadmium concentration over time is further increased with the presence of EDTA in the seawater throughout larval development (Figure 3.7).

Nickel and arsenic are typically anthropogenic, non-essential elements for bivalve larvae, and arsenic is known to be highly toxic and bioavailable for developing larvae (Zamble, 2015; Moreira et al., 2018). In this study, EDTA treated larvae had significantly more nickel and arsenic than control larvae on D22 (Figure 3.7). This corresponds with previous results where arsenic concentration is increased for *P. canaliculus* D-stage larvae (up to 2 days PF) (Chapter 2). Therefore, another cost of using EDTA treatment of seawater for larval development is the potential to increase arsenic bioavailability. Therefore, alternatives to EDTA for reducing the toxicity of metals while not increasing the bioavailability of arsenic should be considered.

3.5 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 later stages of larval development. Consequently, the effectiveness of using EDTA in 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 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.

4. Biodegradable chelating agent improves the survival of early larvae for shellfish aquaculture

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4.1 Introduction

The widespread use of EDTA continues to be of significant economic importance to the aquaculture industry, underpinning greater reliability and productivity from shellfish hatcheries. Unfortunately, EDTA is not very biodegradable and will accumulate in the environment, leaching and redistributing toxic metals along with it (Bucheli-Witschel & Egli, 2001; Oviedo & Rodriguez, 2003; Tandy et al., 2004; Nowack & VanBriesen, 2005). Hatchery facilities typically discharge their used seawater, which would contain the dosed EDTA, back into the marine environment, where it could be expected to persist. While the use of EDTA in aquaculture is only minor compared to extensive use in household and industrial applications, its widespread importance in terms of ensuring effective hatchery production makes it prudent to be assessing alternative options in the event of future regulatory interventions.

From a close examination of the scientific literature this study, appears to be the first to evaluate the effectiveness of a biodegradable alternative to EDTA, ethylenediaminedisuccinic acid (EDDS) for use as a metal chelating agent in shellfish hatcheries. EDDS has a higher binding affinity than EDTA at pH 7 for copper and zinc, both metals for which shellfish larvae are typically highly sensitive (Tandy et al., 2004; Pinto et al., 2014). EDDS has increasingly been used for industrial processing by detergent, pulp, and paper manufacturers, and is commercially available under the name Enviomet™ by Innospec (Pinto et al., 2014). One reason why EDDS has not replaced EDTA in all applications is because it has a poor affinity for calcium and magnesium (Takahashi et al., 1997). Calcium and magnesium concentrations are only problematic for shellfish at unnaturally high concentrations, therefore binding of these metals with a chelating agent is not necessary during shellfish larval development. The SS-isomer of EDDS is considered biodegradable according to the OECD guidelines (Takahashi et al., 1997; Jaworska et al., 1999; Nörtemann, 2005). For example, a concentration of 0.8 mM can be completely degraded in 16 days when exposed to activated sludge, which is a commonly used step in wastewater treatment plants and would result in the chelated metals being complexed

by the organic sludge (Takahashi et al., 1997), and a concentration of 1 mg kg^{-1} can be completely degraded in soil within 28 days (Schowanek et al., 1997).

In this study the feasibility of using the biodegradable SS-isomer of EDDS at the same concentrations that EDTA is applied in shellfish hatcheries was evaluated in raising larval embryos of New Zealand Greenshell™ mussels (*Perna canaliculus*), a regionally important aquaculture species, over 48 h, from fertilised eggs to the D-shaped veliger larval stage (Jeffs et al., 1999). This first stage of larval development is the most vulnerable to metal toxicity in this species, and for many other shellfish species (Calabrese et al., 1973; Calabrese et al., 1977; Ringwood, 1990).

4.2 Materials and Methods

4.2.1 Adult mussel collection and spawning

Adult *P. canaliculus* in reproductive condition were collected from mussel farms in the Marlborough Sounds near Nelson, New Zealand on 29 November 2018 and taken to a nearby shellfish hatchery operated by SPATNZ Ltd. At the hatchery the outer shells of the mussels were gently scrubbed clean of biofouling. The mussels were then placed in shallow plastic spawning trays and subjected to thermal cycling until spawning began (Helm, 2004). The spawning female mussels were removed from the spawning trays and placed in individual spawning containers (5 L buckets containing 25 ± 1 °C seawater).

4.2.2 Experimental design and treatments

Fifty 2.15 L bullet-shaped larval rearing tanks that are highly effective for raising *P. canaliculus* larvae were used for the larval rearing experiment (Ragg et al., 2010), and the tanks were prepared by filling with filtered seawater (FSW), i.e., 5 µm, activated carbon and UV treated. The source seawater was pumped from an intake located 200 m off the coast from the hatchery and stored in high density polyethylene holding tanks until filtration and use in this experiment. Air was delivered at the base of each larval rearing tank to create gentle bubbling through the seawater for the duration of the experiment, circulating the seawater and maintaining the larval embryos in suspension.

Stock solutions (200 mL) of disodium EDTA (Sigma Aldrich CAS: 6381-92-6) and trisodium EDDS (Sigma Aldrich Product Number 92698) at 100 mM in type 1 deionised water were

prepared in acid-cleaned glass bottles. Two concentrations (3 and 12 µM) of the two chelating agents were each used in ten randomly selected tanks by adding the appropriate volume (64.5 µL or 258 µL, respectively) of stock solution. In this way four different chelating agent treatments (i.e., EDTA at 3 and 12 µM, EDDS at 3 and 12 µM), each with ten replicates per treatment, were prepared. In addition, ten randomly selected larval rearing tanks were used as controls and had no chelating agent added. All the tanks were kept at a temperature of 16 °C throughout the experiment.

4.2.3 Fertilisation

An aliquot of 150 µL of mussel ova from the pooled suspension and 10 µL of pooled mussel sperm was added into fifty 100 mL beakers each containing 50 mL of the seawater from each larval rearing tank. After 10 minutes allowing fertilisation to occur in these high density beakers, the 50 mL of seawater was then poured into its respective larval rearing tank.

4.2.4 Larval yields

After 24 h of development post-fertilisation, three 1 mL aliquots of seawater were sampled at random from each larval rearing tank and a drop of Lugol's iodine was added, and the sample was examined under binocular microscope to count the numbers of embryos. After 48 h post-fertilisation, three 1 mL aliquots were obtained from each rearing tank, a drop of Lugol's iodine added, and the number of D-shaped larvae were counted under a microscope. The percentage D-yield for each tank was estimated by dividing the mean of the counts of D-shaped larvae by the mean initial total counts of embryos and multiplying by 100. Larval yield is defined as the percentage of embryos that develop into D-veliger larvae with no shell deformities and a straight D-hinge.

4.2.5 Development

The fertilised embryos were allowed to develop over a period of 48 h over which time they would normally progress to the D-shaped, veliger larval stage of development. After 48 h, for each of the five seawater treatments, 4 mL of seawater was pipetted from each of the ten replicate tanks and combined into one 50 mL centrifuge tube. Then the contents of all ten tanks from each treatment were poured through a 40 µm nylon mesh sieve. Retained D-larvae were

removed from the sieve with a Pasteur pipette and some were transferred into their respective 50 mL centrifuge tube for each treatment, containing 40 mL of the respective seawater. The 50 mL tubes were refrigerated at 4 °C until use in full for X-ray Fluorescence Microscopy (XFM). The remaining larvae from each tank within each treatment were put into 1.7 mL micro centrifuge tubes. These larvae were allowed to settle before the seawater was decanted off. The tubes were then snap frozen in liquid nitrogen prior to transport to the laboratory and remained frozen at -20 °C until stored in an oven overnight at 60 °C to dry before proceeding to analyses for metal content with Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).

