Marine Invertebrate Sperm as an Indicator of Metal Toxicity

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I, Antony Lockyer, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Elevated concentrations of metals have been reported in the marine environment globally where they have the capacity to be toxic to marine organisms. Of concern are the fertilisation and early development of marine invertebrates which are vulnerable to metal toxicity. Scientific and technological advances have enabled the development of numerous rapid, accurate, and semi-autonomous methods for the assessment of sperm. Consequently, sperm parameters are being recommended as rapid alternative endpoints to fertilisation success. For these parameters to be used as endpoints in water quality guideline derivation, ecological relevance needs to be demonstrated via a proven adverse outcome pathway (AOP).

This research assessed the effects of metals (Cu, Zn, Cd and Pb) to fertilisation success in the marine invertebrate *Galeolaria caepitosa* and found that the primary cause of toxicity was through effects on sperm. When the impact of a toxicant is to sperm, current protocols for fertilisation assays could underestimate toxicity. This study found up to three-fold differences in toxicity estimates at sperm densities that all yield >80% fertilisation in controls. More appropriate toxicity estimates would be achieved by assessing toxicity using low sperm densities, for example, those which achieve only 50% fertilisation success. Alternatively, sperm endpoints could be used to provide sensitive (and conservative) toxicity estimates, provided an AOP has been established.

For Cu, effects on sperm motility (%) could account for the effects of metals on fertilisation success, and thus can be used as an indicator of Cu toxicity. For Zn, effects on the ability for sperm to undergo the acrosome reaction can be used as a rapid indicator of effects to fertilisation success. However, For Cd and Pb, effects on fertilisation could not be appropriately represented by any of the sperm endpoints tested here. This research highlights the importance of understanding the mechanism of toxicity to fertilisation success and provides recommendations for future ecotoxicological assessments.

IMPACT STATEMENT

This thesis and research presented highlights the importance of mechanisms of toxicity in ecotoxicological testing. It demonstrates that standard approaches to toxicity tests using fertilisation as an endpoint may not be appropriate when the impact of a toxicant is to sperm. Toxicity estimates here were shown to vary depending on experimental design by up to threefold. Thus, metal toxicity data based on fertilisation tests could potentially be mis-informing national water quality guidelines.

The development of adverse outcome pathways can allow the use of new scientific and technological advancements in ecotoxicological testing. Rapid and sensitive sperm toxicity methods could reduce time, cost and labour associated with current protocols. The results presented in this thesis provide a basis for further research into the use of flowcytometry as a rapid alternative to fertilisation assays.

This research has strong implications for future ecotoxicological tests using fertilisation success as an endpoint and provides the following recommendations; the mechanism of toxicity for any given toxicant should be determined prior to ecotoxicological testing as toxicity estimates are dependent upon this; sperm endpoints can provide sensitive measures of toxicity, but should be related back to fertilisation success where possible; natural spawning densities should inform experimental designs where

possible. These recommendations not only apply to Australia but are also relevant on a global scale and are increasingly relevant as metal concentrations in the marine environment are likely to increase.

The initial findings of this research have been communicated through a peer-reviewed journal article (Lockyer et al., 2019) and results have been presented at several international conferences. The article was published in collaboration with the Commonwealth Scientific and Industrial Research Organisation which has resulted in further ecotoxicological test development. Chapters from this thesis are currently in preparation for submission to Aquatic Toxicology and Environmental Science and Technology.

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Funnily, I have found this section of the thesis to be the hardest to write. This PhD has been an invaluable journey for me, one that has seen me move across the world, develop lifelong friendships and explore some exciting opportunities. Putting into words the gratitude for the people that have generously shared their time with me, and contributed in various ways to my PhD, has been extremely difficult. But here goes…

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To my family, Mike, Becky, Leian and Nan, for knowing not to ask too many questions, for somewhat expanding your interests to include that of worm sperm, and for your unconditional support. Thank you.

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CHAPTER 1

Marine Invertebrate Sperm as an Indicator of Metal Toxicity

The contamination of aquatic ecosystems with natural and anthropogenic chemical substances has been identified as a key global threat to water security and biodiversity (van Dam et al., 2014); with declining water quality identified as one of the most serious issues affecting Australia's marine and coastal environments (Ross and Bidwell, 2006; Diggles, 2013; McDowell and Pfennig, 2013; Great Barrier Reef Marine Park Authority, 2014; Thompson et al., 2014). Among the many substances that are released into the marine environment, metals are amongst the most pervasive and the rate at which they are added to the environment seems unlikely to reduce any time soon. The global demand for metals is increasing rapidly (Halada, Shimada and Ijima, 2007) and is expected to more than double or even triple by 2050 to meet the needs associated with urbanisation in developing countries, increasing population growth, widespread use of electronics, and transitions to renewable energy technologies (Elshkaki and Graedel, 2013; Elshkaki et al., 2018). Global demand for metals will drive increased metal extraction and processing, and will subsequently lead to similar increases in associated environmental impacts (Van der Voet et al., 2018).

1.1 Metals in the Marine Environment

Metals occur naturally in the marine environment. Most natural sources (~80%) are from crustal material that is either dissolved or eroded from the Earth's surface, or from volcanic eruptions into the atmosphere. The remaining 20% can be accounted for by forest fires (10%) and biogenic sources (10%; Nriagu 1990) For some metals, however, inputs to the environment from human activities greatly exceed those of natural sources. Anthropogenic

emissions can be up to two times (Cu), five times (Zn, Cd), and 33 times (Pb) greater than the natural emissions of metals to the atmosphere (Callender, 2013). Anthropogenic emissions of metals in the environment are mainly associated with mining and the metal processing industry (Nriagu, 1990; Callender, 2013). Other sources of metal contamination in the marine environment include industry, sewage and storm water discharges, antifouling paints, commercial fertilizers and pesticides, animal waste, fossil fuel combustion, municipal waste incineration and cement production (Depledge, Weeks and Bjerregaard, 1998; Gaylard, 2004).

Resource extraction

Whilst mining activities are generally localised, they can have a large impact on the environment via mine tailings, waste rock deposits, waste waters, refinery and processing and atmospheric deposition (Reichelt-Brushett, 2012; Rotmann and Thomas, 2012; Pappa *et al.*, 2018). Physical and chemical weathering of wastes become a source of contamination in rivers (Li *et al.*, 2006), lakes and groundwater (Becker *et al.*, 2001; Kerfoot *et al.*, 2004; Yellishetty, Ranjith and Kumar, 2009), soils (Li *et al.*, 2006, 2014; Batista, Abreu and Pinto, 2007; Yellishetty, Ranjith and Kumar, 2009), plants (Li *et al.*, 2006; Batista, Abreu and Pinto, 2007), and sediments (Rowan *et al.*, 1995; Batista, Abreu and Pinto, 2007; Villa *et al.*, 2011; Kusin *et al.*, 2017). Streams from resource extraction operations contain high trace metal concentrations that eventuate in the marine environment (Edinger, Siregar and Blackwood, 2007; Valavanidis and Vlachogianni, 2010; Reichelt-Brushett, 2012). Metals

are also released into the atmosphere as a result of high-temperature refining processes, i.e. smelting (Callender, 2013). In the lead industry, Pb–Cu–Zn–Cd are released in substantial quantities; during Cu and Ni smelting, Co–Zn–Pb–Mn as well as Cu–Ni are released; and in the Zn industry, large releases of Zn–Cd–Cu–Pb occur (Adriano, 1986). Atmospheric contamination of metals is eventually deposited either directly in the sea, or on land where it has the potential to be washed into rivers and streams. Many mining operations are also located near coastlines to reduce transportation costs, which can directly contribute to elevated metal concentrations in marine sediments (González, Ramírez and Torres, 1997; Edinger, Siregar and Blackwood, 2007), and in the tissues of marine organisms (Jones, Mercurio and Olivier, 2000; Peng *et al.*, 2006; Edinger, Siregar and Blackwood, 2007; Prouty, Hughen and Carilli, 2008; Angel *et al.*, 2010). Increasing interest in deep-sea mining, including sulphide chimneys (Hoagland *et al.*, 2010), natural gas (Christie *et al.*, 2011), and trace metals (Hein, Conrad and Staudigel, 2010) are likely to create additional direct sources of marine contamination.

Shipping activity

Increased resource extraction would drive associated increases in port development and operations. This would put further pressure on the local marine environment and enhance metal availability through capital and maintenance dredging and vessel activity (Clark and Johnston, 2016). Port operations, such as dredging, can mobilise metals that are bound to sediment leading to greater concentrations in the water column and in the tissues of

marine organisms (Reichelt and Jones, 1994; Nayar *et al.*, 2003; Hedge, Knott and Johnston, 2009; Erftemeijer *et al.*, 2012). Additionally, contaminants from shipping activities typically include fuels and oils, anti-fouling paints and biocides (Gopinath, Nair et al. 2010). Marine vessels are commonly coated with copper based antifouling paints, which have been used for over 200 years (Woods Hole Oceanographic Institute, 1952). High metal concentrations, particularly copper, have been measured in and around shipping ports (Reichelt and Jones, 1994; Haynes and Johnson, 2000; Srinivasan and Swain, 2007). It has previously been estimated that naval vessels coated with antifouling paints alone contributed 7,200 kg of copper into San Diego Bay each year (Seligman, 1998).

Urban waste streams

Today, 55% of the world's population live in urban areas. This is set to increase to 68% by 2050 (United Nations, 2018). Increased urbanisation causes increased surface run-off, resulting in greater volumes of urban waste streams with complex chemical compositions (McKenna, Richmond and Roos, 2001; Ramos, Inoue and Ohde, 2004; Gopinath *et al.*, 2009; Wang, Chen and Xia, 2010). Stormwater discharges and diffuse run-off are significant sources of pollution in estuaries and coastal environments (Marsalek *et al.*, 1999; Ahn *et al.*, 2005). Urban run-off typically contains high sediment loads and nutrient levels, and a wide range of pollutants, including domestic wastes and litter, pesticides, heavy metals, faecal bacteria, hydrocarbons, PCBs and organic matter (Marsalek *et al.*, 1999; Ahn *et al.*, 2005).

Agriculture

Agriculture can also contribute considerable amounts of trace metals into the marine environment; industrial waste containing high cadmium and lead concentrations was illegally imported to Australia and sold as agricultural fertiliser (Molloy *et al.*, 2005). Many guano-based phosphate fertilisers contain high cadmium concentrations (Loganathan and Hedley, 1997).

Effects of Metals in the Marine Environment

Some metals, (e.g. Cu and Zn) are essential for the survival, growth and reproduction of marine organisms, whereas other metals (e.g. Cd and Pb) are of no known use for biological functions (Valavanidis and Vlachogianni, 2010; Callender, 2013). Both essential and non-essential metals can be toxic to marine organisms when present above threshold concentrations (Lumoa, 1983). This threshold concentration is dependent upon the metal, the environment and the sensitivity of the organism. When marine organisms are exposed to toxic concentrations of metals, they can be vulnerable to cell damage (Li *et al.*, 2016; Ubrihien, Taylor and Maher, 2017), growth inhibition (Satoh *et al.*, 2005), DNA damage (Campbell *et al.*, 2014; Chiarelli and Roccheri, 2014) and mortality (Brown and Ahsanullah, 1971). Most marine organisms have developed several mechanisms for the uptake, excretion, regulation and detoxification of both essential and non-essential metals (Lumoa, 1983; Chiarelli and Roccheri, 2014). These abilities differ between species, making some species more sensitive to metal toxicity than others.

Whilst mechanisms for detoxification exist, when metal concentrations are too high, these can be insufficient, and organisms may present effects (Callender, 2013; Chiarelli and Roccheri, 2014). Areas that have experienced high metal contamination for decades or centuries, such as Port Pirie, South Australia, are likely to contain populations that are more tolerant to pollution when compared to the same species in relatively uncontaminated environments (Bankar *et al.*, 2018).

Once in the marine environment, metals are partitioned in dissolved and particulate phases (Callender, 2013). Thus, marine organisms can be directly exposed to metals through both ingestion and solution. Within each phase, an organism can be exposed to various physiochemical forms of a metal, and each form or speciation may differ in its accessibility and toxicity to an organism (Hudspith, Reichelt-Brushett and Harrison, 2017). The nature, effect and interaction of metals with marine organisms, populations and communities are essential for understanding the impact of increased metal concentrations in the marine environment.

Mechanisms of Metal Toxicity

Sperm cells are directly exposed to metals in the water column where they can be transported into the cell across the cell membrane. The hydrophobic cell membrane is a barrier to most simple chemicals which are hydrophobic and charged, exceptions including H_2O, CO_2, N_2, NH_3 and O_2 which diffuse freely through the membrane (Williams, Coombs and Tinker, 1981). The affinity of

Cu, Zn, Cd and Pb for organic ligands offers them a route across the membrane by binding with transport ligands and proteins (Rainbow, 1997; Langston and Bebianno, 1998). As well as metals that are essential for cellular processes (Cu, Zn), transport proteins may recognise non-essential metals (Cd, Pb) or other compounds , effectively acting as molecular mimics (Jaishankar *et al.*, 2014). The strong affinity of trace metals for organic ligands additionally promotes a concentration gradient of free metal ions across the membrane, even though the total metal concentration within the cell is high. Within the cell, trace metals like Cu, Zn and Cd bind strongly with intracellular organic ligands and there is relatively little release of free metal ions back out of the cell, and thus increasing the intracellular metal concentration (Rainbow, 1997).

Metals have become so central to cellular function that the collection of metal-binding proteins accounts for over 30% of all proteins in the cell. Metals are known to be involved in over 40% of enzymatic reactions, and metal-binding proteins carry out at least one step in almost all biological pathways (Monosson, 2012). Some non-essential metals, such as Cd and Pb, can bind to proteins and replace essential metals in cellular processes causing metal toxicity (Jaishankar *et al.*, 2014). For example, Pb metal ions can replace other bivalent cations like Ca_2+, Mg_2+, Fe_2+, the replacement of these ions with Pb causes significant changes in various biological processes such as intra and inter-cellular signalling, protein folding, maturation, apoptosis, ionic transportation and enzyme regulation. Heavy metals that are bound to

oxygen, nitrogen, and sulfhydryl groups in proteins, can result in alterations of enzymatic activity (Jaishankar *et al.*, 2014).

Disruption of metal ion homeostasis may also lead to oxidative stress. Oxidative stress is caused by an increase in the formation of reactive oxygen species (ROS) which overpowers antioxidant protection and subsequently induces DNA damage, lipid peroxidation and protein modification(Jomova and Valko, 2011). The effects of Cu, Zn, Cd and Pb on ROS generation and oxidative stress are listed in Table 1.1.

Table 1.1: Mechanisms of metal-induced oxidative stress (Jomova and Valko, 2011)

Metal	**Effects on ROS generation and oxidative stress**
Cu	Copper can induce oxidative stress by two mechanisms. First, it can directly catalyse the formation of ROS via a Fenton-like reaction. Second, exposure to elevated levels of copper significantly decreases antioxidant (glutathione) levels.
Zn	Zinc is a redox inert metal and does not participate in oxidation-reduction reactions. A zinc deficiency has been associated with increased levels of oxidative damage including increased lipid, protein and DNA oxidation. Zinc's function as an antioxidant involves two different mechanisms: (i) the protection of sulphydryl groups of proteins against free radical attack and (ii) antagonism of redox-active transition metals, such as copper.

Cd	Cadmium itself is unable to generate free radicals directly, however, it can indirectly result in the formation of ROS and RNS involving the superoxide radical, hydroxyl radical and nitric oxide. Cadmium can replace iron and copper in various cytoplasmic and membrane proteins, thus increasing the amount of unbound free or poorly chelated copper and iron ions participating in oxidative stress via Fenton reactions.
Pb	Free radical-induced damage by lead is accomplished by two independent, although related mechanisms. The first involves the direct formation of ROS including singlet oxygen, hydrogen peroxides and hydroperoxides and the second mechanism is achieved via depletion of the cellular antioxidant pool. Interrelations between these two mechanisms exist so that the increase in ROS on one side simultaneously leads to depletion of antioxidant pools on the other.

1.2 Marine Ecotoxicology

Metals are permanent additions to the marine environment as they are not subject to bacterial degradation and so concentrations are likely to increase. As such, there is a pressing need for methods to assess environmental and ecological quality that are rapid, reliable and cost-effective (Stark, Riddle and Simpson, 2003; Jiang, Xu and Warren, 2014; Xu *et al.*, 2014). Thus, ecotoxicological research and accurate, scientific information about the likely impacts of metals on organisms are crucial to support the assessment and management of anthropogenic pollutants in the marine environment (ANZECC and ARMCANZ, 2000).

Ecotoxicological testing is well established for environmental impact and risk assessment of pollutants in the marine environment and forms a key component of the Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC and ARMCANZ, 2000). Australian water quality guidelines are derived from species sensitivity distributions (SSDs; Warne et al., 2014) which take toxicity test results (single species) from multiple species and extrapolate to the community level to estimate an environmental concentration of a chemical that can be deemed "safe' (Aldenberg & Slob 1993). Bioassays that identify sensitive, sub-lethal effects, are commonly incorporated into SSDs to predict effects at the population level and provide a realistic assessment of toxicity across a suite of species from an environment (Dam & Chapman 2001). The assumption that sublethal methods are more sensitive to contaminants than acute (lethal) methods has

led to increasing interest in their use in guideline derivation and setting of safe environmental concentrations. Sublethal methods include various endpoints such as reproduction, inhibition of growth, or enzyme activity (ANZECC, 2000; Greenstein *et al.*, 2008). This thesis will focus on reproductive endpoints for marine invertebrates as effects on reproductive success can be directly linked to effects at the population level.

Many fish, invertebrates and algae reproduce by broadcast spawning their eggs and sperm freely into the water column where unprotected reproductive cells are directly exposed to toxicants in the water (Marshall, Styan and McQuaid, 2009). Consequently, fertilisation success is vulnerable to metal toxicity with potential consequences for the ongoing population dynamics of affected species (Marshall, 2006). Fertilisation and early development in marine invertebrates are widely considered to be the most sensitive life history stages to environmental toxicants (His, Beiras and Seaman, 1999; Xie *et al.*, 2005). This has led to extensive use of early life stages in ecotoxicological testing (Lewis and Watson, 2012; Hudspith, Reichelt-Brushett and Harrison, 2017) under the premise that toxicity tests using the most vulnerable life history stage would also offer protection to all other stages of life in the natural environment (Mohammed, 2013). Fertilisation success can also be directly linked to higher level effects on recruitment and population success (Lewis and Watson, 2012) thus lends itself as a sensitive and ecologically relevant endpoint. Standard protocols exist for the effects of toxicants to fertilisation success in sea urchins (USEPA, 1995; Simon and Laginestra, 1997; ANZECC

and ARMCANZ, 2000; United States Environmental Protection Agency, 2002; Environment Canada, 2011). These tests are used to assess the toxicity of metals to individual species, inform SSD's and subsequently evaluate the status of coastal marine waters and estuaries globally.

A recent review on the factors affecting the toxicity of trace metals to fertilisation success in marine invertebrates indicated that marine invertebrate sperm were sensitive to metal toxicity and could be affected in a range of ways, depending on the metal or species involved (Hudspith, Reichelt-Brushett and Harrison, 2017). When marine invertebrate sperm are exposed to metals, they can present impaired sperm motility (Au et al., 2000; Fitzpatrick et al., 2008), ultra-structural damage and an inhibition of the acrosome reaction (Zhang et al., 2010). Additionally, there was evidence to suggest that unfertilised eggs were relatively unaffected by metal exposure (hollows et al., 2007; Fitzpatrick, 2008; Gopalakrishnan et al., 2008; Reichelt-brushett and Hudspith, 2016). Thus, if the mechanisms of metal toxicity to fertilisation success are through effects on sperm, then sperm endpoints show promise for the development of rapid and sensitive ecotoxicological assays.

Scientific and technological advances have enabled the development of numerous rapid, accurate, and semi-autonomous methods for the assessment of sperm. For example, sperm motility can be measured using computer assisted analysis (CASA; Wilson-Leedy and Ingermann, 2007; Fabbrocini, Di Stasio and D'Adamo, 2010; Boryshpolets *et al.*, 2013; van der Horst, Bennett

and Bishop, 2018) or rates of sperm accumulation against surfaces (SAAS; Falkenberg, Havenhand and Styan, 2016). Sperm viability, mitochondrial membrane potential and acrosomal integrity can be measured using flow cytometry (Binet *et al.*, 2014; Fallis *et al.*, 2014; Kekäläinen *et al.*, 2015; Peña *et al.*, 2018). Comet assays can assess damage to DNA (Lewis and Galloway, 2008, 2009; Lacaze *et al.*, 2011; Barranger *et al.*, 2014) and other microscopy methods can be used to determine the status of various sperm processes (Pillai *et al.*, 1997; Zhang *et al.*, 2010; Lisa *et al.*, 2013). However, none of these methods can currently be used to derive toxicity data for routine guideline derivation as there are no clear links to effects on fertilisation success and higher levels of biological organisation (ANZECC and ARMCANZ, 2000; Warne *et al.*, 2014). Presently, only endpoints that are 'ecologically relevant', that measure detrimental effects on populations, communities and ecosystems are used to derive guideline values. Toxicity data that measure effects below the individual level of organisation cannot be used.

If sperm methods/endpoints were able to be adopted for routine toxicity testing, data could be more easily obtained which would increase the data available for inclusion in SSDs. A requirement of the ANZECC guidelines are that a minimum of eight species, from at least four taxonomic groups, are used to derive national water quality guidelines for a toxicant. Similar requirements are stated in the EU Water Framework Directive (2000/60/ EC) where a base-set of taxa belonging to three different trophic levels should be used in ecotoxicological testing. Species sensitivity distributions are based on the

extrapolation of single species toxicity data to the community or ecosystem level. Thus, rapid methods that can easily provide data from multiple species could potentially increase the accuracy of SSDs in predicting ecosystem toxicity effects (Garner et al., 2015).

New, rapid and sensitive methods of sperm assessment could be used to derive water quality guidelines for metals, if their ecological relevance can be demonstrated and provided they are scientifically rigorous and defensible (Warne *et al.*, 2014). A relatively new concept has been proposed to facilitate the transition to more mechanistically based approaches of toxicity assessment - adverse outcome pathways (AOP; Groh *et al.*, 2015; Knapen *et al.*, 2015; Bal-Price and Meek, 2017; Vinken *et al.*, 2017; Carusi *et al.*, 2018). An adverse outcome pathway is an increasingly popular risk assessment method that aims to link molecular effects thought to initiate the toxic response in an organism to adverse outcomes at a biological level of organization necessary for risk assessment (e.g. reproduction, growth; Ankley et al., 2010; Vinken et al., 2017). Each AOP begins with a molecular initiating event (MIE) in which a chemical interacts with a biological target, leading to a sequential series of key events (KE) that span multiple levels of biological organisation, to produce an adverse outcome (AO) at the population level (Figure 1). A key event relationship (KER) also gives a summary of the weight of evidence that establishes the causal nature of the relationship between two measurable biological events (Becker et al., 2015). The KER defines the biological plausibility of the relationship between the pair of KEs and highlights the

important biological context and processes that need to be considered in the relationship (Wittwehr *et al.*, 2017). The use of AOP frameworks in ecosystem-level risk assessment is promising for two reasons: 1) once fully developed, they will limit the amount of toxicological testing necessary, limiting use of experimental organisms and allowing contaminant-based risks to be assessed using more model based approaches (Ankley et al. 2010); and 2) they can enhance the number of contaminants assessed and endpoints evaluated, essentially providing faster, more cost-effective means of ecological risk assessment (Maloney, 2018; Murphy *et al.*, 2018). However, for AOPs to truly capture the complexity and ecological relevance of biological effects at the molecular level, they must be quantitative, with specific emphasis on establishing quantitative dose–response relationships between MIEs and various KEs to the adverse outcome of concern (Gust *et al.*, 2016). One major criticism of the AOP framework is the implication that effects at the individual level will result in impacts at the population level. Effects at the individual level may not translate to population because of compensatory processes at several levels of organization which create a complex and nonlinear linkage (Gust *et al.*, 2016; Leist *et al.*, 2017). Adverse outcome pathways are presented as stand-alone linear events, yet the reality is likely to be much less straightforward. It may be that a toxicant can have one MIE that trigger multiple KE simultaneously to lead to toxic effects. It is important that the overall toxicological scenario does not become lost when developing AOPs. Thus, highlighting the importance of understanding the physiological and biochemical processes that are involved in the natural function of an endpoint

and obtaining toxicity data at each KE likely to lead to an AO. Adverse outcome pathways that are heavily based on modelling approaches, with little toxicity data ground truthing the models, are likely to have a large scope for error magnification, every time two functions are modelled together, and may over extrapolate effects at the molecular level to the population level.

The development of AOPs are still in their infancy, with many studies identifying mechanistic effects of contaminants to marine invertebrates in order to establish an AOP (Khan et al. 2018). The AOP framework has been successfully used to predict the effect of pulp and paper mill effluents on the fecundity of white suckers (*Catostomus commersonii*) by measuring testosterone levels in exposed fish and developing population recovery trajectories to predict population-level effects (Miller et al. 2015a). This study used individual toxicity data, alongside a long-term effects-based monitoring program and population models to successfully project alterations in population status, therefore ground truthing model outputs with real scenarios. There are currently no AOPs established for the effects of metals to fertilisation success in marine invertebrates. To develop AOPs for the effects of metals to fertilisation success, an understanding of the biological processes involved in fertilisation success is required, such that the mode of toxicity can be accurately identified. There are a number of distinct processes in both eggs and sperm that need to occur for successful fertilisation.

1.3 Effects of Metals to Fertilisation of Marine Invertebrates

Many marine invertebrates reproduce via broadcast spawning sperm and eggs in the environment, where external fertilisation takes place (Marshall, 2006). Metals have the potential to impact any of the physiological processes involved in fertilisation success. How metals disrupt these processes and cause toxicity in external fertilisation is not clearly understood (Victor and Richmond, 2005; Hudspith, Reichelt-Brushett and Harrison, 2017). The effect of trace metals on the function and morphology of gametes has been studied in some marine invertebrate species, with results indicating that sperm are particularly sensitive to metal exposure (Dinnel *et al.*, 1989; Cam F. Hollows, Johnston and Marshall, 2007).

Once spawned, the likelihood that gametes will interact is a key determinant of fertilisation success (Styan, 1998; Styan and Butler, 2000; Levitan, 2017) which is dependent upon a number of variables, including; the number of sperm immediately surrounding an egg (Levitan, Sewell and Fu-Shiang Chia, 1991; Levitan and Petersen, 1995; Styan and Butler, 2000; Marshall, Styan and McQuaid, 2009); sperm age (Williams and Bentley, 2002; Pizzari *et al.*, 2008); sperm swimming ability (Levitan, 2000; Kupriyanova and Havenhand, 2002a; Naud and Havenhand, 2006; Lewis and Galloway, 2010); gamete size (Riffell, Krug and Zimmer, 2002); chemotaxis (Riffell, Krug and Zimmer, 2002; Krug, Riffell and Zimmer, 2009; Zimmer and Riffell, 2011); compatibility (Levitan, 2012) and environmental conditions (Crimaldi and Zimmer, 2014; J. P. Crimaldi, 2012). Whilst all these processes are extremely important in

determining fertilisation success; metals are only likely to disrupt those that are related to sperm or egg physiology. Whilst adult exposure to metals can impact gametogenesis and the provision and number of viable gametes (Myint and Tyler, 1982; Siah *et al.*, 2003), this is not within the scope of this study. This thesis will focus on the direct effects of metals to the physiological changes that occur in gametes once spawned into the marine environment, that are essential for successful fertilisation (Figure 1.1).