4.2.6 Metal content analysis

All D-larvae samples were microwave acid digested as follows. A weighed sample of the oven dried larvae was placed in a 100 mL Teflon tube. One mL of H_2O_2 and 3 mL of concentrated HNO_3 (69%) was added to each tube, and screw-on caps used to seal the tubes, before 70 min of digestion in a microwave digestion system (Milestone Srl, Sorisole, Italy), at a temperature of 180 °C and 1800 W microwave power. Then 36 mL of type 1 deionised water was added to each tube. Each resulting solution was then analysed with ICP-MS (7700x, Agilent, Santa Clara, CA) and the concentrations of chromium (Cr), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) were back-calculated for the original samples. The ICP-MS was operated in helium 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) as well as a blank matrix-matched solution containing no single element standards. An online internal standard of 20 ppb yttrium (Y) & terbium (Tb) was used to monitor and correct for instrument drift and matrix effects.

4.2.7 X-ray Fluorescence Microscopy

Sample Preparation

Silicon nitride (SiN) windows with a 2 mm^2 aperture were prepared by the Melbourne Centre for Nanofabrication for mounting the samples of D-larvae. A simple method was used for the preparation of samples ready for examination under XFM given that previous research has found that minimizing the preparation steps of biological samples for analysis of metal content is vital to ensure that the metals are observed in as close to their native state as possible (New

et al., 2018). Using a Pasteur pipette ~ 0.5 mL of concentrated suspension of D-larvae were transferred from the bottom of the refrigerated larval sample tubes (50 mL) and into 1 mL of deionized water in an Eppendorf tube to decrease the salt concentration. The Eppendorf tube was then shaken and 0.5 µL of the resulting suspension was extracted with an autopipette and was carefully placed in the centre of a SiN window. The window was dried by placing it on a clean Kimwipe tissue on a hot plate set to 40 °C. The dried droplet was observed under a binocular optical light microscope to ensure sufficient numbers of isolated D-larvae were present for each sample. This was repeated four to five times for the D-larvae from each treatment.

Sample examination

The monochromatic x-ray beam on the XFM was focused down to a spot size of approximately 2 × 2 µm using the KB-mirror system at the XFM beamline at the Australian Synchrotron. Using an incident photon energy of 15.8 keV enabled the analysis of elements ranging from phosphorus (P) to selenium (Se) via K-shell excitation and mercury (Hg) and lead (Pb) via L-shell excitation (Paterson et al., 2011). For each measurement, D-shaped larvae were located on the SiN window and coordinates for each specimen were obtained and set for scanning. Each larva was scanned continuously through the focused beam and x-ray fluorescence spectra were collected using the Maia 384D22 detector (C. Ryan et al., 2014). The sample holder allowed scanning of each larva with 1 µm accuracy and fluorescence events were recorded automatically from an area of 1 × 1 µm at a time to obtain the areal distribution of elements in the D-larvae. All D-larvae were scanned at a rate of 10 µm s^{-1}. The analysis of the raw fluorescence spectra was done using GeoPIXE software (C. G. Ryan et al., 2005). Standards were scanned and fluorescence counts for the known concentrations in the standards were used in conjunction with the software fitting of fluorescence spectra to determine concentrations of calcium (Ca), chromium (Cr), iron (Fe), nickel (Ni), copper (Cu), zinc (Zn) and arsenic (As) in each larva examined. Using GeoPIXE software, the area covered by an individual D-larva was selected and the concentration of each of these seven metals for that area was recorded in units of µg g^{-1}, for ten randomly selected larvae per treatment. The mean concentration ± S.E. from the ten larvae was then calculated for each metal in each treatment. The possibility of artefacts resulting from X-ray induced damage was assessed through inspection of large-area scans taken before and after fine detailed scanning. Negligible differences in count numbers between the before and after scans were interpreted to indicate that there was no significant elemental redistribution resulting from the measurement procedure.

4.2.8 Statistical analyses

Larval D-yield data were tested and confirmed for normality and homogeneity of variance and were subsequently used in a one-way analysis of variance (ANOVA) to test for differences in larval D-yield among the five seawater treatments. Where the ANOVA was significant, pairwise comparisons of means were conducted with Tukey's tests.

All larval metal concentration data from ICP-MS and XFM were tested for normality and homogeneity of variance prior to analyses and if compliant a one-way ANOVA was used to test for differences among the treatments. If the data were found to be noncompliant (i.e., failed either Shapiro–Wilk's or Bartlett's tests) they were log transformed and rechecked for compliance. If they remained non-compliant, a Kruskal-Wallis test was used to compare the results among treatments. Where the ANOVA was significant, pairwise comparisons of means were conducted with Tukey's tests.

4.3 Results

4.3.1 Larval yields

A one-way ANOVA analysis showed that the yield of D-larvae after 48 h was significantly different among the five treatments ($F = 4.426$, $p < 0.05$) (Figure 4.1). The control and EDDS 3 µM treatments had the lowest mean yield of D-larvae, while the mean yield for EDTA 3 µM, EDTA 12 µM, and EDDS 12 µM treatments were all significantly higher but not different from one another (Figure 4.1, $p < 0.05$). The mean yield of D-larvae in the control treatment without the addition of any chelating compound (i.e., 37.4 ± 9.7%) was less than half the yields in the EDTA 12 µM, and EDDS 12 µM treatments (i.e., 89.6 ± 12.9% and 90.2 ± 9.6% respectively) (Figure 4.1). The mean yield of D-larvae in the EDDS 3 µM treatment was not different to any other treatments, being intermediate to the low yields of the control treatment and the high yields of the EDTA 3 µM, EDTA 12 µM, and EDDS 12 µM treatments (Figure 4.1).

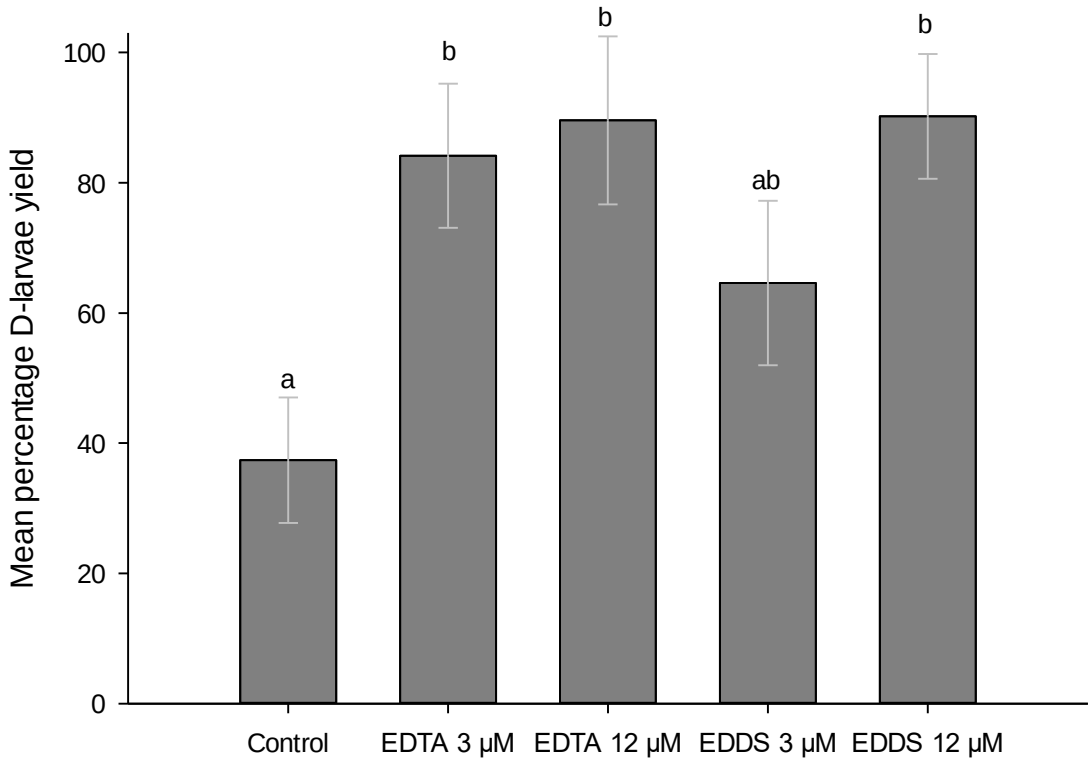

Figure 4.1. Mean yield of D-larvae ± S.E. of *P. canaliculus* after 48 h after rearing in seawater with five different treatments of metal chelating agents. Different letters above bars indicate a significant difference between treatments (Tukey's test, $p < 0.05$).

4.3.2 Metal content in larvae

ICP-MS

For the ten heavy metals assayed in the experimental D-larvae with ICP-MS there were only significant differences among treatments detected for cobalt (Co, $F = 7.256$, $p < 0.05$), zinc (Zn, $F = 6.055$, $p < 0.05$) and mercury (Hg, $F = 9.734$, $p < 0.05$) (Figure 4.2). Cobalt (Co) concentrations in D-larvae were lower in the control treatment compared to all other treatments except the 12 µM EDDS treatment ($p < 0.05$, Figure 4.2). Concentrations of zinc (Zn) in D-larvae were higher in the control treatment compared to the 12 µM EDDS and 12 µM EDTA treatments, but not compared to the 3 µM EDTA treatment (Figure 4.2). Concentrations of mercury (Hg) in D-larvae were lower in both EDDS treatments compared to both EDTA treatments, but not compared to the control ($p < 0.05$, Figure 4.2). Overall, the metal with the highest concentration of the ten metals measured in larvae was iron which ranged from 15 - 65 µg g^{-1} regardless of treatment (Figure 4.2). The metal with the lowest concentration was cobalt which ranged from 0.1 – 0.3 µg g^{-1} regardless of treatment (Figure 4.2).

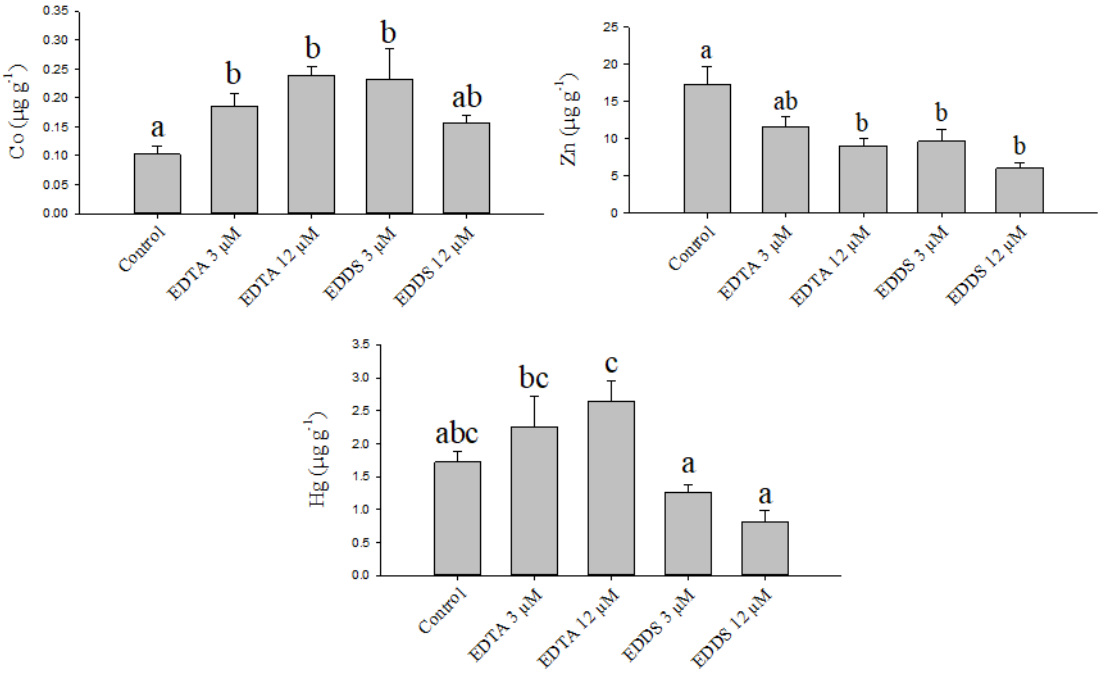

Figure 4.2. Mean concentrations ± S.E. of cobalt (Co), zinc (Zn), and mercury (Hg) in D-larvae of *P. canaliculus* after 48 h of rearing in non-treated control seawater and seawater with four different treatments of metal chelating agents. Letters indicate a significant difference between treatments for each metal (ANOVA, $p < 0.05$).

XFM

Abnormal shell formation was rarely found in the chelating agent treated samples, whereas most larvae in the control were abnormally shaped, indicating embryo-larval toxicity of the water without chelating agent.

In all samples the concentrations of cobalt (Co), cadmium (Cd), mercury (Hg) and lead (Pb) were consistently below detection limits of the XFM. For the seven metals assayed within individual D-larvae with the XFM there were significant differences among treatments detected for all except copper (Cu, Figure 4.3). Calcium (Ca), chromium (Cr), iron (Fe) and nickel (Ni) concentrations in D-larvae were lower in the control treatment compared to all other treatments except the 3 µM EDTA treatment ($p < 0.05$, Figure 4.3). In contrast, zinc (Zn) concentrations in D-larvae were higher in the control treatment compared to all other treatments except the 3 µM EDTA treatment ($p < 0.05$, Figure 4.3). Arsenic concentrations were lower in the 3 µM EDTA treatment, compared to the 12 µM EDTA and 12 µM EDDS treatments ($p < 0.05$, Figure 4.3), but were not significantly different compared to the control and 3 µM EDDS treatments ($p > 0.05$, Figure 4.3).