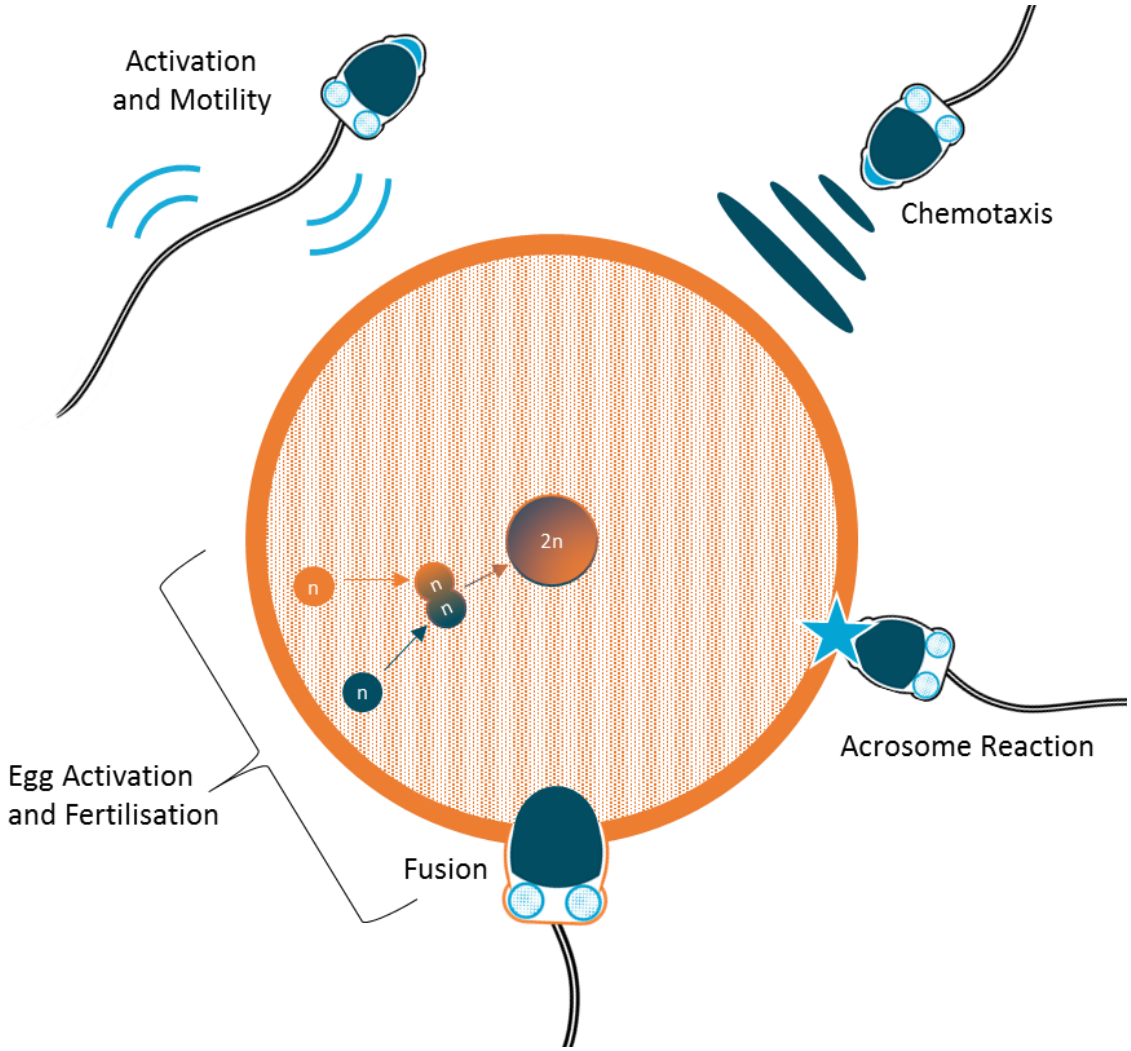

Figure 1.1: Key events for successful fertilisation in marine invertebrates. n= sperm/egg nuclei, 2n = Zygote nucleus.

Sperm activation and motility

Sperm within the gonads are maintained in an inactive state (Tosti and Ménézo, 2016). Initiation of motility is observed immediately after the reversal of environmental conditions. Motility initiation can occur in a filtered seawater solution which indicates that activation is not dependent on chemical stimuli from an oocyte (Aitken, 2000) and that it must be an intrinsic process of the sperm which occurs when diluted out of the gonad. In marine invertebrates, sperm are stored in the gonads at a low pH; sea urchin seminal plasma was reported to have a pH ranging between 7.3 and 7.7 (Rothschild, 1948), similarly the pH of starfish seminal plasma was measured as 6.6 (Shirai et al., 1982). A low extracellular pH may inhibit sperm movement (Aitken, 2000) as this prevents ATP hydrolysis (Tosti and Ménézo, 2016). The release of sperm into seawater causes an increase in intracellular pH and initiates motility (Lee, Johnson and Epel, 1983; Aitken, 2000). This occurs as an uptake of sodium (Na+) into the sperm cell triggering the release of hydrogen (h+) ions (Bibring, Baxandall and Harter, 1984; P. Schlegel et al., 2015). The increase in intracellular pH initiates ATP hydrolysis which fuels the axenomal dyneins in the flagella. Dyneins use the chemical energy from ATP hydrolysis to generate force or movement (Roberts et al., 2013). This movement occurs as active sliding of doublet microtubules which generates flagella bending. The sliding of doublet microtubules by axenomal dyneins through mechanochemical cycles of ATP provides the driving force for flagella motility of sperm (Nakajima, 2005; Inaba, 2011). The regulation of sperm movement is also thought to be dependent upon an increase in intracellular

3′-,5′-cyclic adenosine monophosphate (cAMP) (Jones and Murdoch, 1996). Changes in cAMP mediate dyneins through a protein kinase A (PKA) regulatory pathway (Aitken, 2000). cAMP dependent dynein phosphorylation has been proposed as a central mechanism for triggering motility (Carr and Acott, 1989). Once initiated, sperm must sense the environment and adapt their motility to meet an egg (Miller et al., 2015). Motility is regulated by ion homeostasis, which is under the control of ion channels (Tosti and Ménézo, 2016). Sperm ion channels regulate intracellular pH, membrane voltage and calcium concentration (Ca^{2+}). Rises in Ca^{2+} change the beat pattern of the flagella by inhibiting the activity of dynein arms within the axoneme which alters sperm swimming behaviour (Miller et al., 2015). The opening of Ca2+ channels along the flagella are essential for the maintenance of sperm motility. Sperm motility of the tropical urchin (*Anthocidaris crassispina*) has been found to be negatively impacted by Cd (Au et al., 2001). Au e t al., (2001) observed decreased sperm velocities and an enlarged sperm midpiece with disorganized mitochondrial membranes. It was hypothesised that this may affect the supply of ATP to the flagella and thus disrupting sperm swimming ability. Sperm of the blue mussel (*Mytilus trossulus*) also showed decreased velocities and reduced fertilisation success when exposed to 100 μg L^{-1} Cu. This was hypothetically attributed to Cu induced interference of mitochondrial activity (Fitzpatrick et al.,2008).

Chemotaxis

Once motile, sperm are attracted by chemical substances which are released by the egg (Kaupp, Hildebrand and Weyand, 2006). In broadcast spawning species with, chemotaxis is of crucial importance due to the high dispersion of gametes in the marine environment (Tosti and Ménézo, 2016). In Sea Urchins, a small peptide (resact) contained within the jelly layer of an oocyte induces a calcium dependant swimming pattern and alters the trajectory of sperm (Brokaw, Josslin and Bobrow, 1974; Brokaw, 1979; Kaupp, Kashikar and Weyand, 2008). This process is accompanied by the activation of enzyme activity and cyclic guanosine monophosphate (cGMP) synthesis. It is hypothesised that cGMP hyperpolarises the sperm cell due to potassium efflux, followed by modulation of intracellular calcium (Eisenbach, 1999; Kirkman-brown, Sutton and Florman, 2003; Hildebrand and Kaupp, 2005). Calcium entry is an important factor in regulating sperm chemotactic behaviour and is likely to be common to all species (Strünker *et al.*, 2006; Yoshida and Yoshida, 2011). Calcium dynamics in the flagellum control the swimming trajectory of sperm through an alternating sequence of turns interspersed with periods of straighter swimming, the "turn and run" pattern. This "turn and run" response is seen in sperm of diverse marine species upon exposure to components of an egg of the same species (Guerrero *et al.*, 2010). The curvature of sperm trajectories and asymmetry in flagellar waveforms are used as indices for evaluating chemotactic responses of sperm (Miller, 1982; Böhmer *et al.*, 2005; Guerrero *et al.*, 2010).

The Acrosome Reaction

Once a sperm meets an egg, its next task is to make its way through the egg membrane. This is facilitated, in most marine invertebrates, via the acrosome reaction. The acrosome reaction (AR) is an exocytotic calcium-dependent process, considered to be the major pre-requisite for sperm penetration through oocyte coats (Tosti and Ménézo, 2016). Immediately after initial sperm-egg attachment, the acrosomal vesicle opens and its contents adhere to the egg membrane. The acrosomal filament then projects anteriorly through the egg envelope (Brown, 1976), enabling gamete membrane fusion (Collins and Epel, 1977). Most of the research regarding the AR in marine invertebrates has focused on Echinoderms and Bivalves but, depending on species, the AR can be initiated by one or more of a range of factors, including: a combination of the jelly coat surrounding the egg and calcium ions (Dan, 1952; Dan *et al.*, 1972; Hoshi *et al.*, 1994; Hoshi, Moriyama and Matsumoto, 2012); increasing the extracellular pH (Dan, 1952; Lambert, 1982); egg water (Kekäläinen *et al.*, 2015); increasing the calcium concentration (Brown, 1976; Grant, 1981); or by adding the ionophores A23187 (Morisawa *et al.*, 2004; Fallis *et al.*, 2014) or nigericin (Levine and Walsh, 1979). In general, the AR is species-specific and triggered by signals from the eggs or their accessory structures (Hoshi *et al.*, 1994). It is thought that sugars or glycoproteins on an egg envelope play an important role in induction of the AR (Sato and Osanai, 1990).

Ultrastructural damage to the acrosomal region was observed in sperm of the mud crab *Scylla serrata* after exposure to the trace metals Ag, Cd, Cu and Zn

(Zhang et al., 2010). The acrosome reaction as an endpoint was more sensitive than to Ag, Cd, Cu and Zn toxicity than the widely used sea urchin sperm bioassay. These metals reduced the ability for sperm to undergo the acrosome reaction. It was hypothesised that metals may block the active sites of the acrosomal filaments and potentially inhibit the transfer of calcium into the cell. Opening of the calcium channels in the sperm membrane and the subsequent influx of calcium is an essential initial phase of the AR, and it is thought that metal ions may prevent the AR by blocking calcium channels and inhibiting calcium influx (Liévano et al., 1990).

Egg Activation and Fertilisation

The attachment of sperm to the egg via the AR triggers a variety of metabolic changes referred to as egg activation (Gilbert, 2000). It is hypothesized that a diffusible molecule/package of molecules present in the sperm cytoplasm enters the oocyte cytoplasm after fusion, triggering activation events (Dale et al., 2010; Tosti and Menezo). This is rapidly followed by changes in the electrical properties of the oocyte plasma membrane. Depolarization of the membrane potential is due to ions flowing through the plasma membrane as an ion current, known as the fertilisation current. Depolarisation of the membrane potential prevents other sperm from entering the egg, preventing polyspermy (Jaffe,1976; Rothschild and Swann, 1952). The fertilisation current is due to gating of large non-specific and highly conductive plasma membrane ion channels activated in the oocyte by the fertilizing spermatozoon (Dale and De Felice, 1984; Dale, 1994). The depolarisation of the plasma membrane

prevents other sperm from entering the egg, acting as a primary polyspermy block (Gilbert, 2000; Hudspith et al., 2016). The elevation of free intracellular calcium triggers the cascade of events that leads to oocyte activation. Soon after insemination, a calcium-induced exocytosis of cortical granules gives rise to a dramatic change in the extracellular matrix, leading to elevation of the fertilisation membrane which plays the dual role of avoiding supernumerary sperm entry and then protecting the zygote and the embryo (Wong and Wessel, 2004). Once inside the egg, the sperm undergoes several changes and becomes the pronucleus; the entry of the sperm also initiates the second meiotic division of the egg, resulting in a haploid egg nucleus known as the female pronucleus (Gilbert, 2000). The male pronucleus then migrates toward the centrally located female pronucleus, where they fuse to form the diploid zygote nucleus (Elder and Dale, 2000). Fertilisation is now complete and the zygote undergoes mitosis, followed by further embryonic cell divisions. Research has shown that unfertilized eggs are relatively unaffected by trace metal exposure compared to sperm. Fertilisation success in the polychaete *Galeolaria caespitosa* was not affected by exposing eggs to copper pre-fertilisation (Hollows, Johnston and Marshall, 2007). Similarly, the viability of blue mussel eggs was not influenced by increasing concentrations of copper (Fitzpatrick et al., 2008). Eggs of the polychaete Hydroides elegans were less sensitive to trace metal exposure (Hg, Cd, Pb, Ni, Zn) than sperm (Gopalakrishnan, Thilagam and Raja, 2008). However, once the egg undergoes significant changes in membrane ion permeability, beginning the process of egg activation, metals can interfere with ion channels. Electrical

modifications of the plasma membrane are facilitated by the activation of ion channels, including calcium and sodium channels (Tosti and Ménézo, 2016). Metals can bind competitively or adventitiously to various biological ligands (Cowan, 1997), and can block or disrupt ion channels altering the permeability of cells. Therefore, whilst unfertilised eggs may be relatively insensitive to trace metal exposure, the opening of specific ion channels during egg activation present an opportunity for trace metals to interfere with gamete conductivity (Fitzpatrick et al., 2008). Exposure of sea urchin eggs to copper during the initial stages of egg activation affected Ca^{2+} homeostasis with simultaneous reductions in fertilisation success. Exposure to mercury also disrupts the function of ionic channels involved in egg activation in the ascidian *Phallusia mammillata* (Franchet, Goudeau and Goudeau, 1997). Mercuric ions inhibited the calcium and sodium currents, resulting in an inefficient electrical block to polyspermy and greater incidences of polyspermy. Mercury also prevented the transformation of the male nucleus into the male pronucleus.

1.4 Model Species

Ecotoxicological testing of metals using fertilisation as an endpoint has been extensively researched in Cnidarians and Echinoderms (Hudspith, Reichelt-Brushett and Harrison, 2017). However, only one polychaete species (*Hydroides elegans*) has been tested for the effects of Zn, Cd and Pb to fertilisation success. With a view of expanding our knowledge on the effects of metals to fertilisation in polychaetes (Lewis and Watson, 2012), the intertidal

serpulid, *Galeolaria caespitosa* presents as a model organism for ecotoxicological testing. *G. caespitosa* are easily collected and amenable to laboratory holding, have abdomens swollen with gametes throughout the year (Kupriyanova, 2006) and release large amounts of eggs or sperm when their tubes have been broken or have been disturbed mechanically (Kupriyanova and Havenhand, 2002). *Galeolaria caespitosa* is commonly used in fertilisation biology (Kupriyanova, 2006), has previously been used in ecotoxicological studies (Ross and Bidwell, 2001; Cam F Hollows, Johnston and Marshall, 2007; Lu, Lin and Aitken, 2017) and was recently incorporated into the Australian and New Zealand Environment Conservation Council (ANZECC) water quality guideline for copper (Gadd and Hickey, 2016). Like other serpulids commonly used for toxicity tests, such as *H. elegans* (Gopalakrishnan, Thilagam and Raja, 2008) and *Pomatocerous spp* (Khandeparker, Desai and Shirayama, 2005), *G. caespitosa* reproduce via broadcast spawning (Kupriyanova, 2006) and play an important role in marine communities (Gosselin and Sewell, 2013). Serpulids provide structural complexity and microhabitats that increase diversity of other marine organisms(Haines and Maurer, 1980) and are filter feeders that link the pelagic and benthic food chain (Gosselin and Sewell, 2013).

The spermatozoa of *Galeolaria caespitosa* consist of a cap-like acrosome (A), an oval nucleus (N), a short midpiece containing four round mitochondria (M), and an elongated flagellum (F) that projects from the center of the mitochondrial ring (Grant, 1981; Lu, Aitken and Lin, 2017). In live spermatozoa, the longitudinal length of the sperm head is approximately

3.35µm and the length of flagellum is about 45µm (Lu, Aitken and Lin, 2017; Figure 1.2). The exact role of mitochondria in sperm is not entirely clear (Amaral et al., 2013). Mitochondria are traditionally believed to be the "power plant" of the cell as they play a fundamental role in adenosine triphosphate (ATP) production via oxidative phosphorylation (Peña et al., 2009; Piomboni et al., 2012). However, new research continues to highlight novel ways in which they participate in cellular functions, including; Ca^{2+} signaling, modulating Reactive Oxygen Species (ROS) and cell differentiation (Peña et al., 2009; Piomboni et al., 2012; Amaral et al., 2013). The flagella is the motile machinery of sperm (Inaba, 2011). The flagella has an internal cytoskeletal structure; the axoneme. In *G. caespitosa* the axoneme is of the 9+2 type (Jamieson and Rouse, 1989) composed of 9 outer doublet microtubules (DM) and 2 central singlet microtubules (C1,C2) that are continuous for the length of the flagella (Figure 1; Lodish et al. 2000; Inaba 2011). Each doublet microtubule has an A tubule and a B tubule (AT,BT). Inner and outer dynein arms (IDA, ODA) are attached to each A tubule. The central tubules are connected by periodic bridges (B) and surrounded by a fibrous inner sheath (IS). Doublet microtubules are linked by the protein nexin (N). Doublet microtubles are linked to the central tubules by radial spokes (RS). The bundle of microtubules comprising the axoneme is surrounded by the plasma membrane (PM) (Lodish, Berk and Zipursky, 2000; Inaba, 2011). The acrosome is a large secretory granule that contains hydrolytic enzymes necessary for penetration of an oocyte. The acrosome undergoes exocytosis when receptors on the sperm surface bind to ligands in the egg extracellular

matrix. The nucleus contains the densely coiled chromatin fibres that hold the paternal DNA required for success fertilisation of an oocyte. DNA is densely packed by protamines to allow sperm head condensation and DNA stabilization (Johnson et al., 2010).

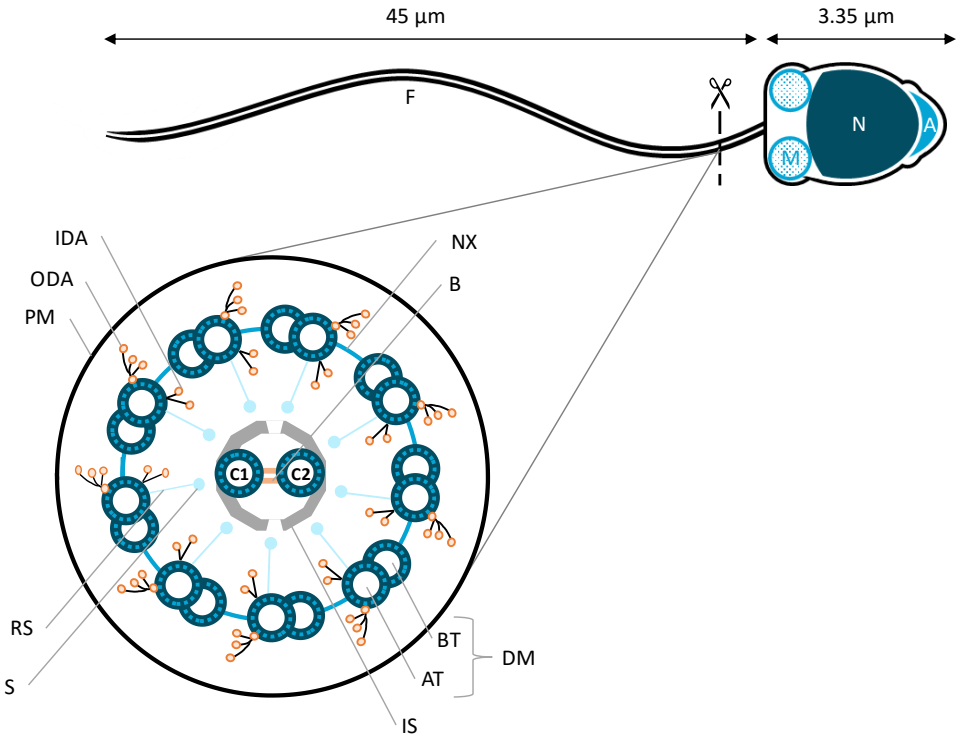

Figure 1.2:Schematic diagram of *Galeolaria caespitosa* sperm with dimensions and cross-sectional diagram of the flagellum showing major structures. A: Acrosome, N: Nucleus, M: Mitochondria, F: Flagella, NX: Nexin, B: Bridge connecting central singlets, AT: A tubule, BT: B tubule, DM: Doublet Microtubule, IS: Inner sheath, S: Spokehead, RS: Radial spoke, PM: Plasma membrane, ODA: Outer dynein arm, IDA: Inner dynein arm. Adapted from Lodish et al., 2000.

1.5 Model Toxicants

In this study, Cu, Zn, Cd and Pb were selected for further toxicity tests. Copper was chosen because it is one of the most commonly tested, and also one of the most toxic trace metals to marine invertebrates (ANZECC and ARMCANZ, 2000). Hence, there is a relatively large amount of existing and comparable

toxicity data (Table 1.1). The other metals were selected because of their prevalence, high toxicity, and associations with mining and smelting activities in South Australia.

Port Pirie in South Australia is the location of one of the largest lead smelting facilities in the world, which has been a long-term source of metal pollution into the Upper Spencer Gulf of South Australia (Edwards et al. 2001). The smelter has been a source of metal pollution since it began operations in 1889. Metals were released from the plant as particulate emissions from the smoke stacks, dust blown from the site, spillage of concentrates during loading of ships, and discharging of liquid effluent (Ward 1983). Marine sediments within the area have extreme elevations in the concentration of heavy metals: over 200-300 times greater than background levels for lead and zinc, and over 1000 times greater for cadmium (Ward and Young 1981). While there has been a reduction in the load of metals entering the Gulf through aerial sources, the waste-water effluent continues to be a significant source of trace metals, including Lead, Zinc, Cadmium, and Copper (Ward and Young 1981; Ross and Bidwell 2001; Ross et al. 2002). National Pollutant Inventory (NPI) data revealed the Port Pirie smelter as the main source of cadmium, lead and zinc in 2002–03 (Butterfield and Gaylard 2005) . Steel operations in Whyalla also discharge lead and zinc to the water, and significant amounts of both these metals and copper to air. Additionally, power stations at Port Augusta discharge copper, lead and zinc to water and air(Butterfield and Gaylard 2005). Whilst the release of these metals has reduced considerably in the last

decade or so, particularly with the introduction of effluent treatment systems (Department of Planning 2013), studies suggest that up to 600 km^2 of marine seabed has been contaminated with heavy metals in Spencer Gulf in South Australia (Dossis and Warren 1981; Edyvane 1995). Concentrations of these metals in South Australian waters frequently exceed the water and sediment quality guidelines prescribed by ANZECC and ARMCANZ (2000; Chakraborty and Owens 2014).

Copper, Zn, Cd and Pb have all been shown to have detrimental effects on fertilisation success. Table 1.1 shows effect concentration data for Cu, Zn, Cd and Pb to fertilisation success in marine invertebrates currently published in the literature. Copper is the most toxic of the metals to fertilisation success in marine invertebrates, followed by Zn. The toxicity of Cd and Pb is interchangeable depending on the species. Whilst these toxicity data document the effect of Cu, Zn, Cd and Pb to fertilisation success in marine invertebrates, they do not determine the cause or mechanism of metal toxicity.

Table 1.2: Cu, Zn, Cd and Pb toxicity data (EC50) for fertilisation success in Marine Invertebrates

	Species	Exposure	Cu	Zn	Pb	Cd	Ref.
Cnidaria	Acropora cytherea	Gametes 4.5h	69				(Puisay, Pilon and Hédouin, 2015)
	Acropora longicyanthus	Gametes 30m + 5h	15		1453		(Reichelt-Brushett and Harrison, 2005)
	Acropora millepora	Gametes 4h	17				(Negri and Heyward, 2001)
	Acropora pulchra	Gametes 4.5h	75				(Puisay, Pilon and Hédouin, 2015)
	Acropora surculosa	Gametes 5h	45				(Victor and Richmond, 2005)
	Acropora tenuis	Gametes 30m + 5h	40		1801		(Reichelt-Brushett and Harrison, 2005)
	Goniastrea aspera	Gametes 30m + 5h	19		2467		(Reichelt-Brushett and Harrison, 2005)
	Goniastrea aspera	Gametes 30m + 5h	15	>500			(Reichelt-Brushett and Harrison, 1999)
	Goniastrea retiformis	Gametes 30m + 5h	25				(Reichelt-Brushett and Harrison, 2005)
	Montipora capitata	Gametes 3h	22				(Hédouin and Gates, 2013)
	Platygyra daedalea	Gametes 30m + 5h	33				(Reichelt-Brushett and Hudspith, 2016)
	Platygyra Acuta	Gametes 30m + 5h	145				(Kwok *et al.*, 2016)
	Lobophytum compactum	Gametes 30m + 5h	261				(Reichelt-Brushett and Harrison, 2005)
	Oxypora lacera	Gametes 30m + 5h				>1000	(Reichelt-Brushett and Harrison, 1999)

Table 1.2: Cu, Zn, Cd and Pb toxicity data (EC50) for fertilisation success in Marine Invertebrates

	Species	Exposure	Cu	Zn	Pb	Cd	Ref.
Echinodermata	Arbacia punctulata	(Sperm 1h) + 20m	12	121	5400	38000	(Nacci, Jackim and Walsh, 1986)
	Arbacia spatulgera	(Sperm 1h) + 10-20m	18	116		140900	(Larrain et al., 1999)
	Asterias amurensis	(Sperm 20m) + 1 h		550		154000	(Lee et al., 2004)
	Dendraster excentricus	(Sperm 1h) + 20m	26	28	13000	8000	(Dinnel et al., 1989)
	Diadema setosum	(Sperm 1h) + 20m	70			950	(Ramachandran, Patel and Colbo, 1997)
	Diadema setosum	(Sperm 10m) + 10m	17	380		6280	(Thongra-ar, 1997)
	Echinometra mataei	(Sperm 1h) + 20m	14			>100	(Ringwood, 1992)
	Paracentrotus lividus	(Sperm 1h) + 20m	57	210	16210	8400	(Novelli et al., 2003)
	Stronglyocentrotus droebachiensis	(Sperm 1h) + 20m	59	383	19000	26000	(Dinnel et al., 1989)
	Stronglyocentrotus purpuratus	(Sperm 1h) + 20m	25	262	8200	12000	(Dinnel et al., 1989)
	Stronglyocentrotus franscisanus	(Sperm 1h) + 20m	1.9	313	1300	18000	(Dinnel et al., 1989)
Polychaeta	Hydroides elegans	(Sperm 20m) + 1 h	10030		30370	94.3	(Gopalakrishnan, Thilagam and Raja, 2007)
	Hydroides elegans	(Sperm 20m) + 1 h		945.3	380.8		(Gopalakrishnan, Thilagam and Raja, 2008)
	Nereis virens	Gametes 4hr	351				(Caldwell et al., 2011)

Table 1.2: Cu, Zn, Cd and Pb toxicity data (EC50) for fertilisation success in Marine Invertebrates. M= Mollusca, U = Urochordata

M	Crassostrea gigas	(Sperm 1h) + 20m	12	444	5500	11900	(Nacci, Jackim and Walsh, 1986)
	Isognomon californicum	(Sperm 1h) + 2h	55				(Ringwood, 1992)
U	Ciona intestinalis	Gametes 20h	37			721	(Bellas, Beiras and Vazquez, 2004)

1.6 Aims and Objectives of this Research

Currently, toxicity data derived from rapid, sensitive and accurate sperm assessment methods cannot be used in water quality guideline derivation as no ecological relevance of sperm endpoints have been demonstrated. Therefore, the aims of this thesis were to determine how metals impact fertilisation success in marine invertebrates; to develop AOPs that enable the use of rapid sperm methods to derive toxicity data that can inform guideline derivation; and ultimately to determine whether marine invertebrate sperm could be used as an indicator of metal toxicity to fertilisation success.

This research starts, in Chapter 2, by assessing the effects of Cu, Zn, Cd and Pb to fertilisation success in *G. caespitosa* across a range of sperm densities, such that the mechanism of toxicity can be determined. Chapter 2 demonstrates that the effect of metals to fertilisation success are through effects on sperm. Thus, in chapter 3, I determine whether effects of these metals on sperm motility can account for the observed effects to fertilisation success. This chapter also explores the relationship between effects on sperm and effects on fertilisation success. Interestingly, sperm motility could not account for the effects of Zn, Cd and Pb to fertilisation success in *G. caespitosa*. Consequently, in chapter 4, I develop a methodology for the assessment of the acrosome reaction and mitochondrial membrane potential in *G. caepsitosa* sperm in response to metal exposure. In chapter 5, the results of my research are summarised to develop adverse outcome pathways for the effects of metals to fertilisation success in marine invertebrates. These AOPs facilitate the use of

rapid sperm endpoints in routine ecotoxicological testing for Cu and Zn. Chapter 5 also provides recommendations for future ecotoxicological tests using fertilisation success as an endpoint.

CHAPTER 2

Metals Impact Fertilisation Success in Marine Invertebrates Through Effects on Sperm

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2.1 Introduction

Fertilisation assays are commonly used to investigate the effects of metals on marine invertebrates. Most studies have found concentration-response relationships with reductions in fertilisation success associated with increased concentrations of metals (see the recent review by Hudspith et al. 2017). Nearly all these studies use a single sperm:egg ratio across treatments that ensures at least 70-80% fertilisation in controls (See Appendix 1). There are previously noted limitations to running ecotoxicological experiments at a fixed sperm:egg ratio(Marshall, 2006). First, in any natural spawning event, the density of sperm is not constant spatially or temporally and so it may be difficult to extrapolate results from laboratory experiments to field conditions(Levitan, Sewell and Fu-Shiang Chia, 1991). Second, metals can impact different aspects of the fertilisation process: for example, by reducing the number of viable gametes, decreasing sperm motility or chemotaxis(Morisawa and Mohri, 1972; Fitzpatrick *et al.*, 2008), disrupting sperm-egg binding processes(Zhang *et al.*, 2010), or affecting the efficiency of polyspermy blocks(Franchet, Goudeau and Goudeau, 1997). However, at a single sperm:egg ratio, all of these effects may present identically as a reduction in fertilisation relative to the control(Marshall, 2006) and so while an effect of the toxicant might be detected, the mechanisms involved will likely remain unclear. Third, the effects of a toxicant can be greater at low sperm densities than at high densities, so the use of just a single sperm density may underestimate toxicity(Dinnel, Link and Stober, 1987). Finally, the use of different sperm:egg ratios for each species complicates the comparison of

toxicity data among different studies and species(Marshall, 2006).