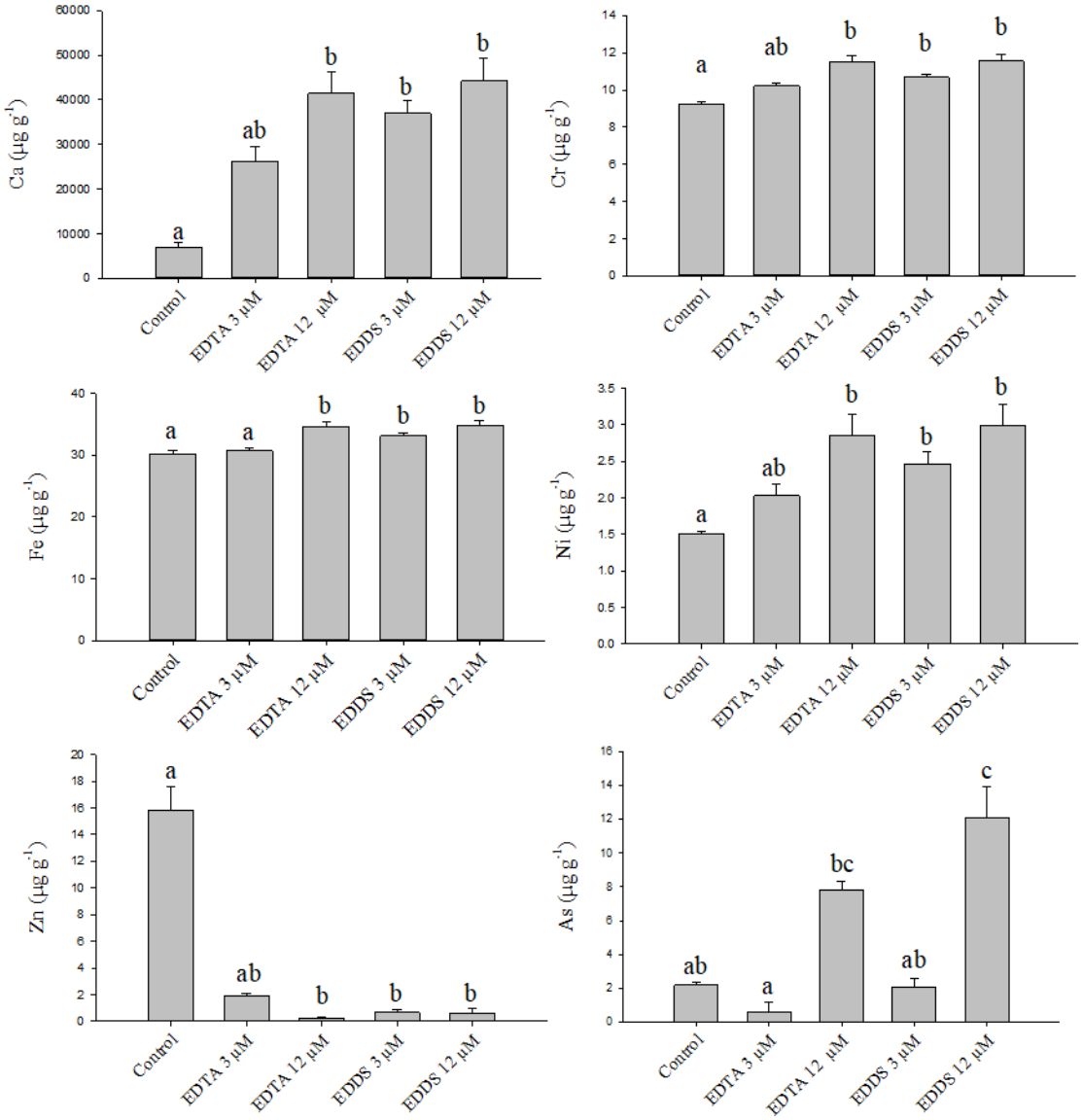

Figure 4.3. Mean concentrations ± S.E. of six different metals in D-larvae of *P. canaliculus* after 48 h of rearing in non-treated control seawater and seawater with four different treatments of metal chelating agents. The six metals with concentrations quantified by XFM were calcium (Ca), chromium (Cr), iron (Fe), nickel (Ni), zinc (Zn) and arsenic (As). Letters indicate a significant difference between treatments for each metal (ANOVA, $p < 0.05$).

4.3.3 Distribution of metals in larvae

The distributions of calcium, copper, zinc and arsenic within individual D-larvae differed depending on the treatment applied, while for the other metals there were no obvious differences in their distribution among the different treatments (Figure 4.4). In general, copper, zinc and arsenic were more widely distributed throughout individual larvae in the control treatment, compared to those raised in the four chelating agent treatments, which tended to have greater localisation of metal concentrations around the visceral region (Figure 4.4). Calcium appeared to be more abundant in larvae grown in both EDDS treatments and was also highly concentrated around the visceral region compared with both the EDTA treatments and the control (Figure 4.4). Zinc appeared to be in overall lower concentrations in D-larvae raised in all four metal chelating agent treatments than in the control (Figure 4.4).

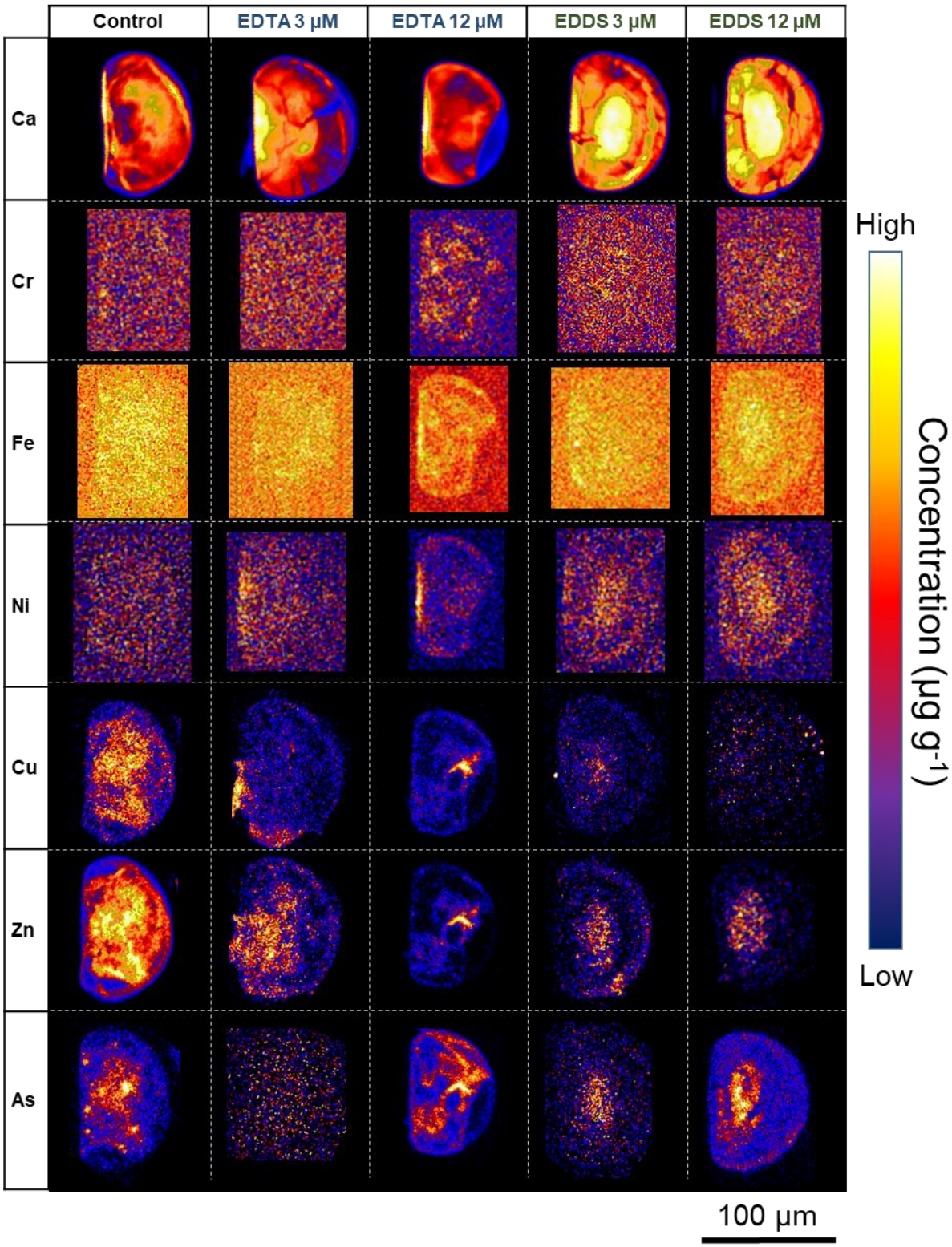

Figure 4.4 . Distributions of calcium (Ca), chromium (Cr), iron (Fe), nickel (Ni), copper (Cu), zinc (Zn), and arsenic (As) within a representative *P. canaliculus* D-larva after 48 h of rearing in seawater with five different treatments of metal chelating agents as determined using x-ray fluorescence microscopy.

4.4 Discussion

The aim of this research was to test the feasibility of using the biodegradable metal chelating agent EDDS as an alternative to EDTA which is commonly used to improve production in commercial larval culture of a wide range of shellfish species. The results of this study strongly indicate that EDDS provides similar improvements in larval survival over the first 48 h as EDTA when used at the same concentration for larval rearing the mussel, *P. canaliculus* (Figure 4.1). Yields of D-larvae after 48 h were doubled in both EDTA and EDDS at 12 µM compared to the control without the addition of a metal chelating agent (Figure 4.1).

There was no direct consistency between the ICP-MS measures of the various metals in the mussel embryos and the serial concentrations of the two chelating agents that would offer an immediately obvious explanation for the differences in embryo survival. However, the toxicity of heavy metal ions in seawater for bivalve shellfish are well known for their non-linear effects over concentration and time (Mandich, 2018).

As reported previously, the addition of chelating agents increased the accumulation of several metals by D-larvae, which resulted in higher concentrations of metals found in their tissues (George & Coombs, 1977; Hung, 1982). Calcium (Ca) in particular, increased in concentration when it was presumably accumulated with EDTA or EDDS. The fate of the chelating agents if they accumulated in the larvae remains unknown, but this did not result in an adverse effect on the survival of D-larvae. It has been previously reported that accumulation of the isolated molecule of EDTA does not present a risk of bioaccumulation, and that the dissolution and bioavailability of heavy metals bound with the EDTA are worthy of far greater attention (Oviedo & Rodriguez, 2003).

The mean concentration of calcium (Ca) in D-larvae measured with XFM was higher when larva were raised with EDDS or 12 µM EDTA treatments compared to the control or 3 µM EDTA treatments ($p < 0.05$, Figure 4.3). This suggests an increase in calcium uptake occurs with the addition of chelating agent. Calcium carbonate shell formation is dependent on the free Ca^{2+} concentration within the developing trochophore and D-larvae over the first 48 h of incubation, starting with formation of amorphous calcium carbonate, which then partially transforms into crystalline aragonite (Weiss et al., 2002; Marin et al., 2008). Successful shell formation is vital for the survival of developing mollusc embryos as it provides support and protection for the tissues of the soft-bodied organisms (Marin et al., 2008), and the benefits of

increased calcium levels within D-larvae are apparent in the improved D-yields observed in this study with chelating agent treatments (Figure 4.1).

Zinc (Zn) concentrations found with both ICP-MS and XFM were markedly lower in D-larvae treated with chelating agent compared with the control (Figure 4.2, Figure 4.3, $p < 0.05$). Zinc is an essential metal for shellfish larvae, however, it can be toxic at sufficient concentrations of the free Zn^{2+} ion state (Brereton et al., 1973; Boyden et al., 1975; Akberali & Earnshaw, 1982; Arkless, 2005; Nadella et al., 2009; Nadella et al., 2013). The concentrations of zinc in the hatchery seawater at the time of this study, may therefore have been at problematic levels for *P. canaliculus* development, and the addition of chelating agent may have mitigated the problems caused by free Zn^{2+} ions in the water, by reducing their bioavailability. Without the addition of chelating agents, the D-yield of larvae was significantly lower, and increased internal zinc concentration may have been a contributing factor towards their morbidity. Furthermore, with chelating agent treatment, zinc was localised in certain areas of D-larvae, especially in the visceral region, rather than being distributed widely throughout their shell and body (Figure 4.4). This is consistent with the finding in the previous study which used only 3 µM EDTA (Chapter 2).

When analysed with ICP-MS, there was no significant difference in mean iron (Fe) concentrations among the five treatments (Figure 4.2). However, when observing individual larvae under XFM, the mean concentration of iron (Fe) in D-larvae was significantly higher when treated with EDDS or 12 µM EDTA compared to the control or EDTA 3 µM treatments (Figure 4.3, $p < 0.05$). Therefore, there seemed to be a greater accumulation of iron when it was bound with EDDS than with EDTA overall. This occurred despite the very similar binding affinities EDDS and EDTA have for iron (Tandy et al., 2004; Pinto et al., 2014). Furthermore, if iron is accumulating more with EDDS, it does not adversely affect the survival of *P. canaliculus* larvae, which may be because it is not present in the free ion state and is accumulating while bound with EDDS. It has been previously reported that the application of EDTA reduces levels of oxidative stress in *P. canaliculus* (Gale et al., 2016). One of the leading causes of oxidative stress is raised reactive oxygen species (ROS) levels, and increases in unbound Fe^{2+} ion concentrations increase production of these ROS (Guerin et al., 2001). Toxic levels of iron for shellfish are not reported in the literature, however, oxidative stress caused by iron could be a contributing factor towards the lower survival rates observed when *P. canaliculus* embryos are incubated without chelating agent in the control treatment (Figure

4.1). As EDDS and EDTA have similar binding affinities for iron, it is understandable that they provide a similar improvement in survival (Tandy et al., 2004).

As observed in the previous study (Chapter 2), cobalt (Co) concentrations were significantly higher in *P. canaliculus* D-larvae incubated with EDTA than without (Figure 4.2). Interestingly, the 3 μM EDDS treatment resulted in a significant difference in cobalt (Co) concentration compared to the control while the 12 μM EDDS treatment had a similar concentration to the control (Figure 4.2, $p < 0.05$). Cobalt is an essential element as a component of vitamin B12, which is not self-produced by animals, and must be ingested (Watanabe, 2007). Vitamin B12 is often accumulated in high amounts by shellfish, through filter feeding of microorganisms in seawater (Watanabe et al., 2001). The marked differences in cobalt concentrations observed here were unlikely to be influential on the survival of the mussel larvae. Reports of the toxic or minimum required concentrations of cobalt for shellfish have not been able to be found in the scientific literature, suggesting they do not exist.

Mercury (Hg) concentrations were significantly higher in D-larvae incubated with EDTA than with EDDS (Figure 4.2, $p < 0.05$). However, concentrations of mercury (Hg) found in D-larvae incubated with either chelating agent were not significantly different from the control (Figure 4.2, $p > 0.05$). Mercury is not an essential element in any lifeform, and its presence at any concentration is a burden on organisms including shellfish (Beiras & His, 1994, 1995a; Q. Wang et al., 2009). Eliminating the increase in concentration of mercury in shellfish larvae due to the addition of EDTA as they develop can only provide benefits, and in this regard use of EDDS could be considered as superior to the use of EDTA.