Marshall (2006) suggested moving away from toxicity testing at single sperm:egg ratios to using fertilisation assays that assess the impact of toxicants across a series of sperm densities. Essentially, his (theoretical) suggestion was to characterise a fertilisation curve and use parameters derived from these to assess concentration-response relationships. This approach provides information regarding the mechanism of toxicity to fertilisation success; additional ecological information that can aid in informing environmental risk assessment and management. Schlegel et al.(Schlegel *et al.*, 2012) adopted this approach to identify the effects of ocean acidification on the fertilisation success of the Australasian sea urchin *Heliocidaris erythrogramma*. To date, however, these approaches have not yet been adopted in routine ecotoxicological testing. A good reason for this might simply be that the extra work involved in running toxicity tests across an extra dimension of sperm:egg ratios is too labour intensive.

This chapter aims to use the approach outlined by Marshall (2006) to determine the mechanism of metal toxicity to fertilisation success. It also aims to quantify the difference in metal toxicity between sperm densities and to assess the value of incorporating multiple sperm densities in routine toxicity testing. Specifically, I assessed the toxicity of four metals (Cu, Zn, Pb and Cd) on fertilisation in the intertidal serpulid, *Galealaria caespitosa* (a tube-building annelid worm), across sperm densities of 10^1 – 10^6 sperm mL^{-1}. The results were

compared with traditional endpoints based on single sperm densities that were subsampled from the data set.

2.2 Methods

2.2.1 Site

Test species and seawater (used as controls and diluent in tests) were collected from the jetty pilings at Grange Beach, South Australia (-34.9026S, 138.4875E). Planned dredging activity in Port Adelaide(Minister for Planning, 2018) has the potential to increase dissolved sea water concentrations of metals and effect the water quality of local Adelaide beaches(Eggleton and Thomas, 2004) and local regulators have identified elevated metal concentrations as a key water quality issue for the area(Environment Protection Authority, 2008). Grange Beach is already impacted by metals(Gaylard, 2004) and the potential impact of further metal increases on local species is unknown. The work we present may be used as part of the baseline data required to help inform risk assessments of metal increases in the area.

2.2.2 Study Species

Galeolaria caespitosa were held in the laboratory for no more than five days. Aggregations of *G. caespitosa* were broken apart and the individuals were carefully removed from their tubes with fine forceps. Forceps were rinsed in reverse osmosis (RO) water after any contact with an animal. Reproductively mature *G. caespitosa* immediately release gametes when their tubes have been broken or have been disturbed mechanically(Kupriyanova and Havenhand, 2002). Extracted worms were rinsed in 0.45-µm filtered seawater (FSW) and

placed in individual containers with 0.5 mL of FSW to encourage sperm release. A pipette was used to collect the spawned gametes which were used for experiments within 15 minutes of collection.

2.2.3 Experimental Conditions

Seawater was filtered (0.45 µm) and refrigerated. All experiments were conducted at a constant room temperature of 20°C to minimise the effects of temperature on fertilisation success and gamete aging(Kupriyanova and Havenhand, 2005). Test salinity was kept at 35±2 ppt, mimicking salinity at Grange Beach. Light quality and intensity were at ambient laboratory levels. The pH of all test solutions was adjusted to 8.0±0.1 using sodium hydroxide (AR grade, Chem-Supply) and nitric acid (69%, Merck).

2.2.4 Experimental Design

Assessing the effects of a toxicant across a range of sperm densities and across enough concentrations of the toxicant to obtain an accurate concentration-response relationship would have required gametes from many worms at one time and, logistically, would have been almost impossible to do simultaneously (~100 sperm density x metal concentration combinations), or before gametes began to age to the point where their viability was reduced(Kupriyanova and Havenhand, 2005). Consequently, we used a paired design, measuring the response ratio between a treatment (seawater with added metal) and a matched control (seawater). We used a common batch of gametes for a pair of treatment and control fertilisation assays (see below) but

tested each toxicant concentration (and paired control) with a new, independent batch of gametes. Thus, each metal treatment level (and paired control) was run as a separate experiment, with a metal requiring about a week to run experiments for seven to eight treatment levels. For each treatment and control pair, we fitted separate fertilisation models to characterise the relationship between fertilisation success and sperm density and derived various parameters from each (see Fertilisation Models, below), normalising the treatment's parameter against the parameter from the associated control fertilisation curve.

2.2.5 Treatment Preparation and Analysis

Metal stock solutions were prepared using Analar grade metal salts of $CuSO_4$, $ZnCl_2$, $CdCl_2$, and $Pb(NO_3)_2$ (99% purity, Sigma-Aldrich©) and Milli-Q water (18.2 MΩ cm^{-1}; Millipore). Glassware was washed prior to use in 10% v/v nitric acid (69%, Merck). Test solutions for each toxicant were prepared on the day of the experiment from refrigerated stock solutions and FSW, no more than one hour prior to test commencement. An adaptive hierarchical approach was taken to determine nominal metal concentrations for each treatment, whereby the results of one paired test (control and one treatment) informed the test concentration chosen for subsequent tests. At the end of each test, sub-samples were collected from each treatment and control, filtered through acid-washed (10% HNO_3) 0.45-µm filters, and acidified to 0.2% HNO_3 (69%, Merck) for dissolved metals analysis. Metal analyses were carried out using

inductively coupled plasma atomic emission spectrometry (ICP-AES; Agilent 720) by CSIRO, Lucas Heights, NSW.

2.2.6 Laboratory Fertilisation Assays

For each experiment, sperm was collected from five to ten males, pooled, and diluted in test solutions via a threefold serial dilution. Similarly, eggs were collected from 5 to 10 females and pooled. Gametes were pooled to minimise the effect of gamete-specific combining abilities(Kupriyanova and Havenhand, 2002). Each metal was tested for toxicity using eleven different sperm densities in fertilisation tests ranging from 10 to 5 x 10^6 sperm mL^{-1} (See Appendix 1 for sperm:egg ratios per treatment). Sperm densities were verified using a haemocytometer at 400x magnification. Sperm were exposed to test solutions for 30 minutes prior to the addition of eggs. The density of eggs was adjusted to 500 eggs mL^{-1}. Eggs were added to sperm in control or treatment solutions to allow development to occur(Cam F. Hollows, Johnston and Marshall, 2007). Development was ceased after 2.5 h, by fixing each sample with formaldehyde solution (4%, Merck). An egg control was also set up alongside each experiment to check for errant fertilisations due to accidental contamination with sperm, i.e. eggs were added to FSW without sperm under the same exposure conditions as the FSW control. Eggs were classed as fertilised if they had begun to undergo cell division(Marshall and Evans, 2005). Each experiment consisted of a FSW control, one test concentration and one egg control. Data were not used if maximum fertilisation in the FSW control was <80% (which is rare in crosses within this species(Styan,

Kupriyanova and Havenhand, 2008)), or if the egg control showed >5% fertilisation.

2.2.7 Fertilisation Models

Fertilisation relationships, based on a theoretical model(Styan, Kupriyanova and Havenhand, 2008; See Appendix 1) of the distribution of sperm-egg interactions using gamete concentrations and characteristics were fitted to the measured fertilisation data for each treatment. Key model parameters, fertilisation efficiency (Fe) and polyspermy block efficiency (Be), were estimated using least squares (Styan and Butler, 2000). Average sperm swimming speed and egg diameter parameters for *G. caespitosa* were those reported by Kupriyanova (2006).

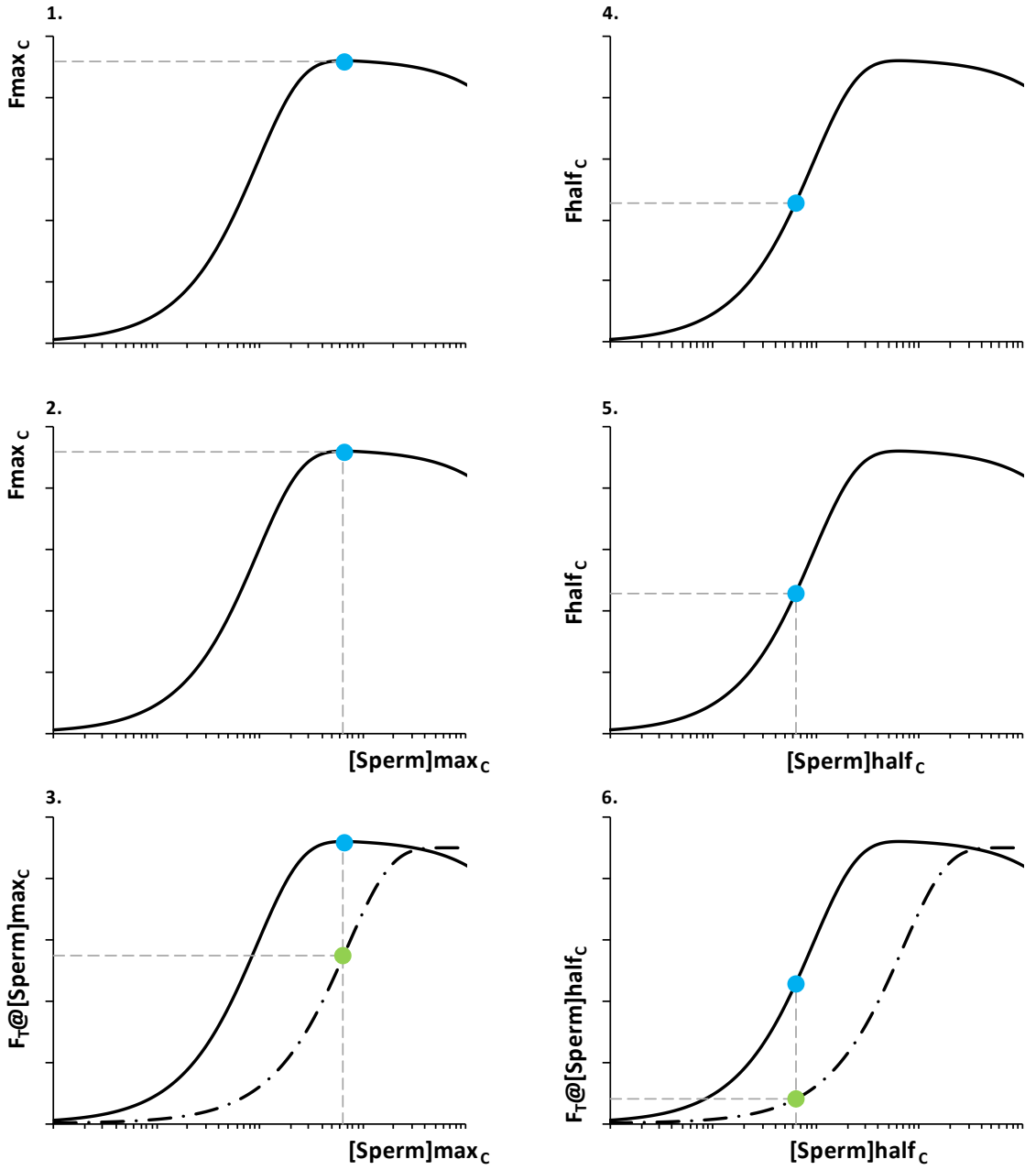
Using the fitted fertilisation models, we calculated fertilisation success at 10^4, 10^5 and 10^6 sperm mL^{-1} for each control and treatment. The percentage fertilisation in each treatment relative to fertilisation in the respective control at these sperm densities was calculated for each concentration of each metal and used to fit concentration-response relationships.

A range of endpoints were defined to characterise different aspects of the fertilisation models. These were derived for each fertilisation assay pair for each concentration of metal and then used to characterise concentration-response relationships for metals (Figure 2.1). We first estimated the maximum modelled fertilisation success (Fmax) and the sperm density that

maximised fertilisation success ([Sperm]max) in each of the controls. In the respective (paired) treatment the fertilisation success at [Sperm]max was then calculated (F_T@[Sperm]$max_{control}$). We also estimated [Sperm]50 which was calculated as the sperm density required to achieve 50% of the maximum fertilisation (F50) in the control and the respective fertilisation success in the paired treatment at the same sperm density (F_T@[Sperm]$50_{control}$). The density of sperm in the treatment required to obtain the same level of fertilisation as the $F50_{control}$ was also calculated ([Sperm]$50_{treatment}$) As well as the modelled values, comparisons were made between the best observed fertilisation in the control ($BestF_{control}$) assay and the observed fertilisation in the treatment ($ObservedF_{treatment}$) at the sperm density ([Sperm]$Best_{control}$) that achieved $BestF_{control}$ (see Figure 2.1).

2.2.8 Statistics

The R package DRC (Ritz and Strebig, 2005) was used to model the test data for each endpoint and calculate toxicity estimates. Regression models tested included logistic, log-logistic and Weibull models with different levels of parametrization. Model comparisons were conducted using the Akaike Information Criterion (AIC) and models that best described the data were applied to determine metal concentrations that elicited a 50% (EC50) and 10% (EC10) decrease in fertilisation success (% control). The associated 95% confidence limits were estimated using the delta method. A ratio test was used to compare EC50 values through the DRC function [EDcomp()] and statistical differences were determined using the method described by Sprague and Fogel (1976)(Sprague and Fogels, 1976).



Step 1: $Fmax_c$
$Fmax_c$ is the maximum modelled fertilisation in the control.

Step 2: $[Sperm]max_c$
$[Sperm]max_c$ is the sperm density that achieved maximum modelled fertilisation in the control

Step 3: $F_T[Sperm]max_c$
$F_T[Sperm]max_c$ is the modelled fertilisation success in the treatment, at the sperm density that achieved maximum modelled fertilisation in the control.

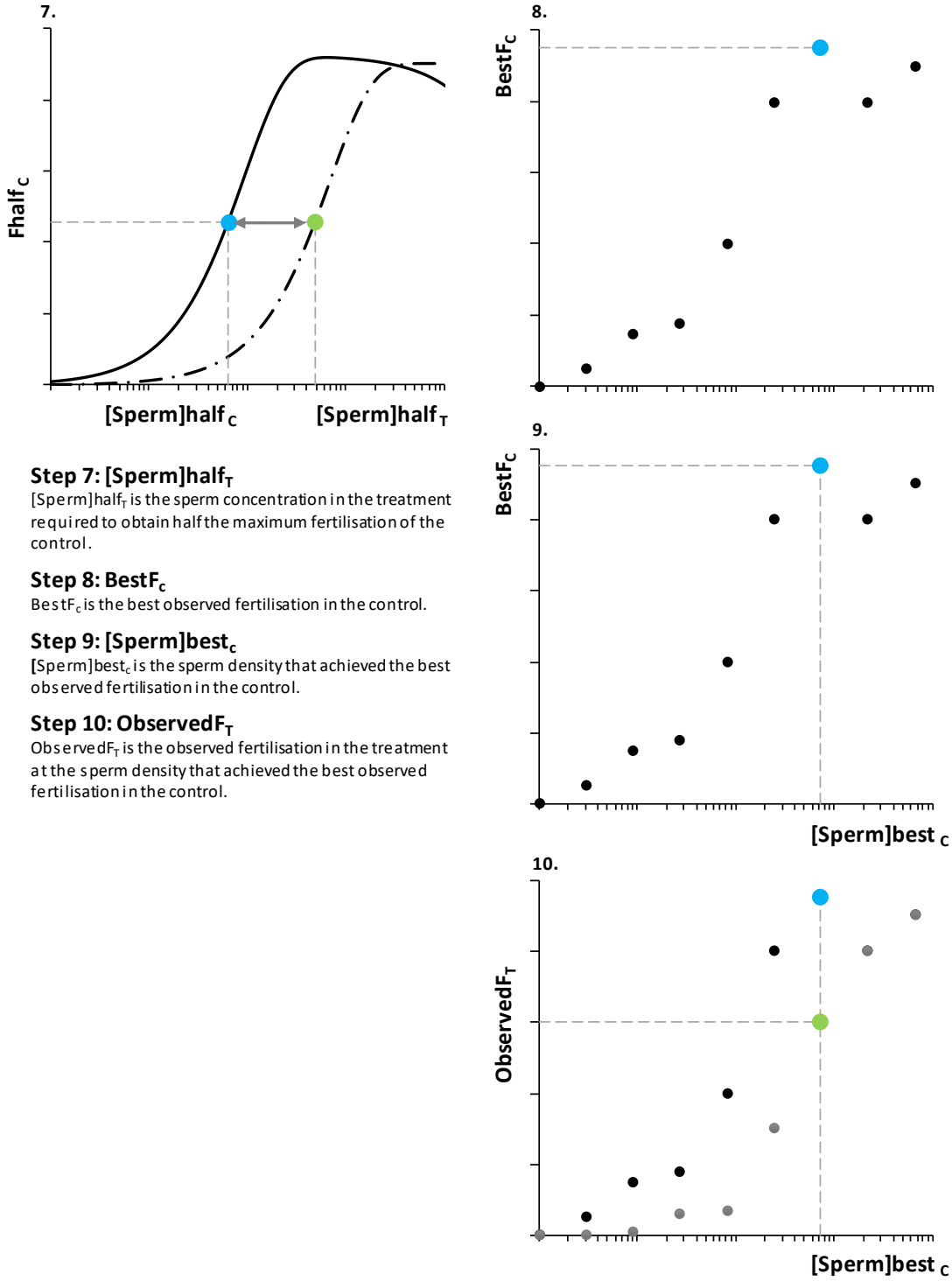
Step 4: $Fhalf_c$
$Fhalf_c$ is the half the maximum modelled fertilisation in the control.

Step 5: $[Sperm]max_c$
$[Sperm]half_c$ is the sperm density that achieved half the maximum modelled fertilisation in the control

Step 6: $F_T[Sperm]half_c$
$F_T[Sperm]half_c$ is the modelled fertilisation success in the treatment, at the sperm density that achieved half the maximum modelled fertilisation in the control.

X Axis: Sperm Density**, Y Axis:** Fertilisation Success

Figure 2.1a**:** A guide to calculating the metrics used to determine toxicity



Step 7: [Sperm]half$_T$
[Sperm]half$_T$ is the sperm concentration in the treatment required to obtain half the maximum fertilisation of the control.

Step 8: BestF$_c$
BestF$_c$ is the best observed fertilisation in the control.

Step 9: [Sperm]best$_c$
[Sperm]best$_c$ is the sperm density that achieved the best observed fertilisation in the control.

Step 10: ObservedF$_T$
ObservedF$_T$ is the observed fertilisation in the treatment at the sperm density that achieved the best observed fertilisation in the control.

X Axis: Sperm Density, **Y Axis:** Fertilisation Success

Figure 2.1b: A guide to calculating the metrics used to determine toxicity

2.3 Results

Thirty experiments passed the acceptability criteria of >80% fertilisation in the FSW control and ≤5% fertilised eggs in the no sperm controls and were used to determine toxicity endpoints. There were eight unsuccessful experiments, seven of which did not exceed 80% fertilisation and one where the no sperm controls had greater than 5% fertilised eggs. These data were not used. For Zn and Cu, concentrations were tested in eight separate paired experiments, while for Pb and Cd, concentrations were tested in seven paired experiments. The concentrations of metals in the controls, some of which were above Australian and New Zealand guideline values (GVs)(ANZECC and ARMCANZ, 2000), were typical of those expected for Grange Beach(Gaylard, 2004) and nearby beaches(Chakraborty and Owens, 2014). In toxicity tests with Cu, Pb, Cd and Zn, controls contained 1-2 µg Cu/L, 16-66 µg Zn/L <4-23 µg Pb/L and <4-16 µg Cd/L (See Appendix 1). Grange Beach water was considered to be of poor quality in 2004, based on exceedances of the GVs for aluminium and zinc(Gaylard, 2004). Therefore, it is likely that the community of worms sampled in this study had undergone long-term exposure to most of these contaminants over several generations, and may well yield different sensitivities to metals than worms collected from pristine environments.

2.3.1 Fertilisation-Sperm Density Relationships

The relationship between fertilisation success and sperm density varied considerably between tests (different batches of sperm). Therefore, each treatment was normalised to the respective control. Figures 2.2-2.5 show the

paired fertilisation assays for Cu, Zn, Cd and Pb (respectively), illustrating the changes in the fertilisation-sperm density relationship with increasing concentrations of Cu. Across controls in all of the experiments, the observed maximum fertilisation (80-98%) occurred at sperm densities ranging between 10^4 and 10^6 sperm mL^{-1} (60:1 to 6000:1, Sperm:Egg). At sperm densities below this, fertilisation success (%) decreased with decreasing sperm density and at higher sperm densities fertilisation success either plateaued or started to decrease slowly with increasing sperm density. However, across paired assays and experiments (i.e. among different crosses), there was considerable variability among controls in the relationships between fertilisation success and sperm density and at a given sperm density fertilisation success could vary by over 50% (Figure 2.2-2.5).

The presence of dissolved metals appeared to affect the relationship between fertilisation success and sperm density. There was a right shift in the fertilisation curves with metals relative to their control assays, i.e. [Sperm]50 increased (Figure 2.2-2.5). At low to moderate sperm densities, fertilisation was lower in metal treatments than in the respective controls. Therefore, more sperm were required in metal treatments than in controls to achieve the same level of fertilisation success. In most cases, fitted maximum fertilisation was slightly less for metal treatments than the controls.

Although in most gamete crosses there were moderate (putative polyspermy) decreases in fertilisation success at high densities of sperm, the presence of

metals did not appear to change this much. Where there were consistent decreases in fertilisation at higher sperm densities, these appeared to occur in both the treatment and control assays (e.g. Copper 11 μg L^{-1} assay; Figure 2.2).

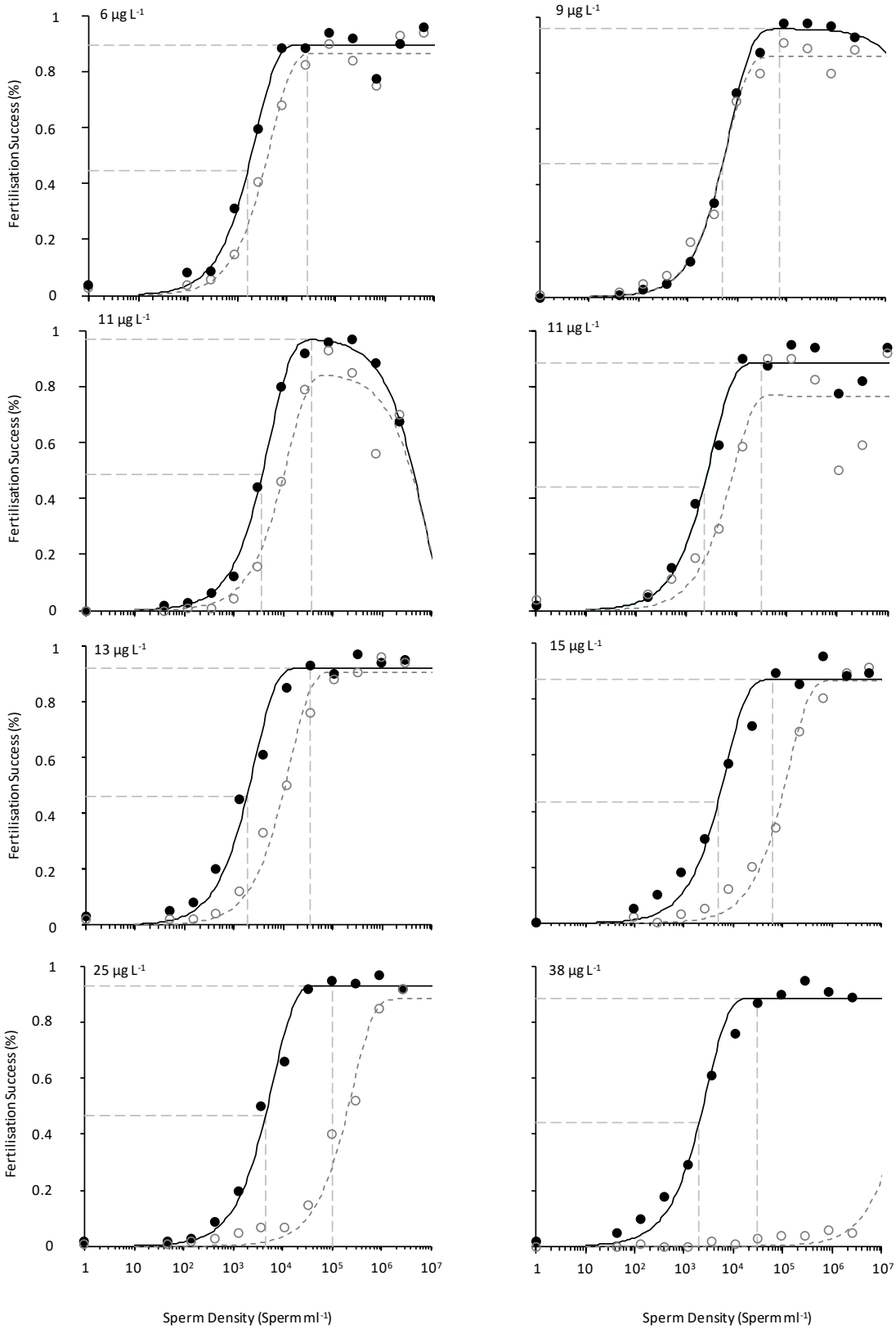


Figure 2.2**:** Fertilisation curves for *G. caespitosa* sperm exposed to Cu prior to fertilisation. Controls are represented by the solid line and treatments by the dashed line. Measured dissolved (<0.45 µm) copper concentration is reported for each treatment in the upper left of the graph.

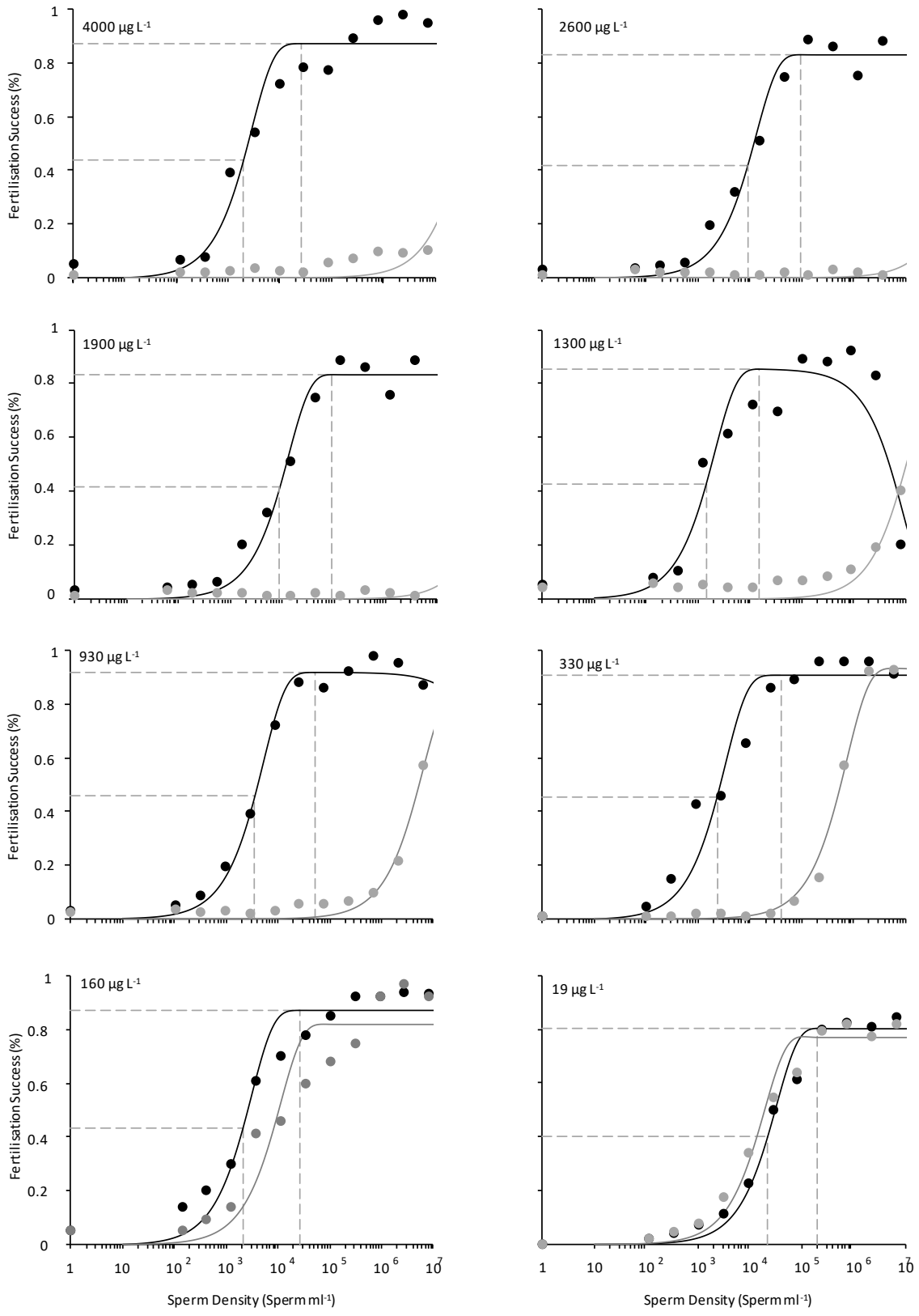


Figure 2.3: Fertilisation curves for *G. caespitosa* sperm exposed to Zn prior to fertilisation. Controls are represented by the solid line and treatments by the dashed line. Measured dissolved (<0.45 µm) Zinc concentration is reported for each treatment in the upper left of the graph.