The previous experiment (Chapter 2) was conducted at a different time of year (March), while this study was conducted in the following November. Furthermore, the previous study had a smaller sample size and embryos were incubated in 170 L tanks at lower larval densities, compared to the 2.15 L tanks and higher larval densities used for this study. The temperature of the room where incubation took place was 18 °C in the previous study, while it was 16 °C in this study, which is believed to be a better temperature for incubation of *P. canaliculus* embryos. In the previous study, densities were around 10,000 embryos per litre, and in this study they ranged from 70,000 to 100,000 per litre. In addition, the percentage larval D-yields for the control were much lower in the earlier experiment (3 ± 1%). The differences in the results between these experiments could have been due to different timing, tank setup, temperature, or the difference in sample sizes. Finally, there is always inherent seasonal

variation in concentrations of heavy metals in coastal water due to increased rainfall and storm water runoff during wetter times of the year (Brown & Peake, 2006).

When analysed with ICP-MS, the concentrations of most heavy metals directly measured in D-larvae did not differ among the five treatments, i.e., control, 3 and 12 µM for both EDTA and EDDS ($p > 0.05$, Figure 4.2). Those metals that differed among treatments were cobalt (Co), zinc (Zn) and mercury (Hg). However, when analysed with XFM, the concentrations of several metals in D-larvae differed significantly among the five treatments ($p < 0.05$, Figure 4.3). These differences among results obtained from the different methods of chemical analyses could be attributed to the numbers of larvae analysed, and the subjective selection of healthy larvae that XFM allows. Acid digestion of hundreds of dried D-larvae per sample, with no control over whether larvae had reached the D-shaped stage of development other than being retained on the 40 µm nylon mesh sieve, provides a potential bias in ICP-MS analysis that can be removed using XFM. Under the XFM, larvae can be selected which have a consistent D-shape, and therefore it is known that the concentrations measured are from D-larvae that have reached a similar stage of development.

It has been reported that EDTA has some antimicrobial properties, and it is possible that EDTA is affecting the microbial populations within the hatchery systems that may in turn impact larval survival (Finnegan & Percival, 2015; Percival et al., 2015). Theoretically, EDDS is capable of having the same effect. However, further research is required to improve understanding of the effect of chelating agents on microbial populations within hatchery systems.

Despite the great benefits the use of EDTA provides for the aquaculture industry, it has a very poor biodegradability and increasingly widespread use in a variety of industrial processes and household products with frequent discharge to the environment in wastewater is of growing concern due to the risk of accumulation in rivers and coastal environments (Bucheli-Witschel & Egli, 2001; Oviedo & Rodriguez, 2003). Accumulation of EDTA will redistribute metals in the environment, which could have significant ecological impacts. The results for the use of EDDS in the larval culture of mussels in this study, suggest it is a strong candidate as a biodegradable alternative to EDTA for use in commercial shellfish hatcheries.

4.5 Conclusion

This study shows evidence that the readily biodegradable chelating agent EDDS works as effectively as the non-degradable EDTA in improving the survival of developing aquaculture shellfish embryos to the D-veliger stage, and provides some greater insight into the role these chelating agents have in reducing the toxicity of heavy metals in seawater. Rather than reduce the accumulation of all heavy metals as a general intervention as previously postulated, the chelating agents appear to result in the D-larvae accumulating more of certain essential metals (e.g., calcium), while zinc had an observable reduction in concentration. This may both improve the rate and quality of shell formation and reduce the toxic effects caused by free heavy metal ions resulting in an improved survival of shellfish embryos overall.

5. General Discussion

The research presented in this thesis improves our understanding about the known benefit of applying chelating agents to shellfish hatchery seawater, particularly at the earliest stages of development. Significant improvements in survival, which were associated with lower concentrations of several toxic heavy metals, were observed with EDTA treatment over the first 2 days of *P. canaliculus* larval development (Chapter 2). These improvements in survival were obtained despite higher concentrations of cobalt and arsenic. However, continuing to raise the larvae beyond the first two days with seawater treated with EDTA did not result in differences in survival, growth, feeding, swimming activity or metal content of mussel larvae (Chapter 3). As EDTA has a poor biodegradability, EDDS was tested as a potential alternative for use in *P. canaliculus* larval rearing (Chapter 4). Similar improvements were observed in the survival of *P. canaliculus* embryos over the first 2 days of development when reared with EDDS added to the seawater to the improvements observed with EDTA, with significantly lower concentrations of zinc (Zn) and higher concentrations of calcium (Ca) observed in the embryos reared with chelating agent added to the seawater compared to embryos reared without chelating agent (Chapter 4).

The first experiment of this thesis measured the concentrations of heavy metals in *P. canaliculus* after rearing for two days from fertilisation with and without EDTA present (Chapter 2). Significant differences in heavy metal concentrations were found, but not for iron, the leading suspect for the cause of oxidative stress. However, copper was significantly reduced in concentration in *P. canaliculus* with EDTA present, indicating that copper was likely to be more problematic for the developing embryos than iron. The measured concentrations of copper in the seawater at the hatchery in the first experiment ranged between 9 and 13 ppb, and it has been reported that at a concentration of 5 ppb or higher in seawater, copper is problematic at the embryonic stage of development for several other species of bivalves, including blue mussels (*Mytilus edulis*) (Hoare, Davenport, et al., 1995). This in conjunction with the fact that the addition of EDTA to the seawater used for larval rearing significantly reduced the concentration of copper in the D-larvae that was accumulated from the seawater, suggested that this in turn may result in less oxidative stress as observed previously by Gale et al. (2016). This may have contributed towards the significant improvement in embryo development success and survival, providing for more efficient larval rearing. However, when copper was not

reduced in Chapter 4 and present in larvae at similar concentrations as control larvae in Chapter 2, there were still the significant improvements in survival with EDTA. It is likely that EDTA provides a combinatorial effect, affecting the toxicity of several metals at once, and not one particular metal. It is also likely that this combination of metals varies depending on the original seawater metal concentrations.

It is well reported in the literature that EDTA is often used for shellfish rearing for the whole larval rearing period, for shrimp (Cook, 1967) and bivalves (Helm, 2004), however, at present EDTA is only used for the first two days of *P. canaliculus* rearing. The earliest stages of the shellfish life cycle are the most vulnerable to metal toxicity (MacInnes & Calabrese, 1979), but it is becoming more common for EDTA to be used for longer as a safeguard against additional losses in yield, even as the shellfish larvae grow and develop a greater natural tolerance to heavy metals (Rainbow, 2002). Buying and using EDTA is a minimal relative cost for hatchery production of shellfish and it is relatively straightforward to apply. However, there has been a lack of research on the use of EDTA for longer periods in bivalve rearing. Questions were raised as to whether using EDTA beyond the first two days of *P. canaliculus* larval development would provide further improvements in overall hatchery spat production efficiency.