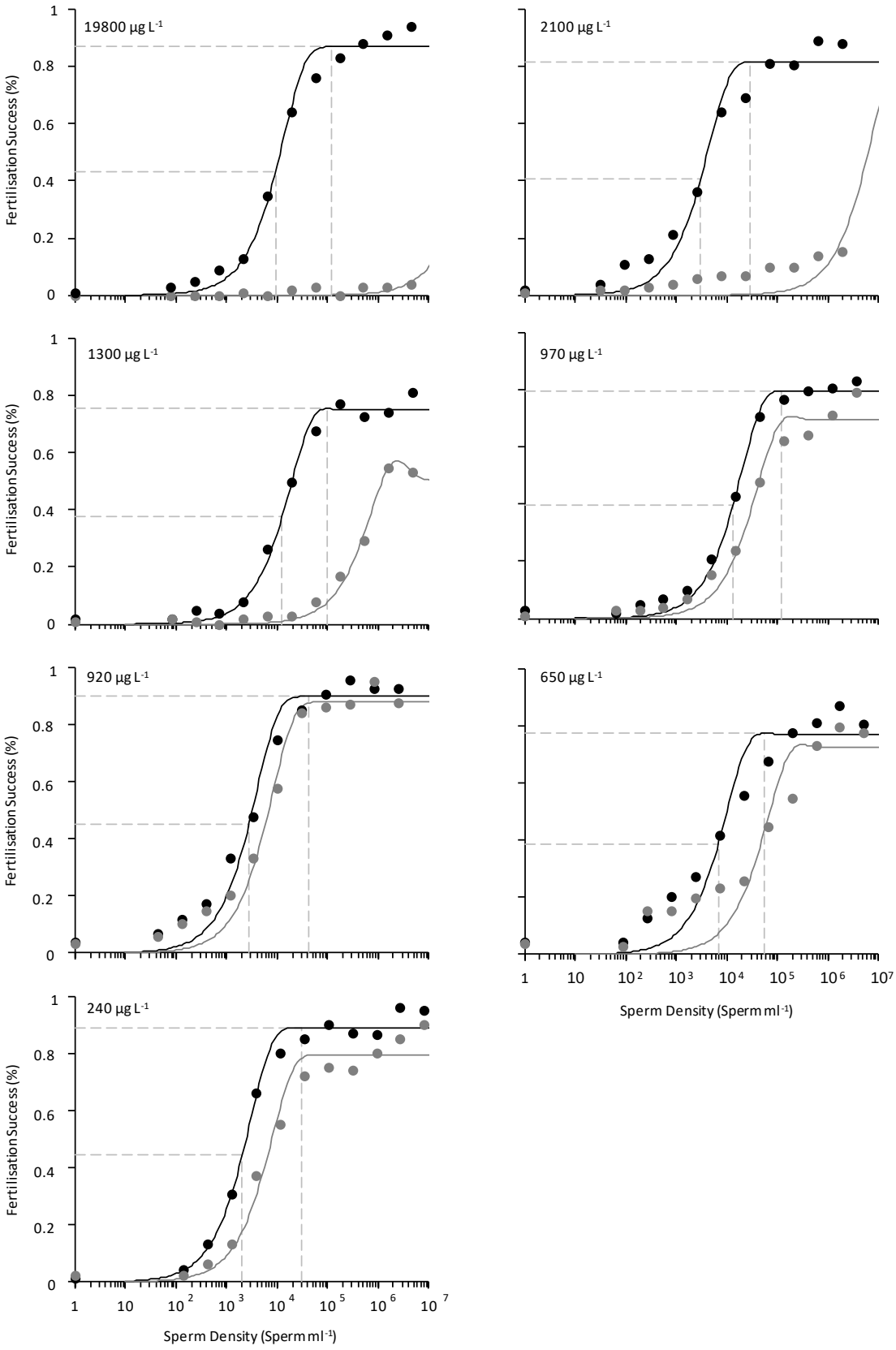


Figure 2.4: Fertilisation curves for *G. caespitosa* sperm exposed to Pb prior to fertilisation. Controls are represented by the solid line and treatments by the dashed line. Measured dissolved (<0.45 µm) Pb concentration is reported for each treatment in the upper left of the graph.

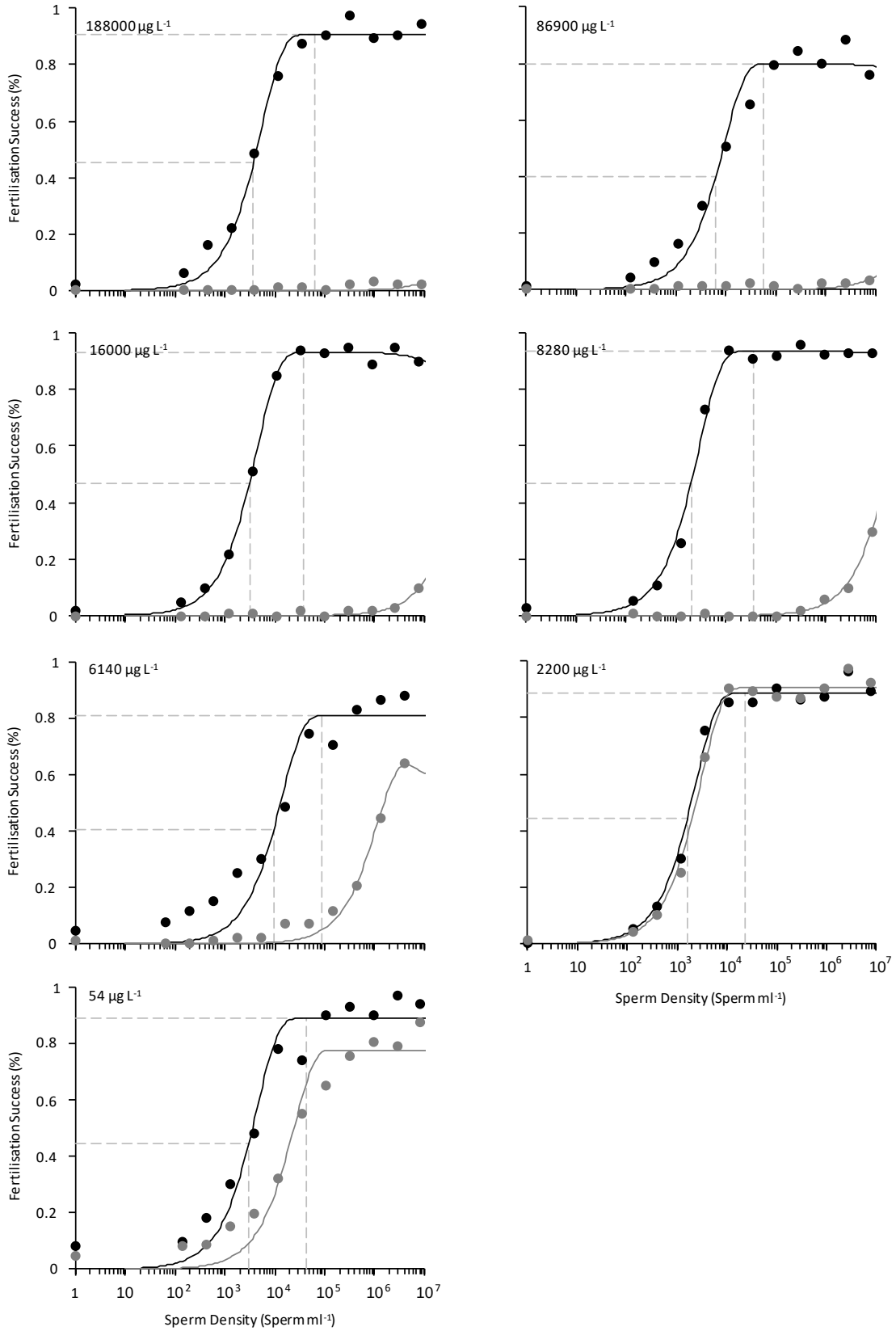


Figure 2.5: Fertilisation curves for *G. caespitosa* sperm exposed to Cd prior to fertilisation. Controls are represented by the solid line and treatments by the dashed line. Measured dissolved (<0.45 µm) Cd concentration is reported for each treatment in the upper left of the graph.

2.3.2 Fertilisation success at specific sperm densities

Based on the fitted fertilisation models to calculate fertilisation success at a given sperm density (10^4, 10^5 or 10^6 sperm mL^{-1}), the concentration-response relationships all showed a decrease in fertilisation with increasing metal concentration (Figures 2.6-2.9). Resulting EC50 values for each metal, measured between 10^4 and 10^6 sperm mL^{-1}, ranged from 12 to 33 µg Cu L^{-1}, from 160 to 550 µg ZnL^{-1}, from 560 to 1500 µg Pb L^{-1} and from 4900 to 6100 µg Cd L^{-1} (Table 2.1). Resulting EC10 values for each metal, measured between 10^4 and 10^6 sperm mL^{-1}, ranged from 8.2 to 27 µg Cu L^{-1}, from 68 to 200 µg Zn L^{-1}, from 65 to 910 µg Pb L^{-1} and from 3900 to 4200 µg Cd L^{-1} (Table 2.1).

For Cu, Zn and Pb, there were significant decreases in toxicity with increase in sperm density evidenced by greater EC50 and EC10 values at higher sperm densities (Table 2.2). For Cd, however, there was no significant difference between the toxicity values derived at each sperm density. Zinc toxicity was most impacted by sperm density with a three-fold increase in EC50 between 10^4 and 10^6 sperm mL^{-1} (Table 2.2). Of the four metals, Cu was most toxic to *G. caespitosa* fertilisation with the lowest EC50 (12-33 µg/L) and EC10 values (8.2-27 µg/L) when tested with sperm densities of 10^4 to 10^6 sperm mL^{-1}. The final ranking of metal toxicity for all three sperm densities tested was Cu>Zn>Pb>Cd.

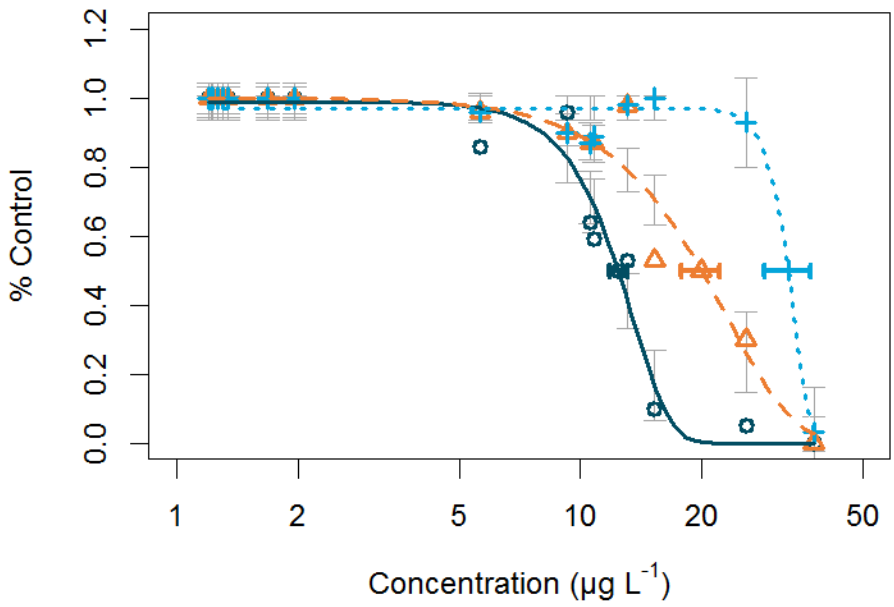


Figure 2.6: Concentration-response relationships when exposing *G. caespitosa* sperm to Cu prior to and during fertilisation. Relationships were fitted at three sperm densities: 10^4 (black, solid), 10^5 (orange, dashed) and 10^6 (blue, dotted) sperm mL^{-1}. EC50 values were calculated for each sperm density (solid horizontal lines) with associated confidence intervals. Error bars in the vertical direction represent confidence in the model.

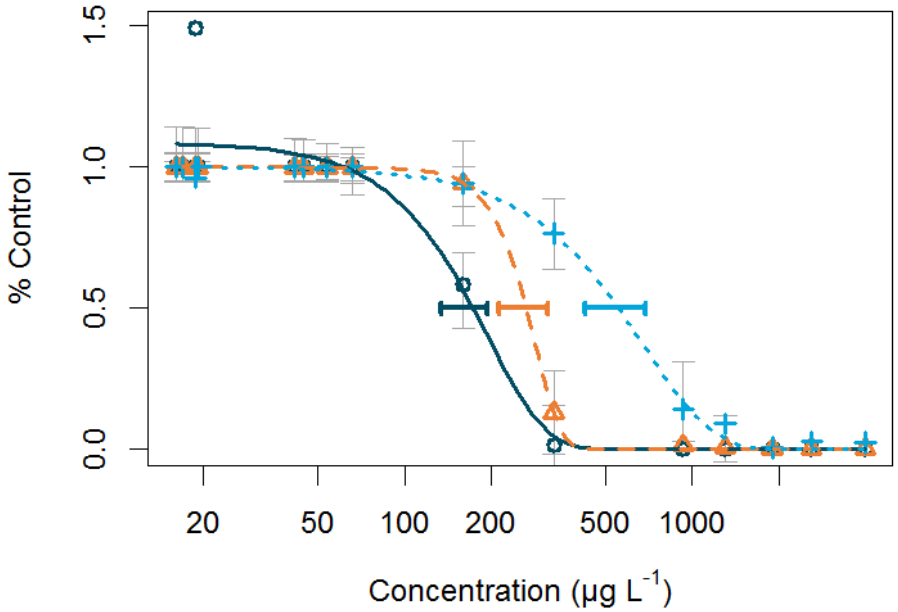


Figure 2.7: Concentration-response relationships when exposing *G. caespitosa* sperm to Zn prior to and during fertilisation. Relationships were fitted at three sperm densities: 10^4 (black, solid), 10^5 (orange, dashed) and 10^6 (blue, dotted) sperm mL^{-1}. EC50 values were calculated for each sperm density (solid horizontal lines) with associated confidence intervals. Error bars in the vertical direction represent confidence in the model.

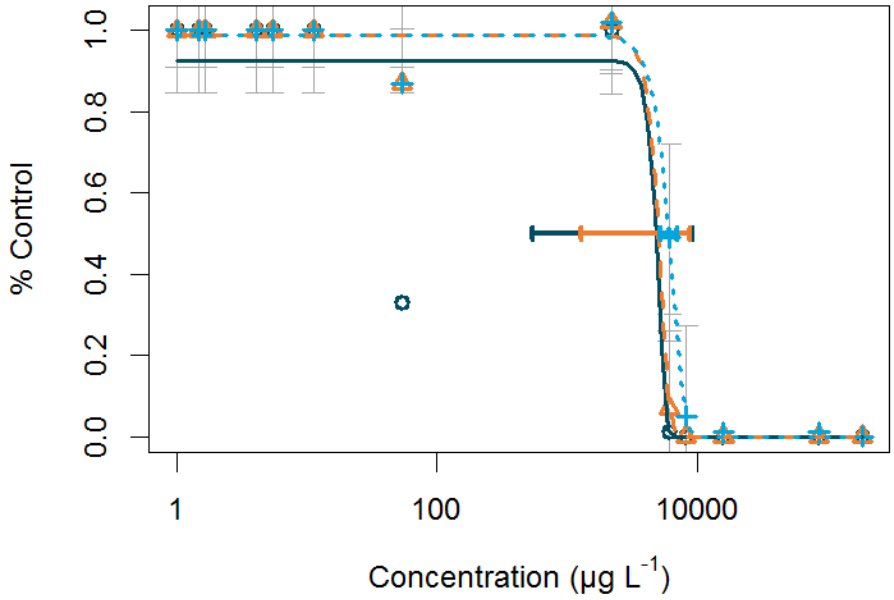


Figure 2.8: Concentration-response relationships when exposing *G. caespitosa* sperm to Cd prior to and during fertilisation. Relationships were fitted at three sperm densities: 10^4 (black, solid), 10^5 (orange, dashed) and 10^6 (blue, dotted) sperm mL^{-1}. EC50 values were calculated for each sperm density (solid horizontal lines) with associated confidence intervals. Error bars in the vertical direction represent confidence in the model.

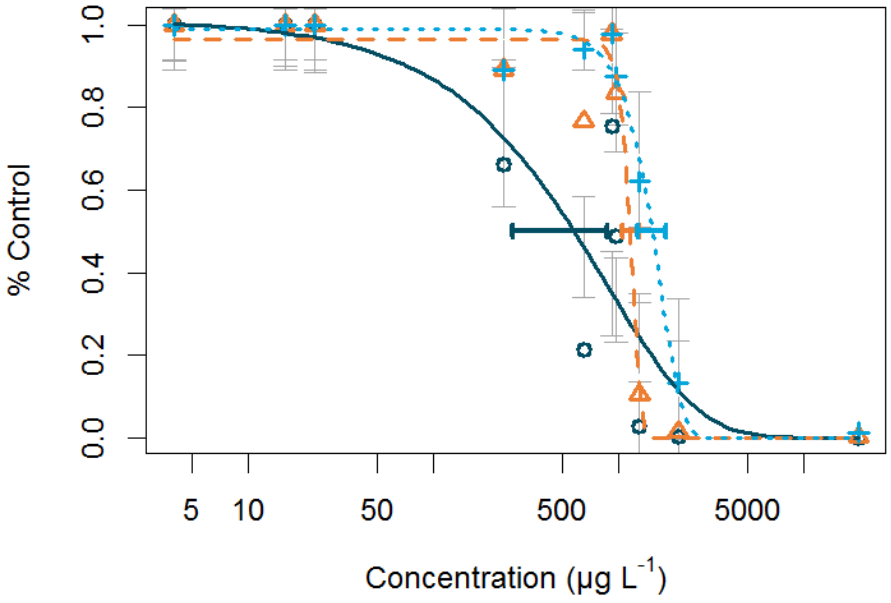


Figure 2.9: Concentration-response relationships when exposing *G. caespitosa* sperm to Pb prior to and during fertilisation. Relationships were fitted at three sperm densities: 10^4 (black, solid), 10^5 (orange, dashed) and 10^6 (blue, dotted) sperm mL^{-1}. EC50 values were calculated for each sperm density (solid horizontal lines) with associated confidence intervals. Error bars in the vertical direction represent confidence in the model.

Table 2.1: EC10, EC50 values and associated 95% confidence limits calculated based on fertilisation success at three standard sperm concentrations for *G. caespitosa* (2SF)a

	Sperm Density (Sperm mL^{-1})	EC10 ($\mu g\ L^{-1}$)	EC50 ($\mu g\ L^{-1}$)
Cu	10^4	8.2 (7.0-9.4)	12 (12-13)
	10^5	9.6 (7.4-11)	20 (18-22)
	10^6	27 (21-34)	33 (29-37)
Zn	10^4	68 (31-110)	160 (130-190)
	10^5	180 (94-270)	260 (210-320)
	10^6	200 (85-310)	550 (420-680)
Pb	10^4	65 (0-180)	560 (270-860)
	10^5	980 (770-1,200)	1,200 (1,000-1,300)
	10^6	910 (620-1,200)	1,500 (1,300-1,800)
Cd	10^4	3900 (0-10,000)	4900 (540-9,300)
	10^5	3900 (3,000-11,000)	5100 (1,300-8,900)
	10^6	4200 (2100-6300)	6100 (5,300-7,000)

[a] Measured dissolved (<0.45 μm) values.

Table 2.2: Statistical comparison of EC50 values at different sperm densities for *G. caespitosa*

	Sperm density	EC50 ratio	Magnitude	Significance
Cu	$10^4 : 10^6$	0.38	2.6	$p < 0.05$*
	$10^5 : 10^6$	0.61	1.6	$p < 0.05$*
Zn	$10^4 : 10^6$	0.30	3.4	$p < 0.05$*
	$10^5 : 10^6$	0.48	2.1	$p < 0.05$*
Pb	$10^4 : 10^6$	0.37	2.7	$p < 0.05$*
	$10^5 : 10^6$	0.76	1.3	$p < 0.05$*
Cd	$10^4 : 10^6$	0.80	1.3	$p > 0.05$
	$10^5 : 10^6$	0.83	1.2	$p > 0.05$

*Significant difference.

2.3.3 Fertilisation model endpoints

To identify which of the fertilisation model endpoints would be most useful to assess toxicity, the fitted fertilisation model parameters Fmax, F50, [Sperm]50 and Observedmax were used to construct concentration-response relationships for all metals (Figures 2.10-2.13). For Cu, Zn and Pb, F50 and Sperm[50] were the most sensitive endpoints, resulting in the lowest EC10 and EC50 values (Table 2.3). For Cd, there was no significant difference between endpoints and a wide range in confidence intervals within the slope of the response curves (Figure 2.13). Effect concentrations (10 and 50%) calculated using [Sperm]50 and F50 were consistently lower than those calculated for standard fertilisation success at 10^6 sperm mL^{-1}.

Table 2.3**:** EC10, EC50 values and associated 95% confidence limits calculated using fertilisation model endpoints for *G. caespitosa* (2SF).

	Endpoint	**EC10 (µg L^{-1})**	**EC50 (µg L^{-1})**
Cu	[Sperm]50	4.5 (0-13)	9.8 (5.5-14)
	F50	9.5 (8.9-10)	11 (10-11)
	Fmax	10 (8.4-12)	17 (14-19)
	Observedmax	26 (22-30)	32 (29-35)
Zn	[Sperm]50	54 (0-110)	120 (70-160)
	F50	68 (18-120)	130 (103-160)
	Fmax	160 (150-160)	200 (190-200)
	Observedmax	120 (90-160)	380 (330-430)
Pb	[Sperm]50	30 (0-75)	180 (22-330)
	F50	36 (0-100)	240 (0-490)
	Fmax	980 (850-1,100)	1,100 (940-1,300)
	Observedmax	1070 (950-1,200)	1,500 (1,400-1,600)
Cd	[Sperm]50	2200 (1,600-6,000)	3,700 (0-9,900)
	F50	2500 (4,000-7,800)	4,000 (0-9800)
	Fmax	3700 (4,600-9,000)	4,600 (880-8,400)
	Observedmax	5400 (4,800-6,100)	6,800 (6,300-7,300)

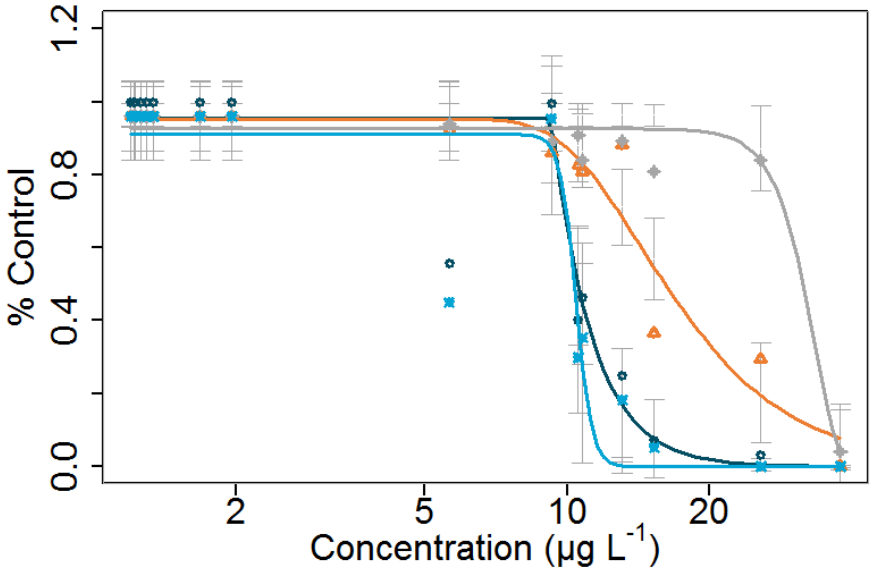


Figure 2.10: Concentration-response relationships for Cu. Curves were calculated using four different endpoints; F50 (navy circles), Fmax (orange triangles), [Sperm]50 (Blue "X") and Observedmax (grey plus). Shaded regions show 95% confidence intervals of the model.

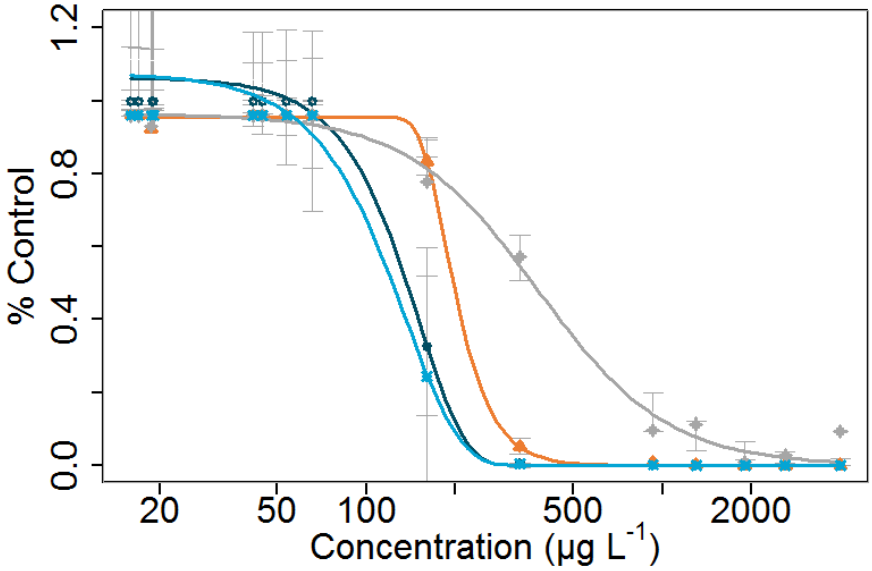


Figure 2.11: Concentration-response relationships for Zn. Curves were calculated using four different endpoints; F50 (navy circles), Fmax (orange triangles), [Sperm]50 (Blue "X") and Observedmax (grey plus). Shaded regions show 95% confidence intervals of the model.

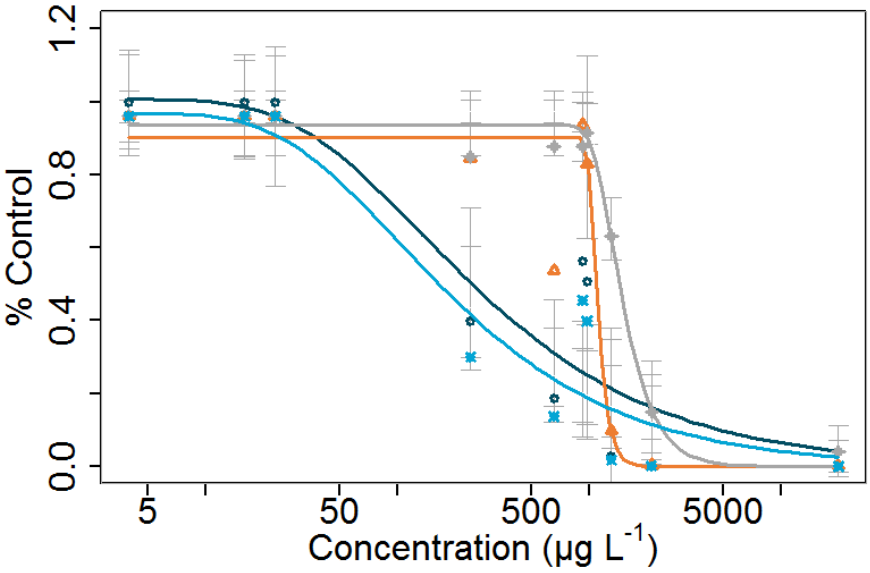


Figure 2.12: Concentration-response relationships for Pb. Curves were calculated using four different endpoints; F50 (navy circles), Fmax (orange triangles), [Sperm]50 (Blue "X") and Observedmax (grey plus). Shaded regions show 95% confidence intervals of the model.