The second study presented in this thesis tested the use of EDTA against common developmental measures including shell growth, activity, and feeding as well as survival of *P. canaliculus* for the first 22 days of rearing into larvae and how this compared to rearing larvae without EDTA. After 22 days of development from fertilisation, the larvae are ready to settle and the hatchery process is complete, providing a reliable high quality seed supply for mussel farmers. Interestingly, the results of this study showed no significant differences in these biological measures with EDTA and that there would be no improvement in efficiency of *P. canaliculus* larval production if EDTA were to be used for longer than the first 2 days of larval development. This suggests the use of EDTA for longer periods in the larval rearing of other species of shellfish may not be necessary, although it may be required for other shellfish species which do not increase their tolerance of heavy metals. Therefore, further research comparing EDTA use at different stages in shellfish development is important for other species of aquaculture shellfish to improve our understanding of the actual requirement for the use of this chelating agent in larval shellfish rearing. In addition, it is important that the concentrations of heavy metals in the seawater used in aquaculture hatcheries are monitored regularly, to ensure any increases in concentration are detected, before they can impact the shellfish. If increases

in concentration are observed in the future, then increasing the concentration or the period of time that EDTA is used could be a method to alleviate the toxic effects of heavy metals. However, more sustainable water treatment methods should be considered first.

Many heavy metals are essential and reducing their bioavailability may result in problematic metal deficiencies. The concentrations of EDTA used and the amount of time they are used should be carefully examined, to ensure that new problems are not created for shellfish later on in their life cycle by using EDTA at their early stages of development.

The chelating agent EDTA is an emerging environmental contaminant that is not readily biodegradable and is accumulating in our waterways due to anthropogenic use since it was first synthesised in 1935 (Oviedo & Rodriguez, 2003). The accumulation of EDTA in aquatic environments is known to have environmental impacts, due to redistribution of heavy metals (Oviedo & Rodriguez, 2003). If heavy metals are redistributed, they could expose previously unaffected ecosystems to their toxicity.

One wider benefit of this research is that it includes a literature review of promising alternatives to EDTA with the potential to be used in larval shellfish rearing for aquaculture. Many of these alternatives have promising attributes and could be tested in the future, especially if awareness and concern around the environmental problems of EDTA use increases, and to ensure that the aquaculture industry has a strong contingency plan with regard to heavy metal toxicity. The most promising biodegradable alternative to EDTA was determined to be EDDS because of its straightforward application method and excellent metal binding properties. In the third study of this thesis EDDS was compared to EDTA when used for the first 2 days of *P. canaliculus* larval rearing. This is thought to be the first time EDDS has been applied in an aquaculture setting, as there are no known published accounts of research on alternatives to EDTA for shellfish larval rearing.

The chelating agent EDDS provided improvements in the survival of *P. canaliculus* whilst developing from fertilised ova to D-veliger larvae consistent with those observed when EDTA was added at the same concentration to the rearing seawater. The results of the study showed that the concentrations of zinc were lower and the concentrations of calcium were higher in the larvae reared with EDDS compared with the control larvae reared without chelating agent. This finding provides impetus toward using EDDS for aquaculture as a biodegradable replacement for EDTA.

The review of potential alternatives to EDTA conducted in the introduction of this thesis also found other chelating agents that could be used in larval rearing in shellfish aquaculture, including IDSA, MGDA and PAA, and depending on which metals are present in the seawater they may also prove useful in the future (Pinto et al., 2014). Techniques for adsorption of heavy metals from seawater were also reviewed using various naturally occurring substrates including clay (Celis et al., 2000), coconut fibre (Shukla & Shukla, 2013), tree bark (Vazquez et al., 2002), $CaCO_3$ shells (Thind, 2013), zeolites and chitosan (Erdem et al., 2004; Ngah et al., 2011). Methods with these more sustainable water treatments could be explored for removing heavy metals from larval rearing seawater in the future, however, they could be more disruptive to apply to a commercial shellfish hatchery and will be likely to increase maintenance costs. As shellfish hatchery production often operates with tight operating margins to ensure overall profitability, it is important to ensure that the introduction of any alternate water treatment process remains cost effective. Based on the results of this literature review and the ease of application in the conducted experiment, EDDS appears to be the most promising candidate. Future research should test the use of EDDS with other species of shellfish including oysters and shrimp, using this study with *P. canaliculus* as an example. By using EDDS in aquaculture worldwide, the pollution of our environment with EDTA could be reduced significantly.

An additional novelty of the research presented in this thesis was the first demonstration of the capability of the synchrotron XFM to map the distribution of several heavy metals and calcium within individual shellfish larvae with 1 µm resolution (Chapters 2 and 4). Synchrotron XFM is an emerging technique allowing the user to measure sub-ppm concentrations and map the distributions of elements with an atomic weight greater than sulphur. One previous study measured and mapped the distribution of copper in *Mytilus galloprovincialis* D-larvae, however, the D-larvae were reared with various concentrations of copper added to the seawater artificially (Deruytter et al., 2014). The larval rearing experiments presented in this thesis always used seawater from a commercial shellfish hatchery, with or without chelating agent added at concentrations that were used by the commercial hatchery operation, to ensure the results were as commercially relevant as possible.

Copper was previously found to accumulate more at the edges of the D-larvae of *M. galloprovincialis* and a clear hotspot was also observed in the centre of the hinge in a D-larva exposed to a copper concentration of 10 µg L^{-1} in the rearing seawater (Deruytter et al., 2014). In the first study of this thesis (Chapter 2), copper was observed to have a similar distribution in D-larvae of *P. canaliculus* that were reared in seawater in which copper occurred naturally

at a concentration measured as 10 µg L^{-1}. The knowledge gained from the initial study using the XFM on mussel larvae, facilitated a greater understanding of sample preparation and instrument capabilities. In the second study a different detector and measurement method to those used in the initial study were applied, which provided greater confidence of the relative distribution of the metals in the larvae. Additional knowledge gained by the current research, was that zinc, iron, arsenic and mercury were often distributed in a similar way within the D-larvae of the mussels to the distribution of copper previously reported by Deruytter et al. (2014).

5.1 Larval incubation temperature

In the first experiment (Chapter 2), larval incubation of *P. canaliculus* took place at 20 °C. The highest yield of D-larvae obtained in any of the tanks in that experiment was with 3 µM EDTA added (i.e., 57% D-yield), which was lower than with 3 µM EDTA added in the subsequent experiments (i.e., ≥ 85% D-yield) (Chapters 3 & 4). After observing the low larval yields in the first experiment, it was hypothesised that the temperature was too high, and in the following experiments the larval incubation temperature was lowered to 16 °C, to optimise conditions for larval rearing. In the subsequent experiments (Chapters 3 & 4), D-yields after 2 days of incubation with EDTA at 16 °C were consistently much improved (≥ 85% D-yield). Furthermore, the D-yield in the control treatment without EDTA was improved by an order of magnitude after reducing the rearing temperature from 20 °C to 16 °C (from 3% to 37%, respectively). It has been previously reported that there is an effect of temperature on metal toxicity for marine and estuarine invertebrates, including shellfish, at any stage of their life cycle and consequently this temperature difference may have contributed to the observed differences in the yields of D-larvae observed among the experiments (MacInnes & Calabrese, 1979; McLusky et al., 1986).

Lowering the incubation temperature to 16 °C not only reduces the solubility of metals in the seawater, but it also provides an environment that is more consistent with natural conditions around New Zealand, and as a result conditions are better suited for *P. canaliculus* biological development. Alternatively, bacterial proliferation in seawater tanks can be expected to escalate with increasing temperature, which may also have affected vulnerable larvae.