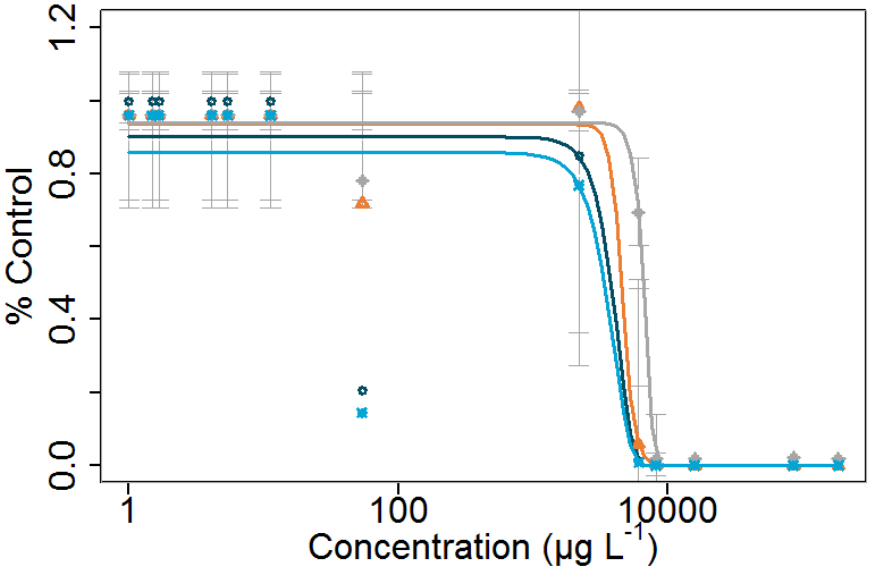


Figure 2.13: Concentration-response relationships for Cd. Curves were calculated using four different endpoints; F50 (navy circles), Fmax (orange triangles), [Sperm]50 (Blue "X") and Observedmax (grey plus). Shaded regions show 95% confidence intervals of the model.

2.4 Discussion

2.4.1 Single sperm densities

The toxicity of Cu, Zn and Pb to fertilisation success in *G. caespitosa* was dependent on sperm density. We found significant differences in EC50 values calculated at fixed sperm densities of 10^4, 10^5 and 10^6 sperm mL^{-1}, with up to threefold differences in toxicity between estimates at 10^4 and 10^6 sperm mL^{-1}. Although similar trends were observed for Cd, there was no statistical difference in EC values with sperm density. These findings are consistent with those of Hollows et al.(Cam F Hollows, Johnston and Marshall, 2007) who found that the effects of Cu to *G. caespitosa* sperm were sperm-density dependent, with stronger effects at low sperm densities, than at high sperm densities(Cam F. Hollows, Johnston and Marshall, 2007). Work on other species, has also found that the sensitivity of sperm bioassays decreases as sperm:egg ratios increase(Dinnel, Link and Stober, 1987).

Fertilisation tests using single sperm densities may underestimate toxicity. While some test protocols expose invertebrate sperm at low sperm densities(Simon and Laginestra, 1997; Williams, Bentley and Hardege, 1997), the majority involve exposing sperm at densities as high as 10^6 sperm mL^{-1} (USEPA, 1995). The review by Hudspith et al.(Hudspith, Reichelt-Brushett and Harrison, 2017) of the effects of heavy metals on fertilisation success across a range of marine invertebrates, suggests that most studies have used single sperm densities of between ~10^5 and ~10^6 sperm mL^{-1}. The sperm densities usually being recommended are because they would likely generate greater

than 70-80% fertilisation success in controls for a given species. Had we only conducted our experiments at 10^6 sperm mL^{-1}, we would likely have underestimated the potency of Cu, Zn and Pb to fertilisation success relative to that if we had run the experiments at 10^5. Both experiments would have generated >70% fertilisation in controls, but the measured toxicity (EC50 or EC10) of metals (Cu, Zn, Pb) would have been significantly lower at 10^5 sperm mL^{-1} ($p < 0.05$;

2.2).

Natural spawning sperm densities can vary due to a range of individual, demographic, species-specific and environmental factors(Levitan, 1998; Havenhand and Styan, 2010; Crimaldi and Zimmer, 2014) and, as such, the use of a single standardised sperm density for toxicity testing may not be suitable. Thus, if spermiotoxicity tests are to be run at a single sperm density, as most are(Hudspith, Reichelt-Brushett and Harrison, 2017), then care is needed in determining what that density should be, as the toxicity results might be conditional on this. Consequently, metrics that are independent of sperm density (but which can be used to predict fertilisation across sperm densities) should also be considered for routine toxicity testing.

2.4.2 Multiple sperm densities

Fertilisation assays across multiple sperm densities can provide information about the mechanism of toxicant impacts on fertilisation. When we examined the toxicity of metals across a range of sperm densities, we observed a shift in the fertilisation relationships to the right relative to controls (see Figures 2.2-

2.5). This suggests that metals impact the fertilisation process mainly through effects on sperm. Figure 2.14 represents how the relationship between fertilisation success and sperm density should change when a toxicant impacts different aspects of the fertilisation process. An impact to sperm viability will reduce the sperm-egg encounter rate, with the effect resulting in a horizontal (right) shift in the fertilisation relationship. In contrast, an impact on eggs or early developing zygotes would produce a consistent decrease in the proportion of fertilised eggs in the treatment relative to the control, resulting in a decrease in the maximum number of eggs that are able to be fertilised (Figure 2.14e). When toxicants disrupt polyspermy blocks, there will not be much of a difference between treatment and control at low sperm densities but an increasing difference at higher sperm densities – thus, there would also be a decrease in the maximum fertilisation and this would occur at a lower sperm density than in the control (Figure 2.14f). Had we chosen to test only one sperm density, while we may have been able to detect an impact on fertilisation (at that sperm density; Figure 2.14a,b,c), we would not have been unable to determine which part of the fertilisation process was affected. Thus, although the fertilisation assays here involved more effort, we believe our work is a good example of the potential value of this added complexity in ecotoxicology assessments where fertilisation is measured as an endpoint.

2.4.3 Fertilisation endpoints

Fertilisation endpoints that are independent of sperm density provided sensitive measures of toxicity. There was a consistent ordering of how

sensitive each endpoint appeared across the four metals; [Sperm]50, F50 and then Fmax, followed by Observedmax. The first three endpoints were all significantly lower ($p < 0.05$) than EC50 values calculated at 10^6 sperm mL^{-1}. The most sensitive were [Sperm]50 and F50, with no significant difference between EC50s using these endpoints. The mode of toxicity of metals on fertilisation (affecting sperm viability) is likely the key factor in determining the relative order of endpoints here. Had metals impacted egg viability we would expect that Fmax (and possibly Observedmax) might then be more sensitive measures of toxicity (see figure 5). Thus, information regarding the mode of toxicity is required to pick an appropriate endpoint.

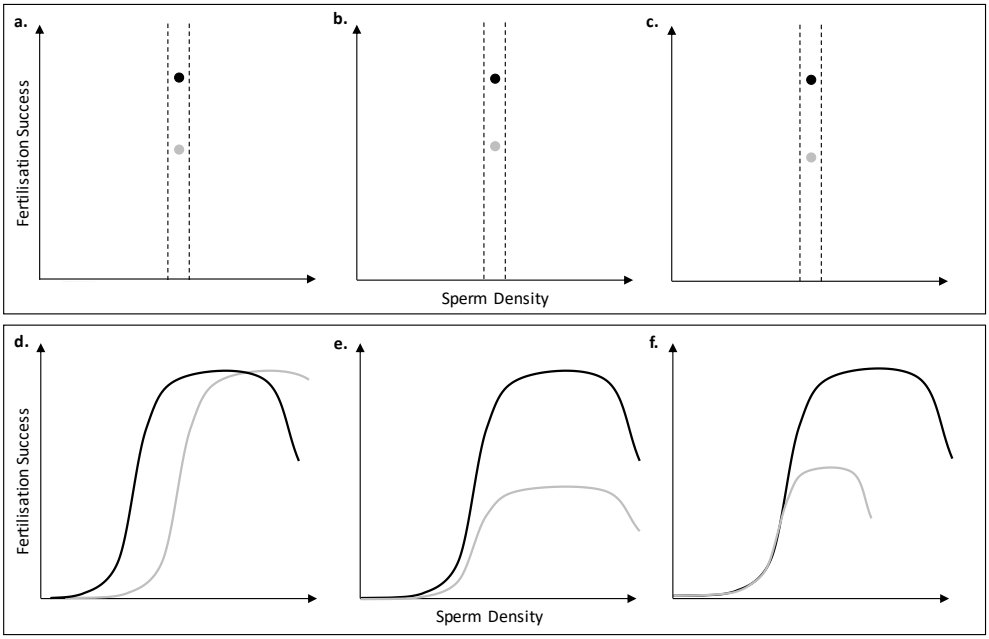


Figure 2.14: The potential effects of a toxicant on fertilisation success: a,b,c) show the impact of a toxicant to sperm, eggs and the efficiency of polyspermy blocks (respectively) when only testing at a fixed sperm density; d,e,f) show the same effects but across a range of sperm densities. Adapted from Marshall(Marshall, 2006).

2.4.4 *Galeolaria caespitosa*

As an ecotoxicological test species, *G. caespitosa* are almost ideal to work with and possess a range of attributes that enabled us to attempt more complex

experimental assessments than might usually be done in assessments of the impacts of metals on fertilisation. Importantly, the worms are easily collected and amenable to laboratory holding and most adults within *G. caespitosa* aggregations have abdomens swollen with gametes throughout the year(Kupriyanova, 2006), from which they immediately release large amounts of eggs or sperm when their tubes have been broken or have been disturbed mechanically(Kupriyanova and Havenhand, 2002). Thus, being able to collect and use ripe animals over a prolonged period enabled us to run the thirty separate crosses we needed to assess the effects of metals using fertilisation assays.

The species also appears to be relatively sensitive to metals which led to clear concentration response relationships. Fertilisation in *G. caespitosa* was sensitive to metals, with effect concentrations (10%) for Cu, Zn, Pb, and Cd estimated at between 4.5-27, 54-200, 30-910 and 2200-4200 μg L^{-1} respectively, depending on the sperm density used in fertilisation assays and on the metric used to assess toxicity. The toxicity values for Cu and Zn are the lowest so far reported for a polychaete, while those for Pb and Cd are within the range of those reported for marine invertebrates by Hudspith et al.(Hudspith, Reichelt-Brushett and Harrison, 2017). This suggests that this test species is a credible candidate for use in risk assessments. The polychaete community we studied is currently persisting in waters where the metal concentrations already periodically exceed the EC10 values we derived for fertilisation success(Gaylard, 2004). Therefore, any further increases in metals

in this area will likely reduce fertilisation success of gametes more frequently, which could lead to population loss and potentially cause community collapse.

It is also possible that *G. caespitosa* collected from more pristine environments would yield different (greater) sensitivities to the metals we tested. For example, the sperm of killfish (*Fundulus heteroclitus*) from a contaminated site showed greater tolerance to metal toxicity (methylmercury) than the sperm of those from pristine environments(Khan and Weis, 1987). Research has indicated that populations living in metal polluted environments can become tolerant to metals(Klerks and Weis, 1987; Weis and Weis, 1989; Durou, Mouneyrac and AmLard-Triquet, 2005; Wang and Rainbow, 2005; Bankar *et al.*, 2018). Therefore, although the data presented here are highly relevant for use in assessing potential impact of increased metals at the study site, they may underestimate the toxicity of dissolved metals to fertilisation in *G. caespitosa* from a pristine environment.

2.5 Conclusion

This chapter provided further evidence that metals impact marine invertebrate sperm during external fertilisation. Therefore, sperm show promise as an indicator of metal toxicity. It also found that the use of single sperm densities to determine the effects of metals to fertilisation can underestimate toxicity. Toxicity tests that evaluate the impact of a metal to fertilisation using multiple sperm densities are more informative than tests using single sperm densities.

CHAPTER 3

Effects of Metals on Marine Invertebrate Sperm Motility

In Preparation for *Aquatic Toxicology*:

"Assessing the impacts of metals on broadcast spawners:

Effects on sperm do not match effects on fertilisation,"

Antony Lockyer, Monique Binet, Laura Falkenberg, Jon Havenhand, Craig

Styan,

3.1 Introduction

As small, physiologically active single cells, gametes have limited protective structure or barriers that might buffer their exposure to a toxicant. Sperm are considered especially sensitive to environmental contaminants and their function can be affected by these in a range of ways. Dissolved metals can disrupt sperm activation, motility and velocity (Caldwell, Bentley and Olive, 2004), prevent sperm-egg binding (Pillai *et al.*, 1997), and damage genetic material (Lewis and Galloway, 2009). Sperm motility and velocity are likely to be heavily impacted by increases in dissolved metals as these processes are regulated by ion homeostasis (Tosti and Ménézo, 2016). Thus, increases in metal ion availability could disrupt normal sperm swimming processes. Effects on sperm swimming ability may reduce the effective density of properly functioning sperm, potentially driving sperm-limitation of fertilisation (Cam F. Hollows, Johnston and Marshall, 2007).

Sperm limitation occurs when sperm availability limits successful fertilisation and zygote production (Levitan and Petersen, 1995). Sperm limitation can be an issue in natural (or overfished) populations when spawner numbers are low or sparsely distributed. In both cases, eggs and sperm may be diluted to low densities before their paths cross each other (Levitan and Petersen, 1995; Styan, 1998). Where metals have an effect on sperm this may act to increase sperm dilution effects (Cam F Hollows, Johnston and Marshall, 2007). The extent of dilution effects depend on structured stirring by the flow field (at large scales) and sperm motility and taxis (at small scales) to bring gametes

together (John P Crimaldi, 2012; Crimaldi and Zimmer, 2014). Both theoretical and experimental evidence has shown that the number of motile sperm in the water immediately surrounding a free-spawned egg is a key determinant of fertilisation success (Styan and Butler, 2000; Marshall, Styan and McQuaid, 2009; Crimaldi and Zimmer, 2014). Sperm motility appears to be extremely important for fertilisation on small scales, within individual packets of water (Kolmogorov scale) where there are already at least some chance of gametes meeting (Crimaldi and Zimmer, 2014). On these small scales, motility is likely to be important for sperm to follow chemical gradients to find eggs (Riffell, Krug and Zimmer, 2002; Zimmer and Riffell, 2011) to search for binding sites on the egg surface and, in some species, to provide physical propulsion through jelly coats or around accessory cells that surround eggs (Christen, Schackmann and Shapiro, 1983; Reinhart, Ridgway and Chandler, 1998).

As sperm swimming is important for external fertilisation it may be useful as an endpoint for assessing the effects of metals on fertilisation success (Fitzpatrick *et al.*, 2008; Fabbrocini, Di Stasio and D'Adamo, 2010; Caldwell *et al.*, 2011). Previous research has shown that sperm swimming can be negatively affected by metal exposure. When sperm of *G. caespitosa* were exposed to Cu this decreased the distance between a spawning male and female at which fertilisation is assured (Cam F Hollows, Johnston and Marshall, 2007). Thus, the presence of Cu either reduced the number of live sperm, or reduced the number of successful sperm/egg interactions. Similarly, sperm of the blue mussel (*Myulis trossulus*)(Fitzpatrick *et al.*, 2008) and of the Atlantic purple

sea urchin (*Arbacia punctulata*)(Caldwell *et al.*, 2011) showed decreased sperm velocities when exposed to environmentally relevant concentrations of Cu. Sperm motility of the purple sea urchin (*Paracentrotus Lividus)* showed decreased percent motility when exposed Cd (EC50: 2.16 ± 0.4 mg L^{-1}) and pore water (Fabbrocini, Di Stasio and D'Adamo, 2010). Sperm swimming appears to be sensitive to metal toxicity and is relatively simple to measure compared to fertilisation trials involving both eggs and sperm (Lockyer, Binet and Styan, 2019). Thus, measuring the effects of metals on sperm swimming, as a proxy for fertilisation, could potentially be a much quicker and cheaper method of toxicity assessment. Particularly where existing information suggests that the likely mechanism of toxicity of metals on fertilisation is via effects on sperm function (Lockyer, Binet and Styan, 2019). However, for sperm swimming endpoints to be adopted in ecotoxicological testing, a link between effects on fertilisation success needs to be established to validate their ecological relevance. At a minimum, it is necessary to understand whether impacts to sperm swimming are proportional to impacts to fertilisation success and, if so, how to scale predictions based on sperm endpoints to fertilisation.

This chapter aims to test whether sperm swimming can account for the effects of metals on fertilisation, using the serpulid worm *G. caespitosa* as a model species (Ross and Bidwell, 2001). *Galeolaria caespitosa* sperm were exposed to metals (Cu, Zn, Cd and Pb) and swimming traits were measured using Computer Assisted Sperm Analysis (CASA) and Sperm Accumulated Against

Surface (SAAS). Concentration response relationships for each metal were established and used to determine effect concentrations where applicable. The results from this experiment were compared to the fertilisation data previously published (Chapter 2; Lockyer, Binet and Styan (2019)). Using the previously published data, and supplementary information on gamete characteristics (Kupriyanova and Havenhand, 2002), A model is developed to determine how effects on sperm would impact fertilisation success across a range of sperm densities. This model was then used to determine the relevance of effects on sperm as a proxy for effects on fertilisation success.

3.2 Methods

3.2.1 General Methods

Study site, experimental conditions and the collection and spawning of *G. caespitosa* were as described by Lockyer et al. (2019). Briefly, worms were rinsed in 0.45µm filtered seawater (FSW) and placed in individual containers with 0.5mL of FSW to encourage sperm release. Sperm were used within 15 minutes of collection. For SAAS experiments *G. caespitosa* were collected from Grange Beach, South Australia, between September 2016 and January 2017. For CASA experiments worms were collected between February and April 2018.

3.2.2 Treatment Preparation and Analysis

Metal stock solutions were prepared using Analar grade metal salts of $CuSO_4$, $ZnCl_2$, $CdCl_2$, and $Pb(NO_3)_2$ (99 percent purity, Sigma-Aldrich©) and Milli-Q water (18.2 MΩ cm^{-1}; Millipore). Glassware was washed prior to use in 10% v/v nitric acid (69%, Merck). A test solution for each toxicant was prepared on the day of the experiment from refrigerated stock solutions and FSW, no more than one hour prior to test commencement. For each test, only the highest concentration was prepared using stock, which was subsequently used for either two-fold or three-fold serial dilutions in FSW. A total of seven treatment concentrations were used for each test.

At the end of each test, sub-samples were collected from each the highest and lowest treatment and the control, filtered through acid-washed (10% HNO_3)

0.45-µm filters, and acidified to 0.2% HNO_3 (69%, Merck) for dissolved metals analysis. Metal analyses were carried out using inductively coupled plasma optical emission spectrometry (ICP-OES; PerkinElmer 5300 V) by Future Industries Institute, UniSA at Mawson Lakes, South Australia. Test concentrations are presented in Appendix 2.

3.2.3 Sperm Exposures

Sperm were collected from five individuals, pooled and diluted to approximately $5x10^5$ sperm mL^{-1} in 15mL of treatment solutions. The exposure of sperm to the treatment solutions constituted the start of the test and the time was recorded. Sperm were exposed to each treatment for 30 minutes. Sperm concentrations were verified at the end of the test using a haemocytometer.

3.2.4 Computer Assisted Sperm Analysis (CASA)

Slides were arranged such that one slide was placed on the microscope stage, two slides placed on top, perpendicular to the bottom slide, and positioned at either end. After 30-minute exposure to the treatments, 100µL of the sperm/treatment solution was pipetted in the middle of the bottom slide to form a droplet. Another slide was added on top so that the droplet was now compressed between the slides. Slides were placed on a phase-contrast inverted microscope (Olympus CK40) equipped with a digital camera (Canon, Eos 5D Mark IV). Video recording started as soon the sperm suspension was

compressed between the slides. Videos of sperm swimming behaviour were recorded in a central location of the suspension for 5 seconds at a frame rate of 50 fps (frames per second). All videos were recorded at 200× magnification (1920 x 1080 pixel, HD). The microscope was set to focus half way between the top and bottom surfaces (middle of sperm suspension). Once set, the microscope settings were left for each treatment during an experiment. Microscope settings were readjusted prior to each experiment.

CASA Video Analysis

One second of footage was analysed per treatment, which was equivalent to 50 frames. The first 50 frames of each video were imported into FIJI (FIJI Is Just ImageJ)(Schindelin *et al.*, 2012) as an image stack. Once imported the images were converted from RGB to 8-BIT images and thresholded so that only the sperm heads were selected (this varied slightly between experiments; see Appendix 2). The CASA plugin(Wilson-Leedy and Ingermann, 2007) was then used to analyse the image stack and provide estimates of % motility, curvilinear velocity and straight-line velocity. For calibration of the CASA ImageJ plugin see Appendix 2.

3.2.5 Sperm Accumulated Against Surface

Recently, Sperm Accumulated Against Surface was proposed as a novel, simple and objective alternative approach for assessing sperm motility(Falkenberg, Havenhand and Styan, 2016). SAAS measures the accumulation or collision of

motile sperm onto a surface. Such measurement is possible as motile sperm will accumulate on the bottom surface of a well plate or slide, whereas non-motile sperm will stay in suspension since sperm sinking rates are very slow. Therefore, the number of sperm that accumulate is indicative of motility and of sperm:egg encounter rates. SAAS tests followed the methods outlined by Falkenberg et al.(Falkenberg, Havenhand and Styan, 2016). In pilot work prior to conducting ecotoxicological tests, the rate of accumulation of sperm against a surface in control seawater was measured to inform an accumulation period for toxicity tests. Sperm were exposed to filtered seawater for 30 minutes. Three millilitres of the sperm suspension were then pipetted into a well of a 12 well cell culture plate (Corning ®Costar® Not Treated). The well plate was placed on a phase-contrast inverted microscope (Olympus CK40) equipped with a digital camera (Canon Eos 5D Mark IV), and all images were taken at 400× magnification (6720 x 4480 pixel). The microscope was focussed on one central location and photographed every minute for a period of 20 minutes. The time at which sperm accumulation had plateaued (nine minutes) was then taken as the accumulation period (see Appendix 2).

SAAS Toxicity Test

After sperm had been exposed to the treatment solutions for a period of 30 mins, 3 mL of the test solution was pipetted into a well plate and then left to accumulate for nine minutes. After the sperm had been left to accumulate for the accumulation period, images were taken at three central locations of each

well plate. Sperm that had accumulated against the lower surface were then manually counted on all images.

3.2.6 Statistics

The R package DRC (Ritz and Strebig, 2005) was used to model the test data for each endpoint and method and to calculate toxicity estimates. Regression models tested included log-logistic, Weibull and Cedergreen-Ritz-Streibig models with different levels of parametrization. A joint model was used to fit a concentration response curve to each replicate. Model comparisons were conducted using the Akaike Information Criterion (AIC) and models that best described the data were applied to determine metal concentrations that elicited a 95% (EC95), 50% (EC50) and 10% (EC10) decrease in fertilisation success (% control). The associated 95% confidence limits were estimated using the delta method. Effect concentrations from each replicate were averaged and statistical differences in effect concentrations were determined using the method described by Sprague and Fogel (1976)(Sprague and Fogels, 1976). Where dose response models could not be fitted to the data, statistical analysis was conducted via a one-way ANOVA to determine if there was significant variation between the control and treatments. If the ANOVA returned a significant result ($P<0.05$), subsequent Tukey tests were conducted to determine which treatments were significantly different to the control. Full statistical analysis are presented in Appendix 2.

3.2.7 Modelling Impacts of sperm to fertilisation success

Using the fertilisation data from Lockyer et al. (2019), we used a theoretical fertilisation model(Styan, Kupriyanova and Havenhand, 2008) to calculate an average sperm density - fertilisation relationship using *G.caespitosa* gametes in filtered seawater (n = 30). Key model parameters, fertilisation efficiency (Fe) and polyspermy block efficiency (Be), were estimated using least squares (Styan and Butler, 2000). Average sperm swimming speed and egg diameter parameters for *G. caespitosa* were reported by Kupriyanova (2006). We then used this model to estimate how the sperm density - fertilisation relationship would shift with decreases (10%, 50%, 95%, 99%) in effective sperm density.

3.2.8 Terminology

Herein, sperm swimming refers to all parameters measured including; motility (%), straight line velocity (VSL), curvilinear velocity (VCL) and sperm accumulation (SAAS). Motility (%) refers to the number of sperm with VSL greater than 10µm s^{-1} divided by the total number of sperm. Straight line velocity is the straight-line distance between the start and the end points of a sperm's track divided by the time of the track. Curvilinear velocity refers to the actual velocity along the trajectory(Rurangwa *et al.*, 2004).

3.3 Results

3.3.1 The Effects of Metals on Sperm Swimming

Exposure of sperm to elevated concentrations of Zn, Pb and Cd did not exhibit a concentration response relationship in sperm swimming. The motility (%) of *G. caespitosa* sperm showed a positive response when exposed to elevated concentrations of Zn, Cd and Pb (Figures 3.3, 3.4, 3.5, 3.6). Statistical analysis (ANOVA) found significant variation ($P<0.05$) in sperm motility between treatments in all replicates for all metals (See Appendix 3 for full statistical analysis). Sperm motility in some metal treatments were significantly different to sperm motility in the FSW control (see figures 3.3-3.5). There were statistically significant increases in sperm motility when exposed to Zn and Cd in three different replicates. In some replicates, there was a greater than threefold increase in motility when exposed to concentrations greater than 100µg L^{-1} of Zn, Cd and Pb. When exposed to concentrations between 350-400µg L^{-1} Zn, motility increased from 29% to 75%, 7% to 60% and 9% to 73% when compared to controls.

There were no significant differences in sperm velocity (VSL and VCL) between control and treatments for Pb (figure 3.9, Appendix 2). For, Cd there were no significant differences in VCL between control and treatments for all replicates. There was no significant difference in VSL in two of three replicates; in the third replicate all treatments were significantly different to the control (figure 3.10, Appendix 2). However, exposure of sperm to elevated concentrations of Zn resulted in significantly decreased VSL in all replicates

and VCL in some replicates ($P<0.05$; Figure 3.11; Appendix 2). However, these effects occurred at concentrations that exceed the effects of Zn on fertilisation success (Chapter 2).

The effects of Cd and Pb on SAAS varied between replicates. In some replicates, statistically significant increases were observed and others statistically significant decreases (See figures 3.6,3.8). This was not the case for Zinc however, only significant decreases were observed in SAAS when exposed to elevated concentrations (figure 3.7).

When exposed to elevated concentrations of Cu sperm swimming displayed characteristic dose response relationships. We observed low dose stimulation in sperm motility when exposed to low concentrations of Cu and, as such, a hormesis model was fitted to the data (Figure 3.1). In all replicates there was an increase in sperm motility when exposed to ~5µg L^{-1} Cu. This was followed by a sharp decline in motility at approximately 8-10µg L^{-1} (Figure 3.1). Effect concentrations (10, 50%) for Cu were 11 and 13µg L^{-1} respectively (Table 3.1). Copper also exhibited a concentration-response relationship in VSL, VCL and SAAS (Figure 3.1) with evidence of a hormetic response in one the replicates for VSL and VCL. Effect concentrations (10, 50%) for VSL response to Cu were 4 and 6 µg L^{-1}, for VCL were 10 and 40µg L^{-1}, and for SAAS were 18 and 36 µg L^{-1} respectively (Table 3.1). There was a high degree of variability between replicates in VSL, VCL and SAAS and this is reflected in the confidence

intervals (95%) of effect concentration estimates (Table 3.1). For SAAS responses a Weibull model best represented the data based on AIC.

3.3.2 Effective sperm density and fertilisation success

A reduction in effective sperm density shifts the fertilisation curve to the right (Figure 3.3). The extent of this shift is dependent on the percentage of sperm affected. Figure 3.3 shows how the fertilisation curve changes if the effective sperm density decreased by 10%, 50%, 95% and 99%. Fmax represents the modelled maximum fertilisation and F50 represents half Fmax, thus a decrease from Fmax to F50 indicates a 50% effect on fertilisation success. The drop from Fmax to F50 at a sperm density of 10^6 sperm mL^{-1} requires a > 99% effect on effective sperm density. Therefore, a >99% effect on sperm would only induce a 50% effect on fertilisation success. At 10^5 sperm mL^{-1} a >95% effect on effective sperm density would be required to cause a 50% effect on fertilisation success. Smaller effects (10% and 50%) on sperm when tested at high concentrations (10^5 and 10^6) have no effect on fertilisation success. As sperm density decreases the difference between effects on sperm and effects to fertilisation success becomes smaller. At ~$5x10^3$ sperm mL^{-1} the proportion of eggs fertilised was approximately 0.5, a 50% effect on effective sperm density lowers this to 0.35, a 30% effect on fertilisation success. Only at very low sperm densities (<$1x10^3$ sperm mL^{-1}) are the effects to sperm linear to effects on fertilisation success.

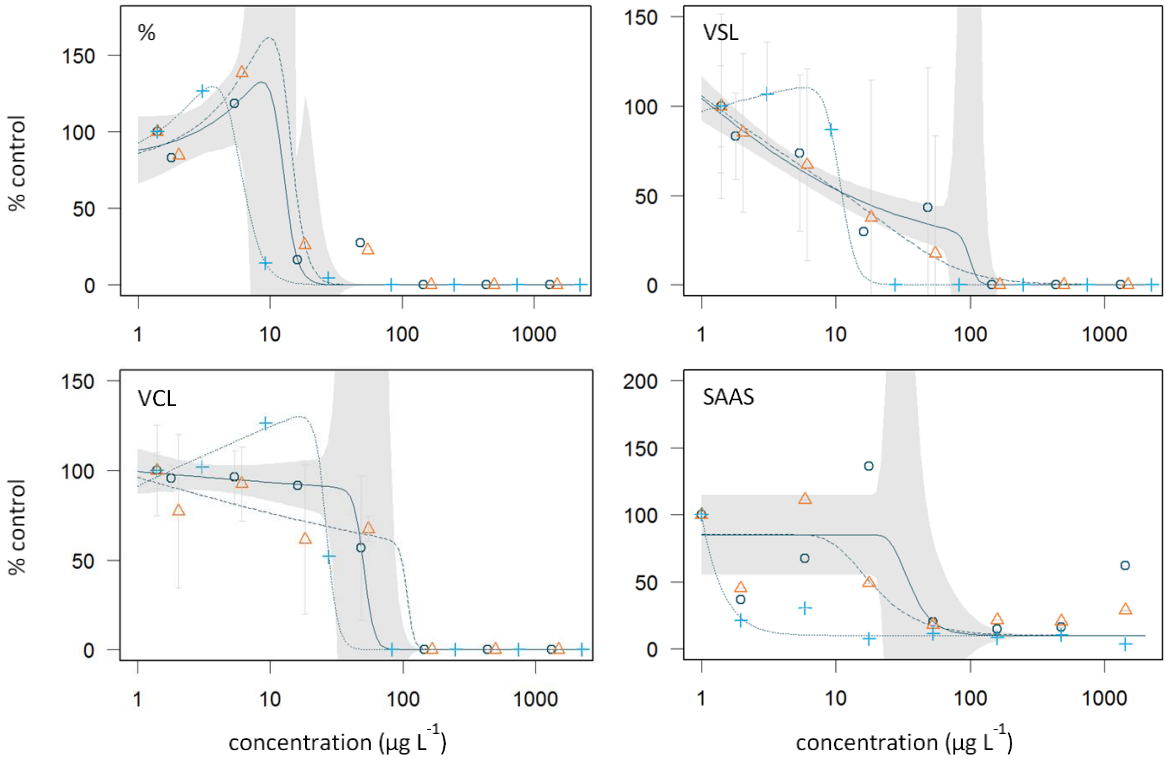


Figure 3.1: Concentration response relationships for sperm exposed to Cu using different endpoints: Motility (%), Straight Line Velocity (VSL), Curvilinear Velocity (VCL) and Sperm Accumulated Against Surface (SAAS). Replicate 1 (Dotted, +); Replicate 2 (solid, o); Replicate 3 (Dashed, Δ). Metal concentrations as Measured Dissolved (0.45µm).

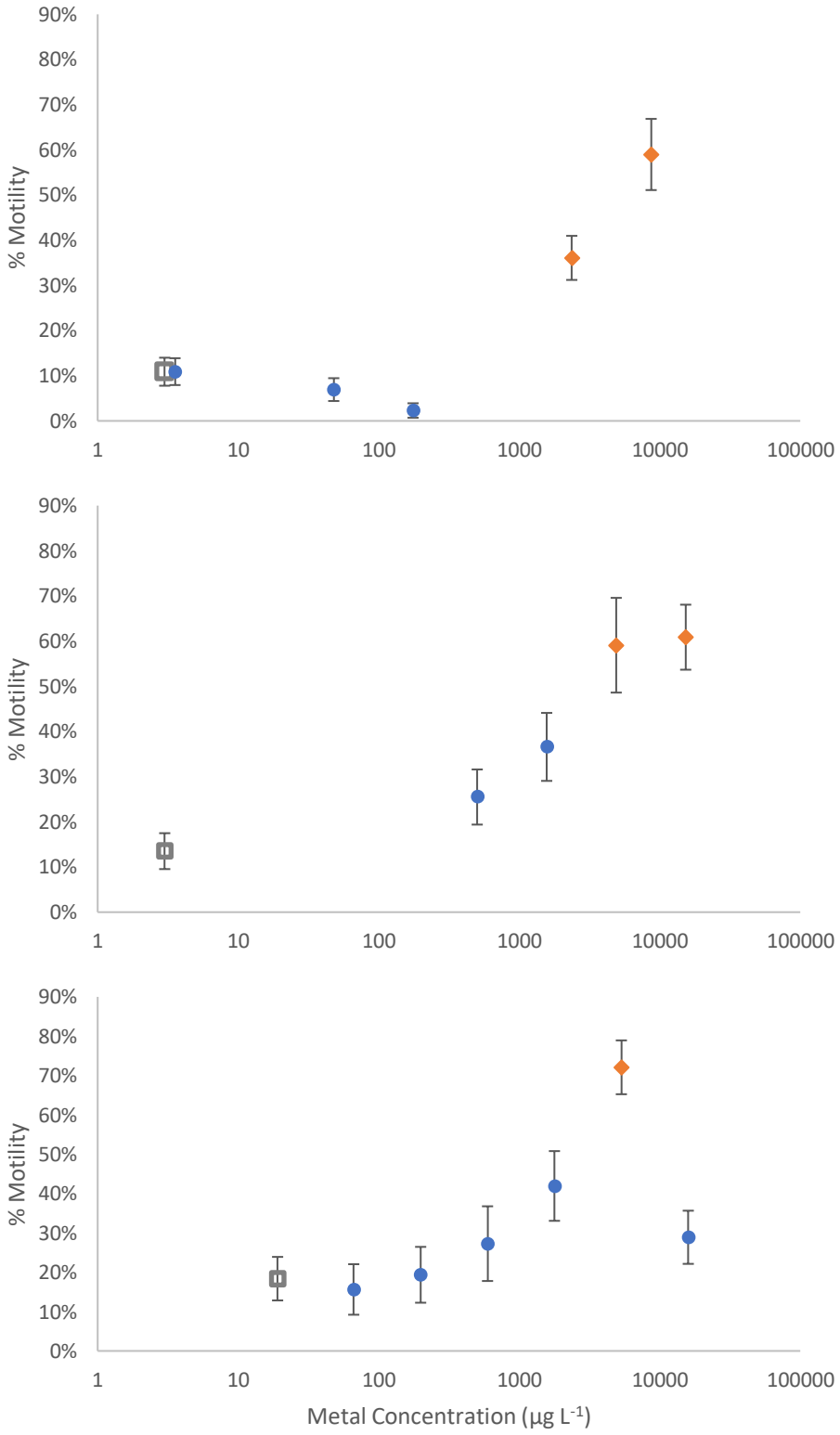


Figure 3.2: Effect of Cd on percent motility of *Galeolaria caespitosa* sperm. Metal concentrations as measured dissolved (<0.45µm). Each replicate (Top, Middle, Bottom) consisted of a FSW control (grey, square) and metal treatments with increasing concentration. Treatments that were not significantly different to the control are represented by blue circles. Treatments that were significantly different to the control are represented by orange diamonds. Standard error associated with each treatment is represented by the error bars.

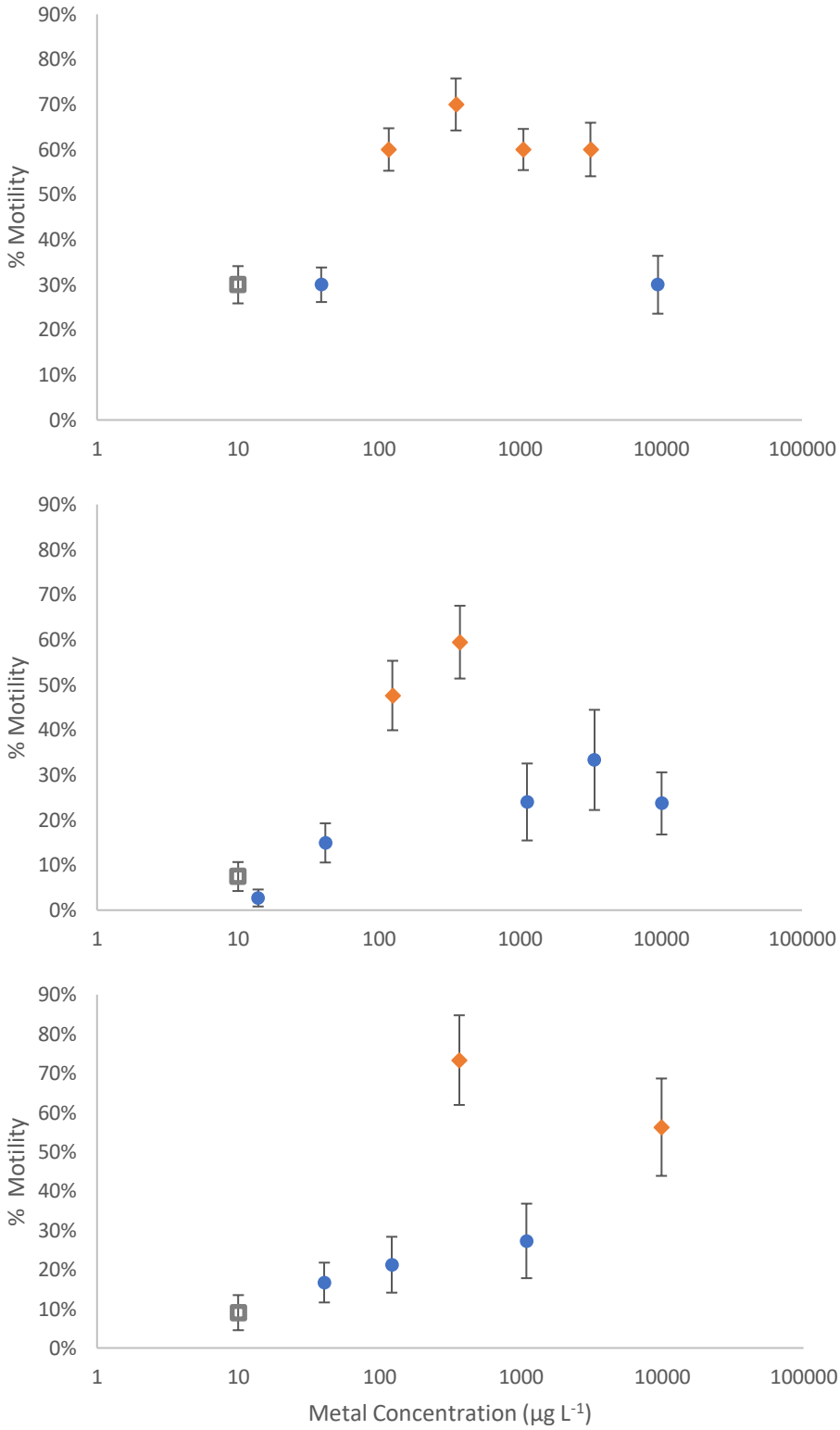


Figure 3.3: Effect of Zn on percent motility of Galeolaria caespitosa sperm. Metal concentrations as measured dissolved (<0.45µm). Each replicate (Top, Middle, Bottom) consisted of a FSW control (grey, square) and metal treatments with increasing concentration. Treatments that were not significantly different to the control are represented by blue circles. Treatments that were significantly different to the control are represented by orange diamonds. Standard error associated with each treatment is represented by the error bars.

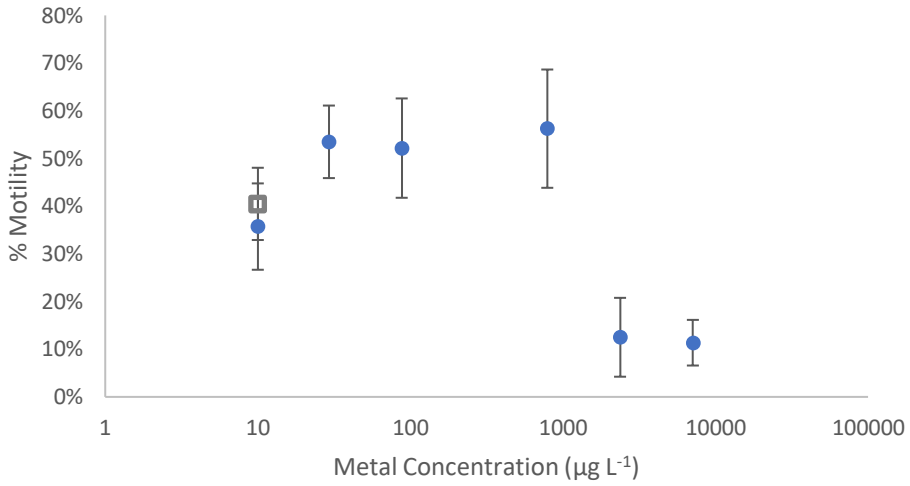


Figure 3.4: Effect of Zn on percent motility of *Galeolaria caespitosa* sperm, replicate 4. Metal concentrations as measured dissolved (<0.45µm). Each replicate consisted of a FSW control (grey, square) and metal treatments with increasing concentration. Treatments that were not significantly different to the control are represented by blue circles. Treatments that were significantly different to the control are represented by orange diamonds. Standard error associated with each treatment is represented by the error bars.

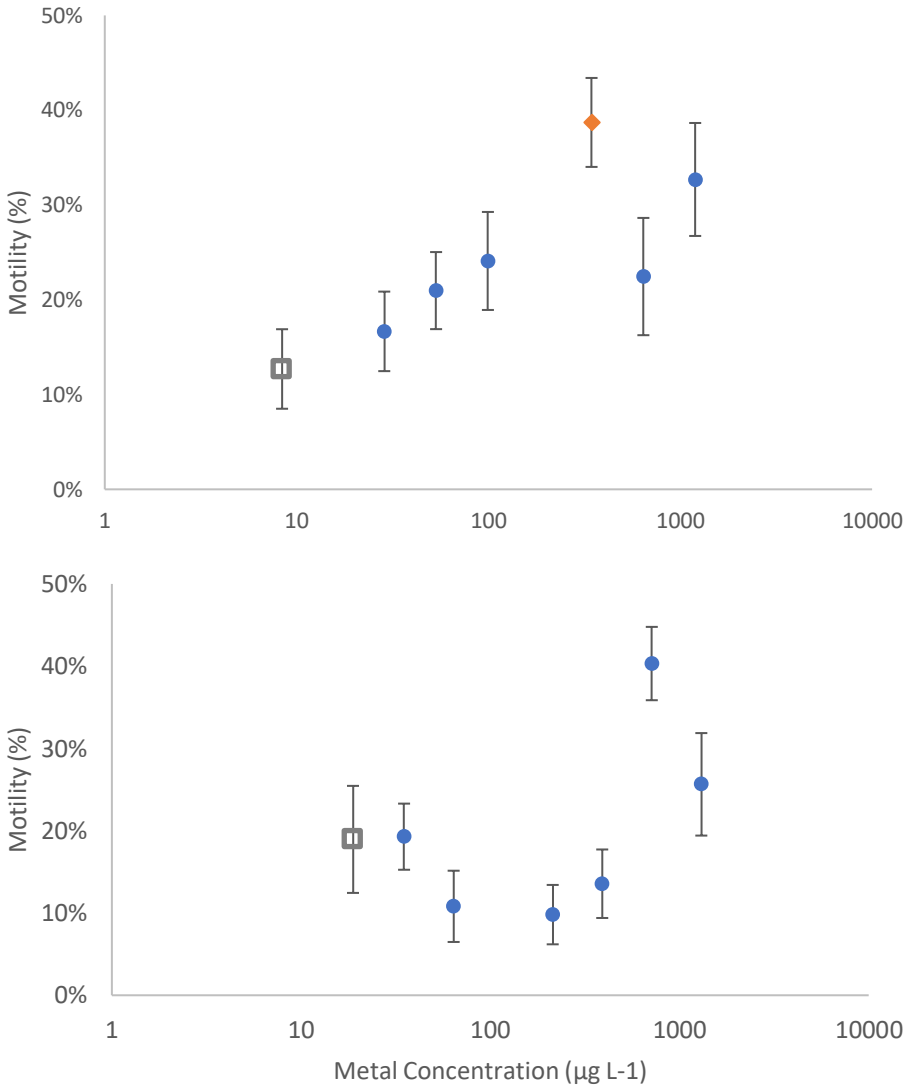


Figure 3.5: Effect of Pb on percent motility of *Galeolaria caespitosa* sperm. Metal concentrations as measured dissolved (<0.45µm). Each replicate (top, bottom) consisted of a FSW control (grey, square) and metal treatments with increasing concentration. Treatments that were not significantly different to the control are represented by blue circles. Treatments that were significantly different to the control are represented by orange diamonds. Standard error associated with each treatment is represented by the error bars.

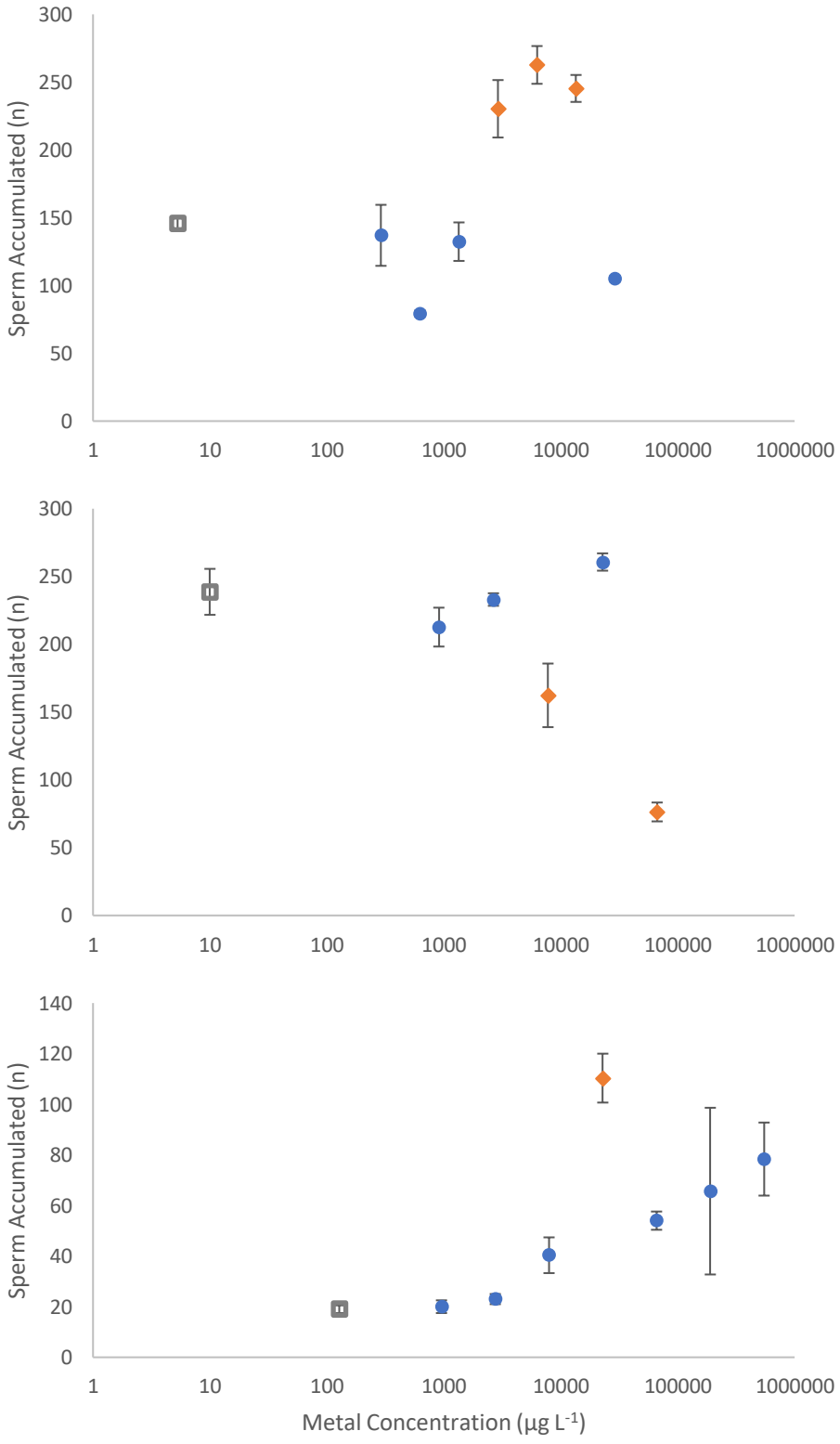


Figure 3.6: Effect of Cd on the accumulation of *Galeolaria caespitosa* sperm against a surface. Metal concentrations as measured dissolved (<0.45µm). Each replicate (Top, Middle, Bottom) consisted of a FSW control (grey, square) and metal treatments with increasing concentration. Treatments that were not significantly different to the control are represented by blue circles. Treatments that were significantly different to the control are represented by orange diamonds. Standard error associated with each treatment is represented by the error bars.

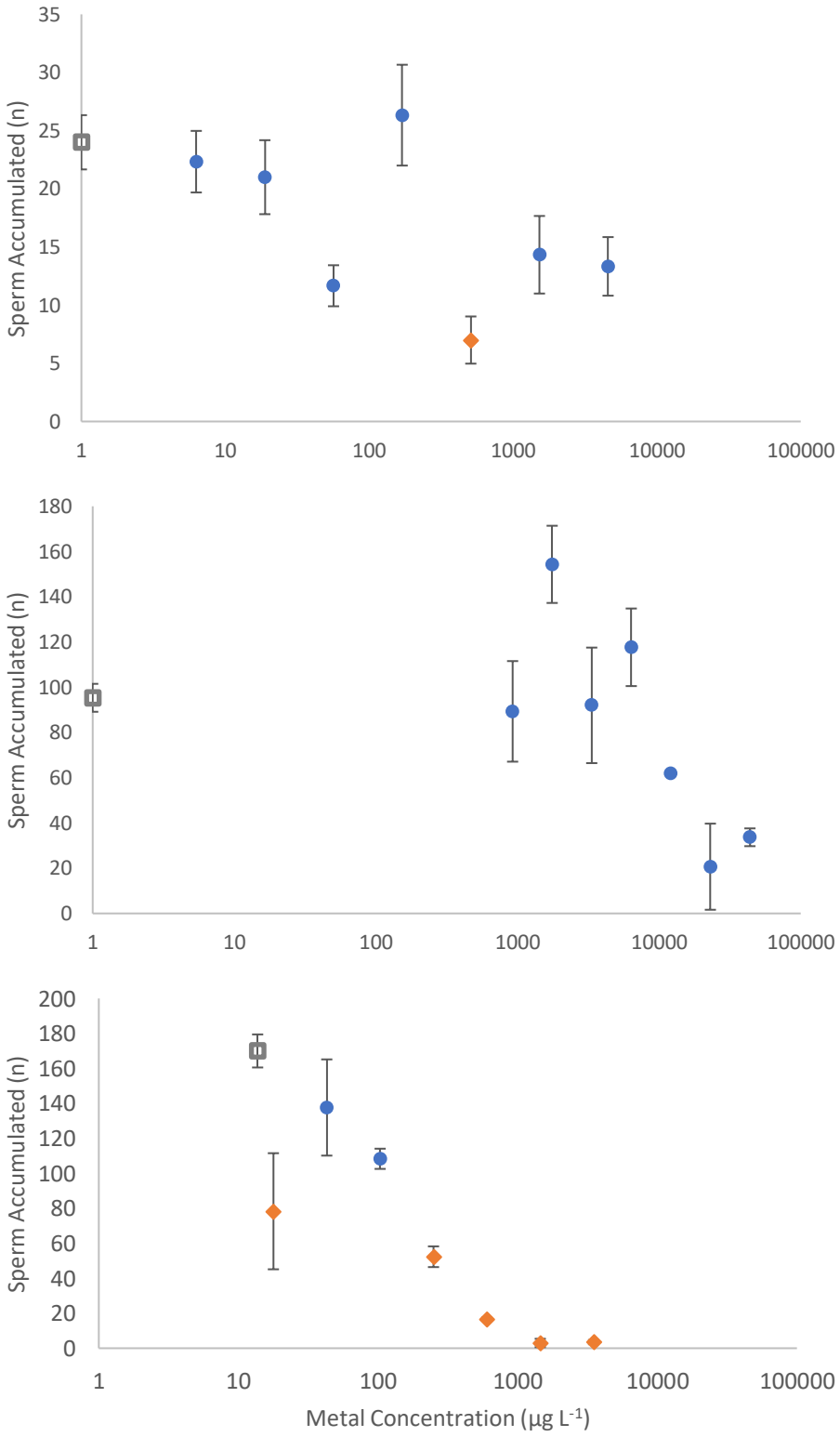


Figure 3.7: Effect of Zn on the accumulation of *Galeolaria caespitosa* sperm against a surface. Metal concentrations as measured dissolved (<0.45µm). Each replicate (Top, Middle, Bottom) consisted of a FSW control (grey, square) and metal treatments with increasing concentration. Treatments that were not significantly different to the control are represented by blue circles. Treatments that were significantly different to the control are represented by orange diamonds. Standard error associated with each treatment is represented by the error bars.

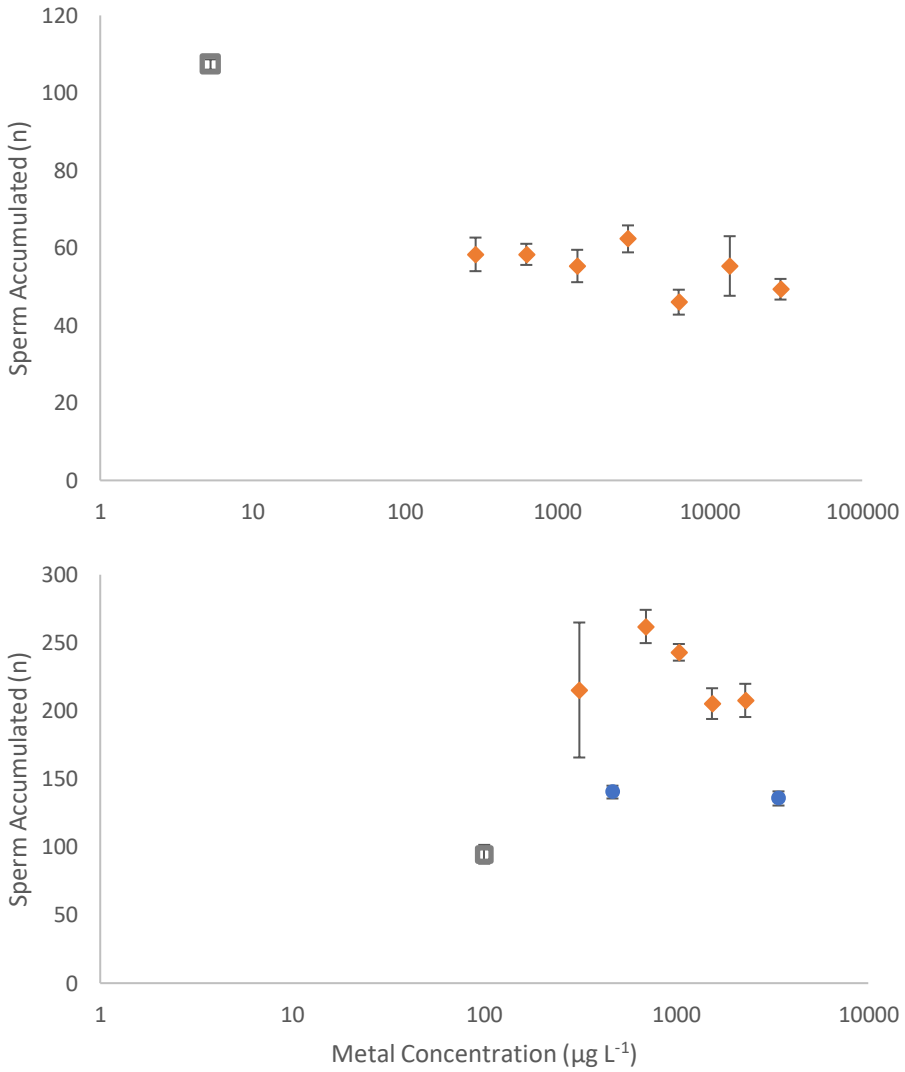


Figure 3.8: Effect of Pb on the accumulation of *Galeolaria caespitosa* sperm against a surface. Metal concentrations as measured dissolved (<0.45µm). Each replicate (Top, Middle, Bottom) consisted of a FSW control (grey, square) and metal treatments with increasing concentration. Treatments that were not significantly different to the control are represented by blue circles. Treatments that were significantly different to the control are represented by orange diamonds. Standard error associated with each treatment is represented by the error bars.