5.2 Larval rearing tank setup

In the first experiment (Chapter 2) D-larvae were reared in 170 L tanks at a density of at most 10,000 D-larvae per litre, while in the third experiment (Chapter 4) D-larvae were reared in 2.15 L tanks at a density of between 70,000 and 100,000 per litre. This change in rearing setup was made so that more replicates could be done for each of the treatments in the third experiment. Despite the increased density, and therefore increased competition in the tanks, D-yields were not adversely affected. It is possible that higher numbers of D-larvae resulted in lower amounts of heavy metals accumulated from the seawater for each individual, because more D-larvae were sharing the burden of the heavy metals in each tank overall. It is also likely that the circulation of seawater within the 2.15 L tanks which was more vigorous than in the 170 L tanks improved the suspension of D-larvae in the seawater and reduced the quantity of D-larvae sticking to the surfaces of the tanks, which would hinder their development of swimming capabilities.

5.3 Future work

Following the research of this thesis, there are many avenues for future work for the benefit of shellfish aquaculture and to improve our understanding of the interaction between heavy metals and shellfish biology. As stated earlier, EDTA is not biodegradable, and this work has highlighted that EDTA could be replaced by EDDS in aquaculture hatcheries, as it has been for several other industrial applications. It is hoped that this work inspires others to scrutinise further the use of EDTA in all of its applications and explore the potential for the use of EDDS and other potential substitutes for EDTA with higher environmental performance.

5.3.1 Chelating agents and the microbiome

The effect of adding chelating agents on the microbiome in the seawater used for shellfish rearing has not been investigated. EDTA has antimicrobial properties, and it is possible that adding chelating agent and reducing the bioavailability of heavy metals is affecting the survival of microbes, leading to a secondary benefit for developing shellfish larvae through less potential for attack by microbial pathogens. A preliminary study investigating the numbers of Colony Forming Units (CFU) and how the numbers were affected by the presence of 3 µM EDTA was attempted during the research for this thesis (Chapter 3). However, the counts of CFU on the agar plates were 'Too Many To Count' (TMTC), and consequently it was difficult to determine any differences between the seawater treated with EDTA and the control. It could be worthwhile using a *Vibrio* selective agar for the bacterial plating, rather than marine agar to reduce the total counts on each agar plate. *Vibrio* is a dominant pathogenic bacterial genus that frequently causes problems in shellfish larval rearing. Further to investigating the effect of EDTA on microbial numbers and pathogenic strains, the effect of EDTA on the composition of the microbiome in larval incubation water could be investigated to determine if certain microbial species are affected by EDTA more than others. This can be achieved using molecular genetic methods for assaying microbiomes. Once it is known if and which microbial species are affected, a study could investigate further with a toxicological assay, attempting to develop shellfish with controlled numbers of the species present in the seawater.

5.3.2 Metal speciation

X-ray Absorption Near Edge Structure (XANES) using a synchrotron XFM beamline has the capability to determine the chemical state of metals in the shellfish larvae. For example, as well as determining the total concentration and distribution of zinc (Zn), the proportions of the chemical states of Zn in samples could be determined. If standards for the different chemical species of Zn, such as Zn solid, Zn^{2+}, Zn(EDTA), Zn(Carbonic Anhydrase) and $ZnCO_3$ are run, a sample with Zn in a combination of these chemical states can be analysed, and the proportions of total Zn in each of these states could be determined using modelling of the x-ray fluorescence spectra.

Preliminary work investigating Zn XANES was attempted for *P. canaliculus* D-larvae alongside the research in this thesis. Individual D-larvae were successfully scanned, and Zn XANES profiles obtained from them. Standards of Zn, ZnO, $ZnCO_3$, and Zn bound with carbonic anhydrase were run, and Zn XANES profiles for these obtained. Due to time constraints from confirmation of beamtime and actual running of the experiment, analytical Zn(EDTA) could not be obtained in time for the experiment. There was no match between the standard profiles and the spectra obtained from the mussel larvae. It is very likely that the profile from D-larvae is a combination of several of these Zn chemical states, or a standard for zinc had not been included such as Zn(EDTA). The standard for Zn(EDTA) needs to be run, and considerable time and effort needs to be put in to developing the methods of modelling and fitting the larval XANES profiles and comparing the profiles between treatments.

At present it is not straightforward to model different combinations of standards in an attempt to match up the profiles with those obtained from the samples, without considerable effort in coding software. Once a more user-friendly software is available, the proportions of Zn in its different chemical states in the samples could be determined. One advantage of being able to do this would be that the concentration of Zn(EDTA) in the D-larvae could be quantitatively determined, allowing us to know more about the mechanism of EDTA in reducing Zn toxicity. If Zn is accumulating in D-larvae while bound with EDTA, its toxicity would be very different to its accumulation as a free Zn^{2+} ion, and this would provide another explanation for the significant improvements in D-yields seen with EDTA treatment. This could also be applied with EDDS treatment, using a standard of Zn(EDDS).

In addition, the other biologically essential heavy metals investigated in this thesis, including chromium (Cr), iron (Fe), nickel (Ni), copper (Cu) and their respective EDTA coordinated

states could be investigated with XANES, providing further information about the concentrations of each of their chemical states within larvae, adding to the current knowledge about the total concentration of metals in the D-larvae reported in this current research.

5.3.3 Adsorption

Instead of adding chelating agents to rearing water, the possibility that shellfish hatcheries could include a metal adsorption step within their seawater filtration setup to remove problematic heavy metals was considered at the outset of this research, and a comprehensive literature review was undertaken to identify any potential materials applicable for this application in theory. The changed filtration method could be tested and shellfish survival, as well as metal content of treated seawater and reared mussel larvae could be measured and compared. The literature review found that several materials have the capability to adsorb an uncontrolled variety of heavy metals and allow removal of them from solution (Chapter 1), however, the selectivity of which heavy metals are adsorbed is poor. These materials frequently also adsorb essential metals from seawater, such as iron, as well as having minimal effect on the trace concentrations of heavy metals. The fabrication of a material that selectively adsorbs a specific metal could be costly and is unlikely to be as cost effective as using a chelating agent. In summary, the result of this research advises that future research effort should be focussed on chelating agents as the best method for reducing heavy metal toxicity in shellfish hatcheries, unless a cheap and selective material is discovered or created.

5.4 Overall conclusions

At the outset of the work of this thesis it was hoped that the metal or combination of metals responsible for larval mortality could be identified, and a clear reduction in the concentrations of those metals would be observed in the presence of chelating agents. In the first experiment (Chapter 2) the addition of EDTA reduced the concentrations of chromium, copper, zinc cadmium and lead that were otherwise accumulated by the control larvae. In contrast, in the third experiment (Chapter 4) only zinc concentrations were significantly reduced, and the concentrations of chromium, copper, cadmium and lead were unaffected, while calcium concentrations were significantly increased. Therefore, the overall result of this thesis suggests that there may be a combination of metal species involved in producing toxicity, and that another benefit of adding chelating agent could be to improve the bioavailability of calcium. The results of this thesis suggest that from the combination of metals involved, zinc is the metal most likely to be an influence on the observed differences in D-larval mortality, and the metal that the addition of chelating agent most likely helps to reduce in toxicity. It has been well discussed in previous chapters the essential nature of zinc for shellfish larval development as well as its pathways to toxicity for shellfish larvae. Further research should perhaps look at dose-dependent effects of metals such as zinc independently and in combination with other metals. In addition, EDDS has been identified as a viable biodegradable alternative to EDTA for shellfish hatchery application.

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