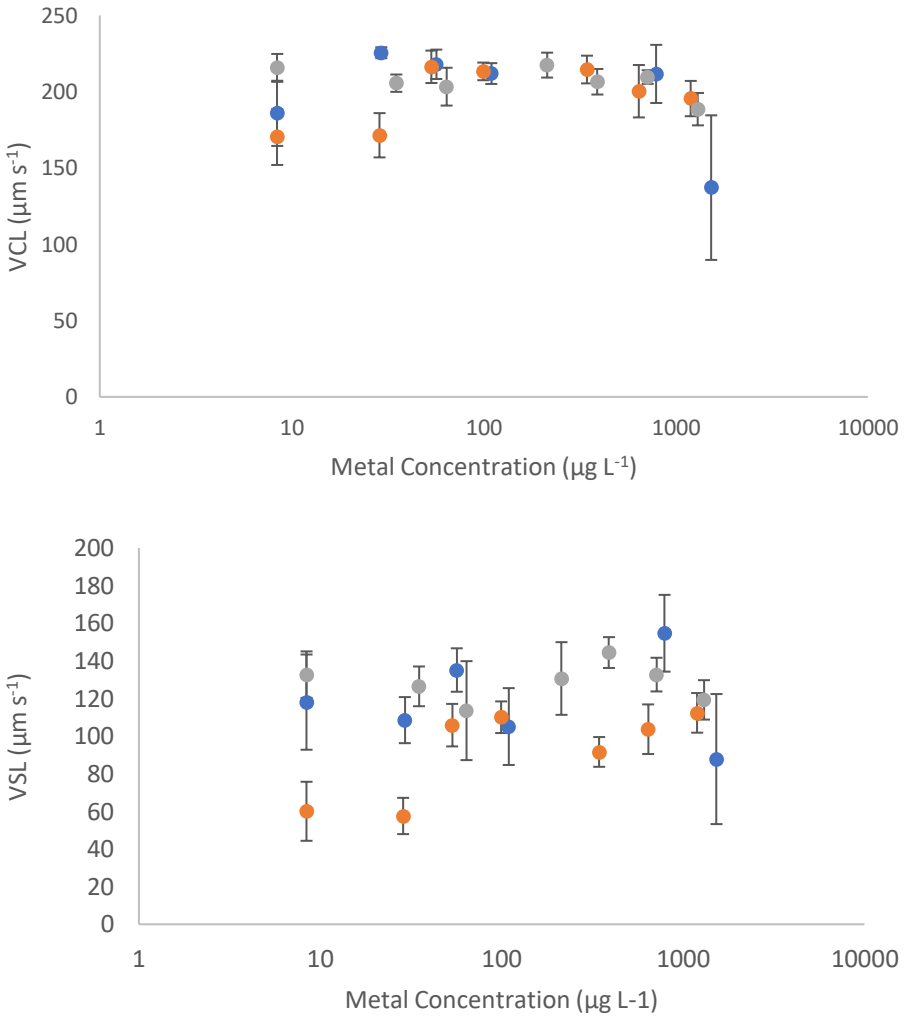


Figure 3.9: Effect of Pb on the velocities of *Galeolaria caespitosa* sperm (VCL; Top, VSL; Bottom). Metal concentrations as measured dissolved (<0.45µm). Each replicate consisted of a FSW control and metal treatments with increasing concentration. No metal treatments were significantly different to the FSW controls. Standard error associated with each treatment is represented by the error bars.

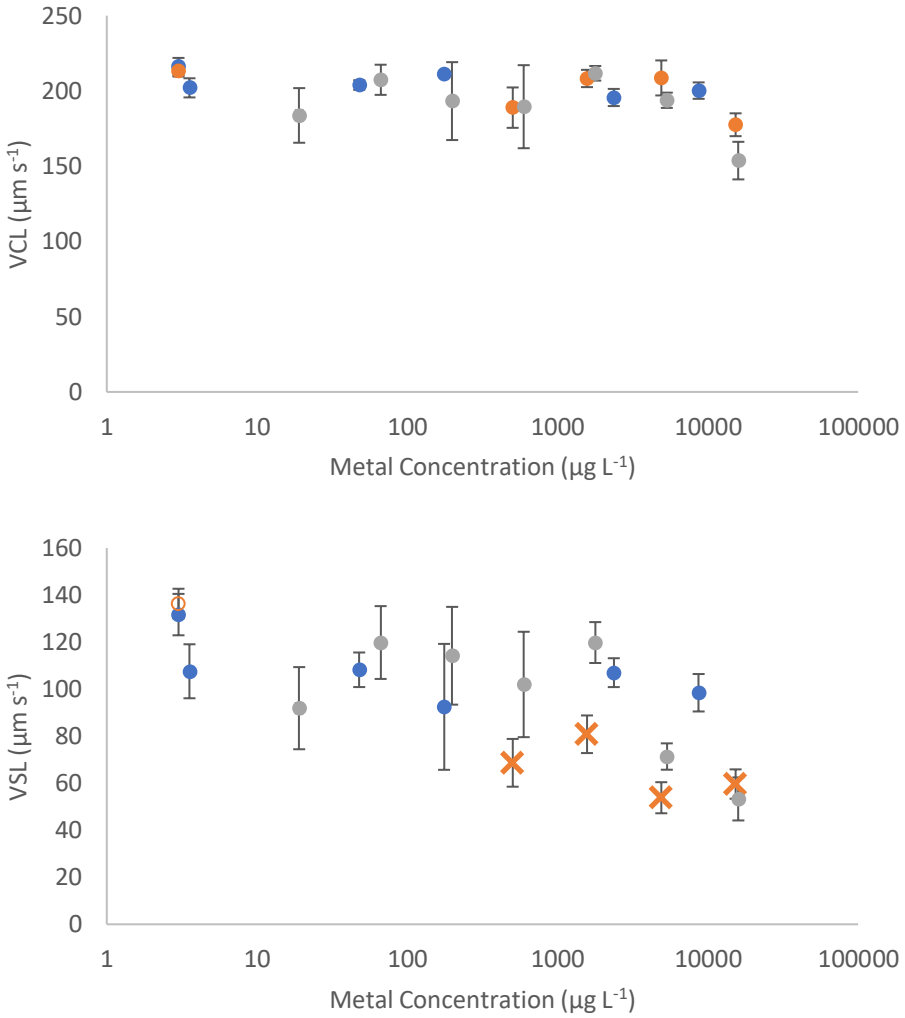


Figure 3.10: Effect of Cd on the velocities of *Galeolaria caespitosa* sperm (VCL; Top, VSL; Bottom). Metal concentrations as measured dissolved (<0.45µm). Each replicate (1; blue, 2; orange, 3; grey) consisted of a FSW control and metal treatments with increasing concentration. Metal treatments that had significantly different sperm velocities than FSW controls are represented by an (X). Standard error associated with each treatment is represented by the error bars.

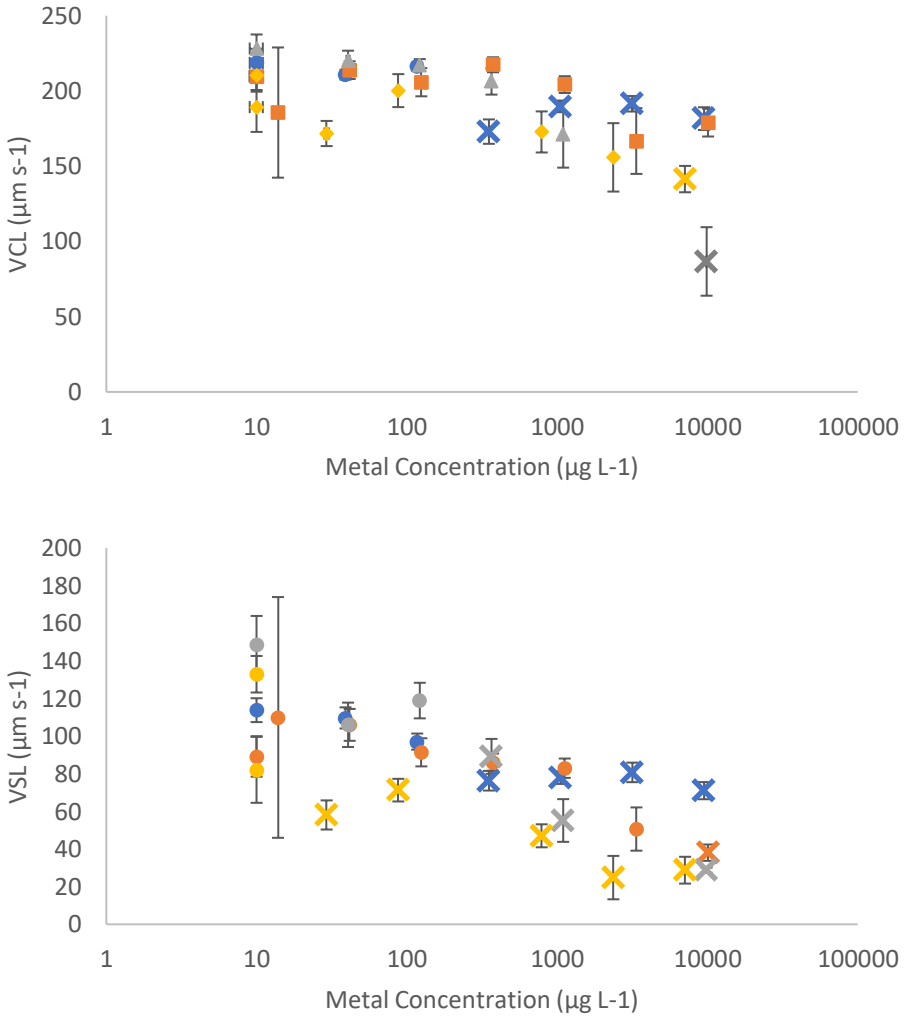


Figure 3.1l: Effect of Zn on the velocities of *Galeolaria caespitosa* sperm (VCL; Top, VSL; Bottom). Metal concentrations as measured dissolved (<0.45µm). Each replicate (1; blue, 2; orange, 3; grey, 4; yellow) consisted of a FSW control and metal treatments with increasing concentration. Metal treatments that had significantly different sperm velocities than FSW controls are represented by an (X). Standard error associated with each treatment is represented by the error bars.

Table 3.1:Average effect concentrations for *G. caespitosa* sperm exposed to Cu, Zn, Pb and Cd based on percent motility (%MOT), straight line velocity (VSL), curvilinear velocity (VCL), sperm accumulation (SAAS) and fertilisation success (FERT).[1]Data taken from Lockyer et al. (2019) at a sperm density of 10^5 sperm mL^{-1}.

	Effect	%MOT	VSL	VCL	SAAS	FERT[1]
Cu	10	11 (6-17)	4 (0-10)	10 (0-22)	12 (0-26)	9.6 (7.4-11)
	50	13 (7-19)	6 (0-12)	40 (0-85)	18 (0-39)	20 (18-22)
	95	19 (12-26)	71 (17-126)	76 (0-161)	50 (0-106)	-
Zn	10	N/A				180 (94-270)
Pb	10	N/A				980 (770-1,200)
Cd	10	N/A				3900 (0-10,000)

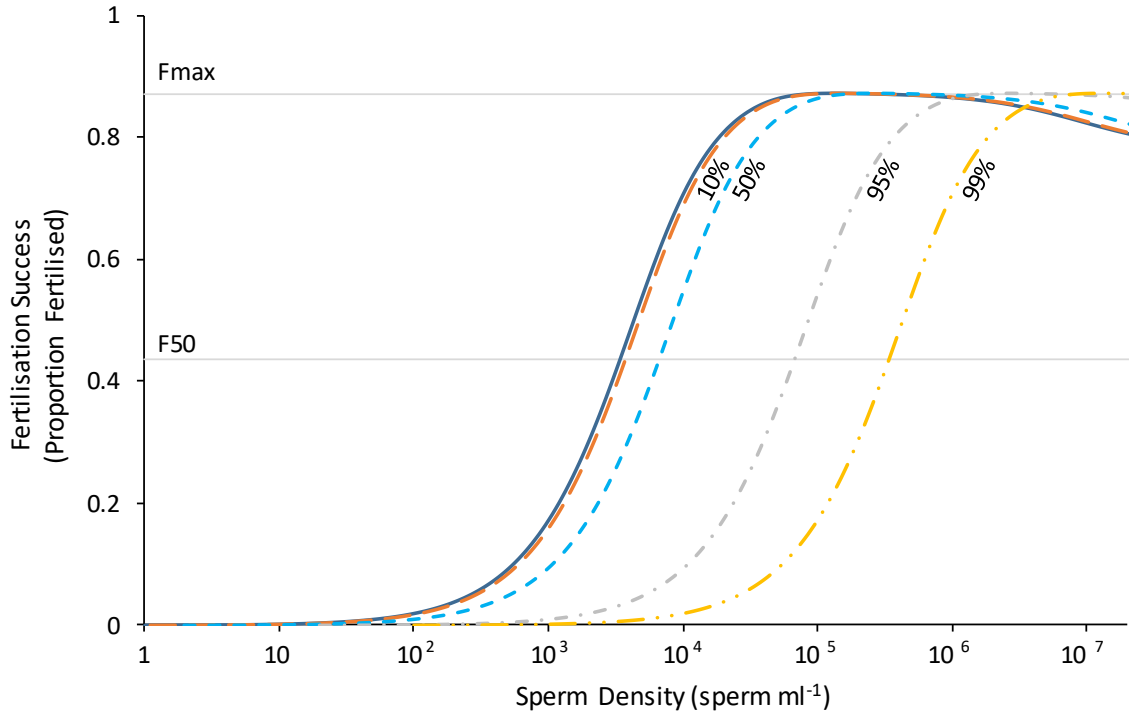


Figure 3.12: Average fertilisation model for *G. caespitosa* showing how the fertilisation relationship changes with decreases in effective sperm density. 10% reduction (orange, long dash), 50% reduction (blue, dash), 95% reduction (grey, dash dot), 99% reduction (yellow, long dash dot dot). Fmax: maximum fertilisation success, F50: 50% of the maximum fertilisation success.

3.3.3 *G. caespitosa* Sperm Analysis

Of the 104 videos recorded, 14 were discarded due to noise and horizontal drift in the footage. All SAAS data were used. Sperm motility and velocity (VSL, VCL) were variable among controls. Across the 12 controls, the average proportion of motile sperm ranged from 7% to 29% (16.34 ± 7.19). The average straight-line velocity (VSL) ranged from 60 to 149μm s^{-1} (114 ± 25). The average curvilinear velocity ranged from 170 to 228μm s^{-1} (205 ± 18).

3.3.4 Seawater Metals Analysis

The measured dissolved metal concentrations in the controls and the highest and lowest treatments are presented in the Appendix 2. The range of concentrations are presented in the dose response data (Figures 3.1, 3.2). All FSW controls were below the limits of detection for each metal. Limits of detection for Cu, Zn, Pb and Cd, were 10, 50, 100 and 50 μg L^{-1} respectively. The limits of detection here were not low enough to detect ANZECC guideline values, however they are acceptable for the effects observed in this study.

3.4 Discussion

3.4.1 Effects of Metals on Sperm Swimming

Sperm motility is a process that responds to signals from the environment (Tosti and Ménézo, 2016)and is dependent on the transfer of ions between the cell and the external environment for both activation and maintenance of motility(Rothschild, 1948; Shirai *et al.*, 1982; Aitken, 2000; Tosti and Ménézo, 2016). This study found that an increase in the dissolved metal concentration in seawater can enhance (Zn, Cd) or disrupt (Cu) sperm motility. For Zn, Cd and Pb fertilisation was more sensitive than motility. Zinc and Cd, increased sperm motility at concentrations that cause a negative effect on fertilisation. EC50s for fertilisation success for Zn, Pb and Cd were 180, 980 and 3,900 µg L^{-1} respectively (Lockyer et al., 2019). Sperm motility at similar concentrations to the EC50's for fertilisation was greater than observed in respective controls.

Zinc had the greatest stimulatory effects on sperm motility (Figure 3.3). Elevated concentrations of dissolved Zn (>100 µg L^{-1}) in seawater increased *G. caespitosa* sperm motility (%)suggesting that Zn may play a role in motility initiation. Stimulatory effects of Zn on sperm motility have also been observed in the purple sea urchin at concentrations of approximately 650 µg L^{-1} (Young and Nelson, 1974). We did observe a reduction in motility when *G. caespitosa* sperm were exposed to extremely high, and not environmentally relevant, concentrations of Zn (10,000 µg L^{-1}, SAAS). Similarly, exposure to high concentrations of Zn have been shown to reduce respiration in *Mytulis edulis* sperm (65,400 µg L^{-1}; 50% inhibition of respiration)(Akberali, Earnshaw and

Marriott, 1985) and result in ultrastructural mitochondrial damage (100,000 μg L^{-1})(Earnshaw *et al.*, 1986). It is worth noting that the concentrations tested in these experiments are very high, and unlikely to be found in contaminated marine environments. However, we did observe decreases in the straight line and curvilinear velocity of sperm when exposed to elevated concentrations of Zn for 30 minutes. These effects occurred at concentrations that were greater than the effects of Zn on fertilisation success. If Zn is involved in the initiation of sperm motility then this may explain the lower velocities after 30 minutes of exposure. Greater concentrations of zinc could potentially increase intracellular pH and initiate motility much more rapidly than at lower concentrations. If sperm are stimulated at an earlier stage than in a respective control, and begin to utilities ATP stores, they may start to age or slow quicker. Future research should look into how Zn effects sperm motility and velocity on a smaller time scale.

Our results support the literature suggesting that Zn could be involved in the regulation of intracellular pH and subsequent motility initiation (David L Clapper *et al.*, 1985) . In broadcast spawning marine invertebrates sperm motility is initiated when sperm are diluted into seawater where a rise in intracellular pH initiates ATP hydrolysis fuelling axenomal dyneins. The sliding of doublet microtubles by axenomal dyneins, through mechanochemical cycles of ATP, provides the driving force for flagella motility of sperm (Nakajima, 2005; Inaba, 2011). When sea urchin sperm (*Lytechninus pictus)* are diluted in seawater they rapidly concentrate Zn(David L. Clapper *et*

al., 1985). Zinc has been found to occur throughout the sperm cell but is mostly concentrated in the mid piece and flagella(Morisawa and Mohri, 1972). Both the flagella and isolated microtubules have been shown to contain Zn in relatively high concentrations suggesting the participation of Zn in cell movement(Morisawa and Mohri, 1972). The rapid accumulation of Zn in the sperm cell likely plays a role in the regulation of intracellular pH(David L. Clapper *et al.*, 1985).

In this study, exposure to Cd concentrations of greater than 100µg L^{-1} increased % motility (Figure 3.2). However, no significant effect was observed on VCL even when exposed to concentrations greater than 10,000µg L^{-1}. There was not conclusive evidence to determine the effects of Cd on VSL and this should be investigated further. However, these results suggest Cd could also play a role in motility initiation in this species. Similarly, positive effects of Cd on velocity have been observed in sea urchin (*Anthocidaris crassispina*) sperm(Au, Chiang and Wu, 2000) after very short exposures (0 minutes; 1,000µg L^{-1}). However, no effects were observed in mussel (*Perna viridis*) sperm after exposures of 50,000µg L^{-1} Cd for up to 60 minutes (Au, Chiang and Wu, 2000). Further, negative impacts to sperm motility from exposure to Cd have been recorded in zebrafish (*Danio rerio*)(Acosta *et al.*, 2016), sturgeon (*Acipenser ruthenus*)(Li *et al.*, 2010), sea urchin (*A. crassispina*)(Au, Chiang and Wu, 2000) and bovine sperm(Kanous, Casey and Lindemann, 1993). Such negative impacts have been attributed to swelling of the mitochondria(Au,

Chiang and Wu, 2000) and the inhibition of microtubule sliding of the axoneme(Kanous, Casey and Lindemann, 1993).

Exposure to Pb showed varied effects on % motility; both positive and negative effects were observed at elevated concentrations (>500µg L^{-1}) suggesting the effects of Pb may be dependent on the individuals from which sperm were obtained. There was no significant effect of Pb on sperm velocity (VSL or VCL). There is very little published data on sperm motility in response to direct exposures to Pb in marine organisms. In the context of humans, however, Huang et al. (2001) exposed sperm to 5,000 µg L-1 of Pb for 2, 4, 6 and 8 hours with no significant effect on motility observed.

Both Cd and Pb have the capacity to interfere with calcium-mediated processes(Wirth and Mijal, 2010) and are considered calcium mimics that can affect a variety of systems(Bridges and Zalups, 2005; Acosta *et al.*, 2016). Cadmium has the potential to disrupt ion homeostasis, particularly Ca, but also sodium (Na) and magnesium (Mg)(McGeer, Niyogi and Scott Smith, 2011). Cadmium can also inhibit the uptake of Ca^{2+} by blocking Ca^{2+} channels(McGeer, Niyogi and Scott Smith, 2011). Pb^{2+} can also be carried into cells via Ca^{2+} channels(Simons, 1988). Ca^{2+} plays a pivotal role in fertilisation, participating in the main functions invertebrate spermatozoa such as maturation, motility, and the acrosome reaction (Darszon *et al.*, 2018). Thus, a disruption to ion homeostasis, or to the transfer of Ca^{2+} into the cell, may prevent sperm activation or the regulation of sperm motility.

Considering that Zn, Cd and Pb impact fertilisation in *G. caespitosa* through impacts to sperm (Lockyer et al., 2019); these metals must have an effect other sperm functions during the fertilisation process, such as the ability to undergo the acrosome reaction. In the case of these metals, motility endpoints cannot account for the effects to fertilisation success and are not appropriate as a sensitive or ecologically relevant measure of metal toxicity.

In contrast to the responses of sperm to Zn, Cd, and Pb, exposure of sperm to environmentally relevant concentrations of Cu caused a negative effect on both sperm motility and velocity. We observed low concentration stimulation followed by a sharp decline in both sperm motility (%) and sperm velocity (VCL and VSL). We observed a 50% decrease in sperm motility when sperm were exposed to 13 µg L^{-1} and a 95% decrease when exposed to 19µg L^{-1}. Reductions of swimming speed have also been found with increased Cu in the blue mussel (*Mytulis trossulus*; 100 µg L-1)(Fitzpatrick *et al.*, 2008), purple sea urchin (*Arbacia punctulata*; 34 % reduction in speed 2.5µEq L^{-1})(Young and Nelson, 1974) and lugworm (*Arenicola marina*)(Campbell *et al.*, 2014).

The mechanism underlying the change in motility under enriched Cu may be associated with mitochondrial activity. Copper has been found to occur in the midpiece (mitochondria) and nucleus of sperm (Morisawa and Mohri, 1972; Earnshaw *et al.*, 1986). Copper is required by mitochondria for the activity of a number of enzymes (Cobine, Pierrel and Winge, 2006) which may explain the

low dose stimulation observed here. An overload of Cu could result in the decreased activity of such enzymes which may result in impaired mitochondrial function(Leary, Winge and Cobine, 2009). Once taken up into the mitochondria, Cu can inhibit ATP production by interfering with electron transport(Ay *et al.*, 1999). Accumulation of Cu in the mitochondria may also decrease mitochondrial membrane potential and cause oxidative damage through the formation of reactive oxygen species(Krumschnabel *et al.*, 2005). Moreover, alternative mechanisms have limited support. For example, exposure to Cu did not alter sperm morphology in the blue mussel, suggesting that the effects of Cu on swimming speeds were not caused by structural damage(Fitzpatrick *et al.*, 2008). Therefore, exposure to Cu likely interferes with mitochondrial activity, reducing sperm velocity and motility.

3.4.2 Relating Effects on Sperm to Fertilisation Success

A toxicant that reduces sperm swimming ability, effectively reduces the density of sperm around an egg, and thus increases sperm limitation (Lewis and Caldwell, 2010). If we assume that non-motile sperm are unable to locate and penetrate an egg, then reductions in sperm motility (%) would correspondingly reduce the total number of effective sperm. If we observed a 50% reduction in sperm motility relative to a control, you would need to double the sperm density in the treatment to have the same number of effective sperm. Thus increasing the total sperm density that achieves maximum fertilisation success, and shifting the fertilisation curve to the right. Figure 3.3 shows how the fertilisation model for G.caespitosa would shift with

10, 50, 95 and 99% reductions in the number of effective sperm. Using this model, it is clear that effects on sperm are nonlinear to effects on fertilisation success and the extent of the effect on fertilisation is dependent upon sperm density. At 10^5 sperm mL^{-1}, a 95% reduction in sperm motility would be required to achieve a 50% effect on fertilisation success. However, at 10^6 sperm mL^{-1}, a 95% reduction in sperm motility would have negligible effects on fertilisation success as there are still enough effective sperm to achieve maximum fertilisation success. As a result of this relationship, sperm endpoints such as motility (%) were more sensitive indicators of Cu toxicity than fertilisation success (figure 3.4). The EC50 calculated for sperm motility (%) (13 µg L^{-1}) was significantly lower than that of fertilisation success at a sperm concentration of 10^5 sperm mL^{-1} (20 µg L-1) (Table 3.1). However, the concentration that elicited a 95% impact to sperm motility (19µg L^{-1}) corresponds with the EC50 for fertilisation success of 20µg L^{-1} (Lockyer, Binet and Styan, 2019). There was no significant difference between the two values ($p < 0.05$). Thus, impacts to sperm motility can account for the effects observed to *G. caespitosa* fertilisation for Cu and an adverse outcome pathway can be demonstrated. For this species, measures of motility could be used as a rapid and sensitive indicator of Cu toxicity, as an alternative to conducting full fertilisation tests.

Fertilisation success is used as an ecologically relevant endpoint as it directly effects recruitment and population health (Hudspith, Reichelt-Brushett and Harrison, 2017). Whilst sperm endpoints may be more sensitive than

fertilisation success, as is the case for Cu here, the relevance of such endpoints could be questioned. If natural spawning densities and fertilisation success rates are high, then effects on sperm would have negligible effects on fertilisation success. In such cases, toxicity estimates (EC10, 50) based on sperm endpoints would overestimate the potency of a toxicant and could be considered too conservative. However, in sperm limited environments these metrics could provide sensitive and representative measures of metal toxicity. Ideally, sperm endpoints would be used in conjunction with a fertilisation model of the test species to determine how effects on sperm would impact fertilisation success across a range of sperm densities. The most accurate toxicity data would be informed by the density of gametes during natural spawning events, which for most marine invertebrates are either unknown or highly variable. Thus, when deriving toxicity data from sperm endpoints, both an understanding of how effects on sperm manifest as effects on fertilisation success, and the sperm density at which to base toxicity estimates, need to be considered.

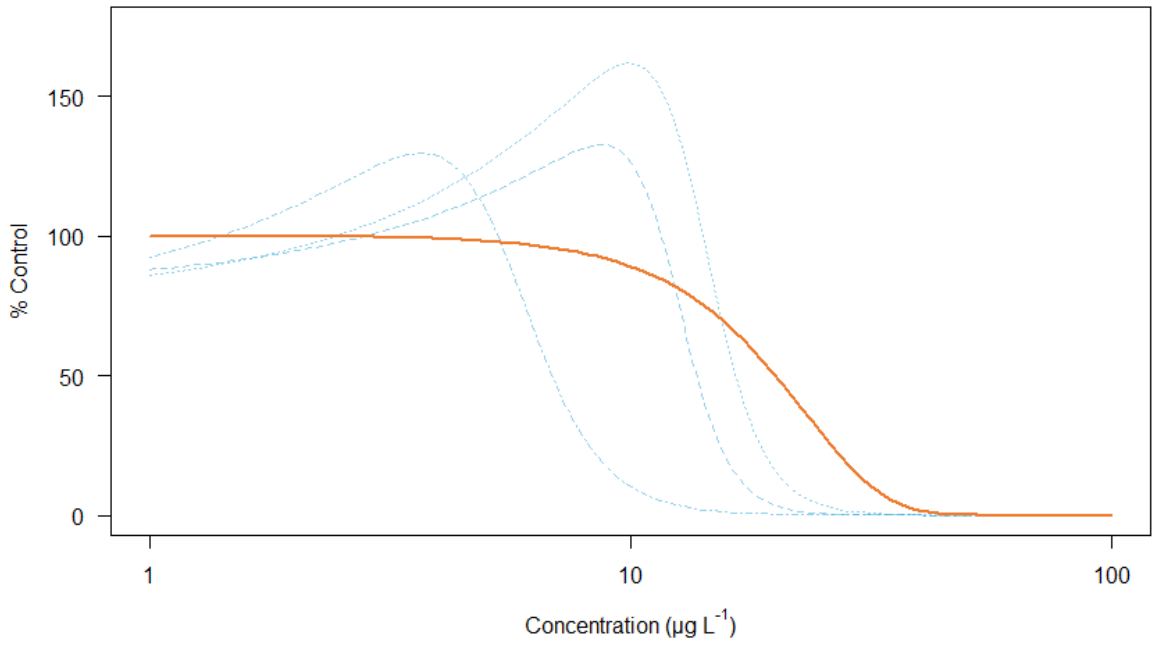


Figure 3.13: Concentration response relationships for the effects of Copper on *G. caespitosa* sperm motility (%; Blue; line types represent replicates) and on fertilisation success at a sperm density of 10^5 sperm mL^{-1} (orange).

3.5 Conclusion

Sperm motility can account for the effects of Cu to fertilisation success. However, exposure of *G. caespitosa* sperm to Zn, Cd and Pb increased motility at concentrations that have negative effects on fertilisation success. As such, impacts to fertilisation from these metals cannot be explained by effects to sperm motility, indicating that these metals must impact other sperm functions (e.g. acrosome reaction). Therefore, sperm swimming endpoints are not consistently an ecologically relevant or sensitive alternative to measuring fertilisation success for the assessment of metal toxicity.

CHAPTER 4

Effects of Metals on the Acrosome Reaction and Mitochondrial Membrane Potential

4.1. Introduction

Sperm of the serpulid tube worm *G. caespitosa* showed a positive response in sperm motility when exposed to dissolved metals (Zn, Pb and Cd) at concentrations that elicit a negative effect on fertilisation. Thus, sperm motility (%) cannot account for the effects of these metals on fertilisation as increased sperm motility should, theoretically, increase the number of sperm:egg encounters and the likelihood of fertilisation success (Styan, 1998; Styan and Butler, 2000). Thus, the effects of Zn, Cd and Pb that decrease fertilisation, must instead be on either the ability of sperm to undergo the acrosome reaction (AR), sperm induced egg activation, or chemotaxis. However, Cu inhibited sperm motility, likely through effects on the mitochondria, at concentrations that correspond with effects on fertilisation success (Fitzpatrick *et al.*, 2008). As such, it is hypothesized that metals interact with cellular functions that exert negative effects on fertilisation success. One approach for the assessment of cellular processes, which is widely used in medical applications, is flow cytometry (FCM).

Flow cytometry is a rapid method for the quantitative measurement of light scattering and fluorescent properties of cells and is increasingly being used to assess the viability and function of marine invertebrate sperm (Favret and Lynn, 2010; Binet *et al.*, 2014; Le Goïc *et al.*, 2014; Kekäläinen *et al.*, 2015). A number of sperm characteristics related to fertilizing capacity (viability, acrosomal integrity, mitochondrial function, DNA/chromatin integrity and reactive oxygen species (ROS) production) can be accurately measured using

FCM (Le Goïc *et al.*, 2013). Sperm cells can be stained with a range of fluorescent dyes (probes) that investigate individual cellular processes. Flow cytometers then analyse thousands of cells per seconds for light scattering and fluorescence properties (Binet *et al.*, 2014). This provides information regarding cell size, shape, structure and fluorescence which can be used to quantify differences in sperm in response to a toxicant; thus allowing a rapid, accurate and sensitive determination of toxicant effect concentrations. Developments in technology and the availability of probes have made flow cytometry affordable, reliable and user friendly, allowing the adoption of such methods in routine toxicity tests (Peña *et al.*, 2018).

The majority of the research using FCM to assess sperm has focused on sperm viability (Paniagua-Chávez *et al.*, 2006; Favret and Lynn, 2010; Akcha, Spagnol and Rouxel, 2012; Le Goïc *et al.*, 2013; Rolton *et al.*, 2015; Gallo, Boni and Tosti, 2018) and mitochondrial membrane potential ($\Delta\Psi m$; (Adams, P. A. Hessian and Mladenov, 2003; Binet and Doyle, 2013; Le Goïc *et al.*, 2013; Binet *et al.*, 2014; Schlegel *et al.*, 2015)), with few studies using flow cytometry to assess the AR (Kekäläinen *et al.*, 2015). Previous research, using microscopy methods, has suggested that metals interfere with the AR in marine invertebrates (Zhang *et al.*, 2010). Thus, flow cytometry in combination with staining for the AR, may be able to account for the effects on Zn, Cd and Pb to fertilisation success.

Prior to fertilisation, glycoproteins on the sperm membrane undergo structural modifications that are determinants of the AR (Diekman, 2003; Jime Ânez *ete al.*, 2003). Lectins that bind to these glycoproteins can be labelled and used to help identify acrosome-reacted sperm. Staining of the AR using lectins has been investigated for bivalves (Favret and Lynn, 2010; McAnlis, Lynn and Misamore, 2010; Fallis *et al.*, 2014; Kekäläinen *et al.*, 2015), echinoderms (Favret and Lynn, 2010; Nakazawa, Shirae-Kurabayashi and Sawada, 2018) and ascidians (Nakazawa, Shirae-Kurabayashi and Sawada, 2018). In bivalves (*Mytilus galloprovincialis; Crassostrea virginica* and *Dreissena bugensis*) the lectins peanut agglutinin (PNA), D*olichos biflorus* agglutinin (DBA), *Lens culinaris* agglutinin (LCH) and wheat germ agglutinin (WGA) have been found to selectively bind to the acrosomal region of acrosome-reacted sperm (Favret and Lynn, 2010; McAnlis, Lynn and Misamore, 2010; Kekäläinen *et al.*, 2015). However, for the mussel *Dreissena polymorpha*, WGA was found to label the entire unreacted sperm (Fallis *et al.*, 2014). In the sea urchins (*Heliocidaris crassispina* and *Temnopleurus hardwikii*) exposed to the calcium ionophore (ionomycin), PNA most intensely labelled the region between the nucleus and mitochondria. There was also light acrosomal staining in *H. crassispina* (Nakazawa, Shirae-Kurabayashi and Sawada, 2018). In ascidians PNA also labelled the region between the nucleus and the mitochondria and not the acrosomal region (Nakazawa, Shirae-Kurabayashi and Sawada, 2018). The binding of lectins to the acrosomal region appears to differ between faunal groups, and in some cases species. No specific lectin can be used to determine acrosomal integrity of sperm of all

marine invertebrates. Thus, appropriate staining of the AR for *G.caespitosa* needs to be determined prior to developing ecotoxicological tests.

As well as disrupting the AR, metals have the potential to interfere with mitochondrial activity which, again, can potentially be labelled and visualised using fluorescent markers. Mitochondrial functions including adenosine triphosphate (ATP) generation depend on an electrochemical proton gradient across the inner mitochondrial membrane(Binet *et al.*, 2014; Little *et al.*, 2018; Nicholls, 2018; Teodoro, Palmeira and Rolo, 2018). As such, a reduced mitochondrial membrane potential ($\Delta\Psi m$) can be equated to a reduced capacity for ATP generation and other mitochondrial processes. In marine invertebrates, $\Delta\Psi m$ is typically measured using the probes Rhodamine 123 (R123) (Zou *et al.*, 2010), MitoTracker (Akcha, Spagnol and Rouxel, 2012; Le Goïc *et al.*, 2013; Nakazawa, Shirae-Kurabayashi and Sawada, 2018) or JC-1 (Binet *et al.*, 2014). JC-1 is considered a better probe to assess mitochondrial membrane potential fluorescence (Peña *et al.*, 2018), but emits dual fluorescence which may interfere with the fluorescence of other probes and may limit the potential for simultaneous staining of different markers. Tetramethylrhodamine methyl esther (TMRM) has been widely used to detect $\Delta\Psi m$ (Distelmaier, Werner J.H. Koopman, *et al.*, 2008; Bosch-Panadero *et al.*, 2018; Little *et al.*, 2018; Nicholls, 2018) and has been recommended as a valid estimation of $\Delta\Psi m$ under carefully controlled conditions (Zorova *et al.*, 2018). When TMRM is taken up by mitochondria it accumulates across charged membranes. TMRM is the least toxic mitochondria probe, exhibits lower

binding than R123 and MitoTracker (Scaduto and Grotyohann, 1999; Distelmaier, Werner J H Koopman, *et al.*, 2008; Kholmukhamedov, Schwartz and Lemasters, 2013), can be used without washing away prior to flow cytometric analysis (Nicholls, 2018) and equilibrates quickly - which is ideal for non-quenching studies. In non-quench studies, TMRM is used at low concentrations (1-30nM) such that fluorescence intensity decreases as ΔΨm is reduced. Low concentrations of TMRM prevent dye molecules from aggregating in the mitochondria and causing fluorescence quenching. Thus, a higher signal equates to a higher membrane potential (Perry *et al.*, 2011). As TMRM does not label species specific lectins, but accumulates across electrically charged membranes, uptake of the stain by mitochondria is not likely to differ between species. However, the concentration of TMRM required to detect differences in ΔΨm needs to be determined for *G. caesptiosa* prior to use in ecotoxicological tests.

In this study, a flow cytometric method is developed to assess the acrosome reaction (AR) and mitochondrial membrane potential (ΔΨm) for the serpulid polychaete, *G. caespitosa*. The main objectives of this study were: 1) to develop a simple and rapid methodology for assessing the AR and ΔΨm in *G. caespitosa*; 2) to determine whether metals (Cu, Zn, Pb and Cd) impact the AR and or/ ΔΨm; and 3) to determine if effects to these cellular processes can explain the effects observed to fertilisation success.

4.2 Methods

4.2.1 General Methods

Study species is as mentioned in the previous chapters. *Galeolaria caespitosa* were rinsed in 0.45µm filtered seawater (FSW) and placed in individual containers with 0.5mL of FSW to encourage sperm release. Sperm were used within 15 minutes of collection. For acrosome experiments *G. caespitosa* were collected from Wollongong Beach, New South Wales, between May and June 2018. For mitochondria experiments worms were collected from Grange Beach, South Australia, between October and November 2018.

4.2.2 Treatment Preparation and Analysis

Metal stock solutions were prepared using Analar grade metal salts of $CuSO_4$, $ZnCl_2$, $CdCl_2$, and $Pb(NO_3)_2$ (99 percent purity, Sigma-Aldrich©) and Milli-Q water (18.2 MΩ cm^{-1}; Millipore). Glassware was washed prior to use in 10% v/v nitric acid (69%, Merck). Seven test solutions for each toxicant were prepared on the day of the experiment from refrigerated stocks and FSW, no more than one hour prior to test commencement. Samples of each test concentration were acidified to 0.2% HNO_3 (69%, Merck) for dissolved metals analysis. Metal analyses were carried out using inductively coupled plasma optical emission spectrometry (ICP-OES; PerkinElmer 5300 V; AR) and inductively coupled plasma triple quad mass spectrometry (ICP-QQQ-MS; Agilent 8800; ΔΨm) by Future Industries Institute, UniSA at Mawson Lakes, South Australia. Test concentrations are presented in Appendix 3.

4.2.3 Sperm Exposures

Sperm were collected from five individuals, pooled and diluted to approximately $5x10^5$ sperm mL^{-1} into treatment solutions. The exposure of sperm to the treatment solutions constituted the start of the test and the time was recorded. Sperm were exposed to each treatment for 30 minutes. Sperm concentrations were verified at the end of the test using a haemocytometer.

4.2.4 Acrosome Reaction

2.4.1 Initiating the Acrosome Reaction

Sperm were exposed to several solutions that have previously been shown to initiate the AR in marine invertebrates; Calcium chloride (Brown, 1976; Grant, 1981), egg water (Kekäläinen *et al.*, 2015), chorion extract (Sato and Osanai, 1990), FSW with an increased pH (9.2; Dan 1952; Dan et al. 1972) and the calcium ionophore A23187 (Morisawa *et al.*, 2004; Fallis *et al.*, 2014). Unless otherwise stated, sperm were exposed to each solution for 30 minutes at a sperm density of ~5 x 10^5 sperm mL^{-1}. We also used a CaMg free seawater as a negative control as this should prevent sperm from undergoing an acrosome reaction. After 30 min exposure to the solution sperm were manually counted under the microscope to determine acrosomal integrity (60 x magnification, Olympus BX43). One hundred sperm from each treatment were manually counted to determine acrosome status (reacted/non-reacted; Figure 4.4). Three replicates were conducted for each treatment.

Calcium Chloride Solution

A 0.34µM Calcium chloride solution diluted 1:19 in sperm suspension.

Egg Water

Eggs were collected from twenty females, suspended in filtered seawater (FSW) to a concentration of ~2,500 eggs mL^{-1} and stirred gently at 20°C for 3 hours. The solution was then 11µm filtered to remove eggs and other particles from the FSW (hereafter referred to as 'egg water'). The eggwater was diluted via a twofold serial dilution in FSW such that the concentrations were as follows: 2500, 1250, 625, 312, 156 eggs mL^{-1} (filtered).

Chorion Extract

Egg envelopes (chorions) were isolated using the methods of Sato and Osanai (1990). Unfertilized eggs were suspended in FSW seawater and then gently homogenized with a Teflon homogenizer. The homogenate was centrifuged at 300 X g for 5 min. After removing the supernatant, the sedimented chorions were resuspended in fresh FSW and centrifuged again. Transparent chorions were obtained by repeating this procedure ten times. Isolated chorions were placed in Milli-Q water (18.2mΩ cm^{-1}, Millipore; 2% V/V) for 3 Hours. The chorion suspension was filtered (11µm) and diluted 1:9 with FSW.

Calcium Ionophore

The Calcium ionophore A23187 was diluted in DMSO to a concentration of 2000µM. Solutions of A23187 were prepared via a threefold serial dilution in

FSW at concentrations of; 50.0, 16.6, 5.6, 1.9, 0.6, 0.2µM. Sperm were exposed to the calcium ionophore solutions for a period of 10 minutes.

2.4.2 Acrosomal Stain Optimisation

After finding optimal conditions for initiating the acrosome reaction in *G. caespitosa,* we had to identify which stain would be most appropriate to label acrosome reacted sperm. Three treatments of 10µM calcium ionophore (A23187) and three FSW controls were each labelled with Fluorescein Isothiocyanate (FITC) – labelled lectins at concentrations of 10µg mL^{-1} (Vector Laboratories, Inc. Burlingame, CA, USA): Peanut agglutinin (PNA), Lens culinaris agglutinin (LCH) and Wheat germ agglutinin (WGA). Sperm were exposed to each of these at approximately 5 x 10^5 sperm mL^{-1} and incubated in the dark at 20°C for 30 minutes. Live sperm were used for lectin labelling to ensure that only sperm surface carbohydrates were labelled (Fallis *et al.*, 2014; Kekäläinen *et al.*, 2015).

Three Images were taken of each treatment using both brightfield and fluorescence microscopy. Each image was taken with the same camera settings, exposure was kept at 200ms. The total number of sperm in the bright field image was then compared to the number of sperm that had taken up the stain. Each image was then analysed in imageJ by defining outlines of the fluorescent acrosomes and measuring the average integral density per pixel within the outline. Background intensity was then subtracted. We then

determined if there was a significant difference in stain intensity between each of the FITC labelled lectins.

Staining Conditions

Once the most effective stain had been determined, the difference in acrosomal stain intensity with increasing concentrations of stain was investigated. We exposed sperm to three treatments of 10 µM A23187 and three controls (FSW). One treatment and one control were exposed to concentrations of 5, 10 and 20µg L^{-1} PNA. Test solutions were then incubated at 20°C for 30 mins. Once the optimal stain concentration was determined we aimed to minimize the incubation period to reduce the time of the test procedure. We exposed sperm to 10µM A23187 and 10µg/L PNA for 5, 10 and 20 mins.

2.4.3 Metal Toxicity Tests for the Acrosome Reaction

After exposure to treatment solutions, a calcium ionophore (A23187; Sigma Aldrigh ©; 10µM) and Flourescein Isthiocyanate labelled peanut agglutinin (FITC-PNA; Vector Laboratories© Inc., Burlingame, CA, USA; 10µg L^{-1}) were added and incubated for 10 mins in the dark. Filtered seawater controls consisted of one with no A23187 to determine % errant reactions, and two exposed to A23187 (one at the beginning and end of the test).

4.2.5 Mitochondrial Membrane Potential

2.5.1 Mitochondrial Membrane Potential Stain Optimisation

The aim here was to determine an appropriate concentration of Tetramethylrhodamine, methyl ester (TMRM) that would stain functional mitochondria. We aimed to use the smallest concentration that gives a large enough differentiation between functional and non-functional mitochondria so that the stain can be utilised in non-quench mode. Non-quenching modes should use the lowest possible concentration (~1–30 nM; Perry et al. 2011). Non-quench mode enables us to see both positive and negative effects to the mitochondria. We tested different ratios of live/dead sperm. For dead sperm, sperm were heated to 50°C for 10mins and motility was checked to ensure no sperm were motile. The % alive sperm in each of the solutions was; 0, 12.5, 25, 50, 100. Each of the solutions were tested with concentrations of 12nM, 25nM and 50 nM TMRM (Figure 4.7). To examine the effects of decreased mitochondrial function as opposed to dead cells, we used aged sperm (>24 hours). Sperm were diluted to ~5 x 10^5 sperm mL^{-1} and left for 6 hours at 20°C. Sperm were then refrigerated to 4°C. Stocks of aged sperm were not used after 36 hours since collection.

2.5.2 Metal Toxicity Tests for Mitochondrial Membrane Potential

TMRM was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 25µM and stored in the dark at 4°C. This solution was diluted in tests such that the final concentration in the assay was 12nM. TMRM was added to test

solutions prior to the addition of sperm. Once sperm were added to the test solution sperm were incubated for 30 mins at 20°C. Filtered sweater controls consisted of one unstained sperm, two stained sperm at the beginning and end of each test, an aged sperm control and a dead sperm control. Following incubation, the test solutions were subject to flow cytometric analysis.

4.2.6 Flow Cytometric Methods

Flow cytometric analyses were conducted using a FACs-Calibur (AR; Becton Dickinson) and a BD Accuri™ C6 Plus (ΔΨm) both equipped with a blue laser (488 nm). As each cell passes through the laser, its volume displacement, light scattering and fluorescent signals are measured. Electrical volume (EV) and side scatter (SSC) signals provide information on cell size and shape. All samples were run on low speed (~14µL/min) for either 2 mins, or until 10,000 counts. The data were analysed as the percentage of sperm found in the gated regions where fluorescence from the dyes were detected. FITC-PNA stained cells were detected in the FL1 channel (530/30 band-pass filter) and TMRM stained cells were detected in the FL2 channel (585/42 band pass filter). Sperm staining of the acrosome and mitochondria was verified using fluorescence microscopy (Olympus BX 43).

4.2.7 Statistics

Statistical comparisons for stain optimisations were made using a Two Sample T-Test. The R package DRC (Ritz and Strebig, 2005) was used to model the test data and to calculate toxicity estimates. Regression models tested

included log-logistic and Weibull models with different levels of parametrization. Replicates were pooled to fit a concentration response curve. Model comparisons were conducted using the Akaike Information Criterion (AIC) and models that best described the data were applied to determine effect concentrations. Concentrations that elicited a 95% (EC95), 50% (EC50) and 10% (EC10) decrease in AR or $\Delta\Psi m$ were calculated. The associated 95% confidence limits were estimated using the delta method. Statistical differences in effect concentrations were determined using the method described by Sprague and Fogels (1976). Where dose response models could not be fitted to the data, statistical analysis was conducted via a one-way ANOVA to determine if there was significant variation between the control and treatments. If the ANOVA returned a significant result ($P<0.05$), subsequent Tukey tests were conducted to determine which treatments were significantly different to the control. Full statistical analysis are presented in Appendix 3.

4.3 Results

4.3.1 Initiating the Acrosome Reaction

The Calcium Ionophore A23187 was the most effective inhibitor of the acrosome reaction in *G.caespitosa* sperm. On visual inspection, exposure of sperm to concentrations greater than 1.9µM A23187 caused over 80% of sperm to undergo the acrosome reaction (Figure 4.3). There was no difference between exposure to 5.6 or 16.7µM A23187. Thus, we chose a concentration of 10µM A23187 for subsequent tests. Whilst egg water, calcium chloride, chorion extract and an increased pH all showed greater numbers of acrosome reacted sperm than respective seawater controls, they did not achieve sufficient levels of acrosome reacted sperm to use as a positive control for the ecotoxicological tests (Figures 4.1;4.2). There was minimal difference between FSW and CaMg Free seawater treatments, as such subsequent experiments used FSW as a negative control.

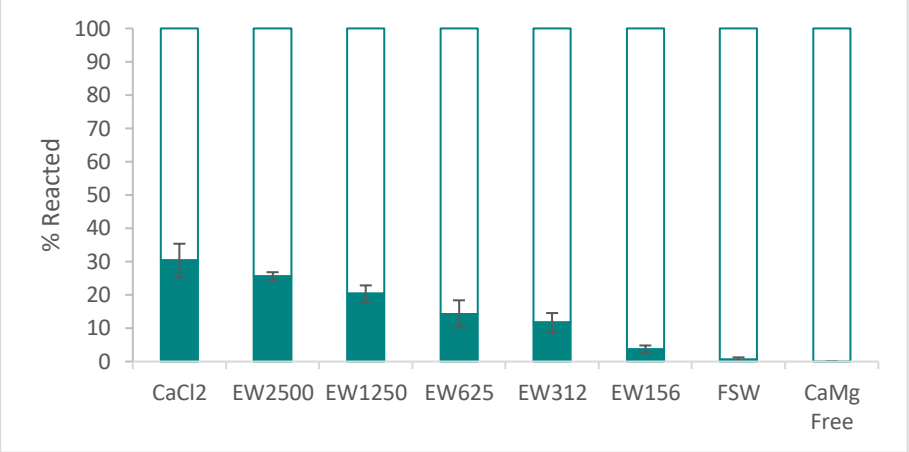


Figure 4.1: Effects of Calcium Chloride and Egg Water solutions on acrosomal integrity of *G. caespitosa* sperm.

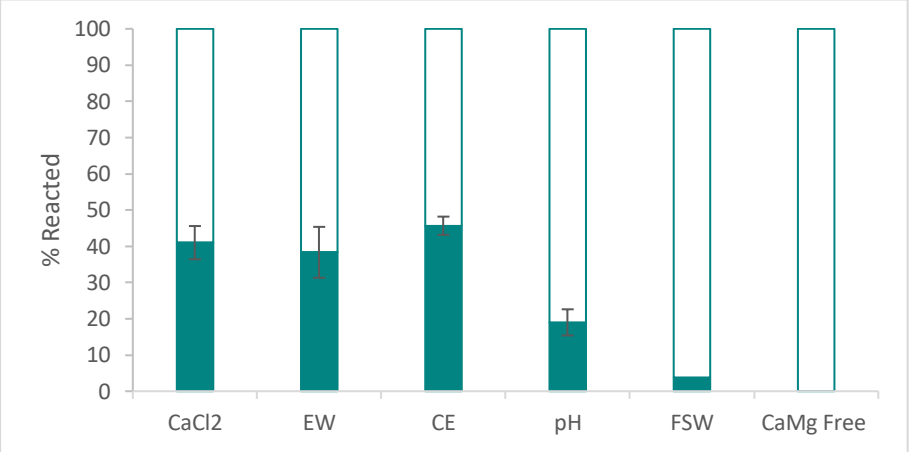


Figure 4.2: Effects of calcium chloride, egg water, chorion extract and increased pH on acrosomal integrity of *G. caespitosa* sperm.

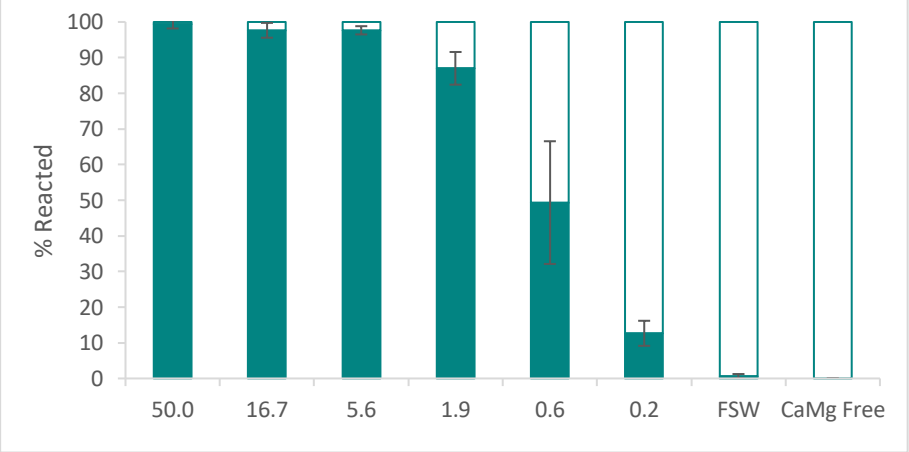


Figure 4.3: Effects of the calcium ionophore A23187 on acrosomal integrity of *G. caespitosa* sperm after 10 minutes exposure.

4.3.2 Acrosomal Stain Optimisation

All three of the stains selectively labelled the acrosome of reacted sperm and did not label unreacted sperm. Acrosome reacted sperm that were labelled with PNA had the most intense staining, almost double that of the other stains (Figure 4.4). There was a statistically significant difference between the stain intensity of sperm stained with PNA when compared to the other two stains (Table 4.1).

Stain Conditions

Sperm were exposed to FSW or A23187 and stained with 5µg L^{-1}, 10µg L^{-1} and 20 µg L^{-1} PNA. No unreacted sperm took up the stain in any of the FSW controls. There was no difference in stain intensity of acrosomes of reacted sperm between concentrations of 10µg L^{-1} and 20 ug L^{-1}. However, the acrosomes of sperm stained with 5µg L^{-1} were significantly more intense than when exposed to 10 or 20µg L^{-1} (Table 4.2). The background intensity increased significantly when exposed to 10 and 20µg L^{-1} (Table 4.2), which may have masked the intensity of reacted acrosomes. These concentrations were tested again on the flow cytometer. There was a greater separation of reacted/non-reacted sperm using 10µg L^{-1} as opposed to 5µg L^{-1} PNA (Figure 4.5). Thus, 10µg L^{-1} was chosen as the optimal stain concentration as we could achieve a better separation on the flow cytometer and minimise background noise. To minimise the incubation period, we exposed sperm to 10µM A23187 and 5µg L^{-1} of PNA for periods of five, ten and twenty minutes. There was a significant difference in stain intensity between 5- and 10-minute incubation

periods. There was no significant difference in stain intensity after 10- or 20-minute incubation periods (Table 4.3). Therefore, we used 10 minutes as the required incubation period for subsequent tests. Altogether the optimal conditions were; 10µM Calcium Ionophore, 10µg L^{-1} PNA and a 10-minute exposure period. These conditions were confirmed using flow cytometry.

4.3.3 Mitochondrial Membrane Potential Stain Optimisation

All three of the concentrations of TMRM gave good separation between live and dead sperm, thus we chose to use the lowest concentration, 12nM TMRM for subsequent tests (Figure 4.7). There was a 93% uptake of TMRM in live sperm with 0% uptake in the dead sperm. Aged sperm showed a decrease in uptake of TMRM when compared to fresh sperm (Figure 4.6), thus indicating a decrease in mitochondrial membrane potential.

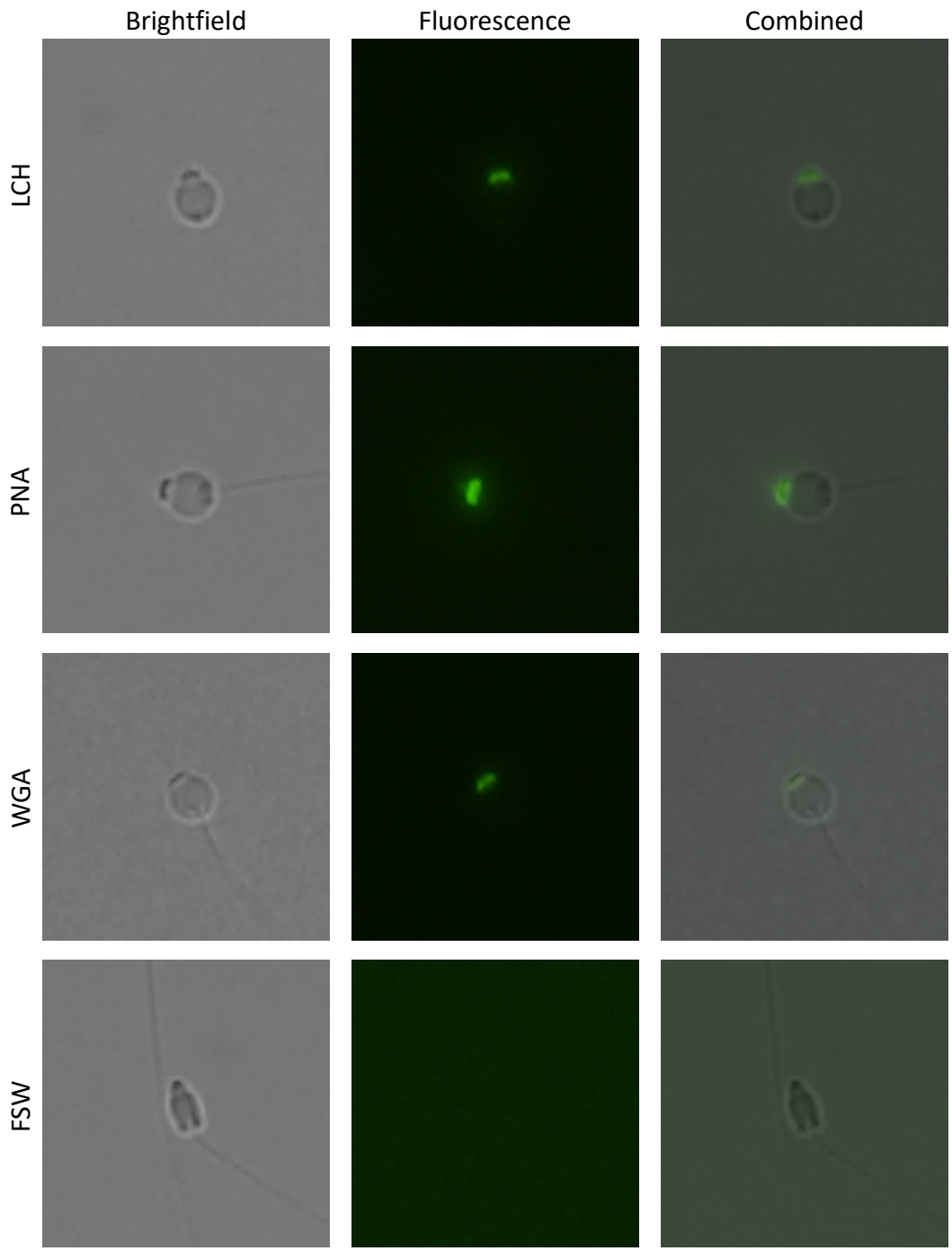


Figure 4.4**:** Fluorescence intensity of *G. caespitosa* sperm when exposed to A23187 and stained with WGA, PNA and WGA (reacted). The FSW control was stained with PNA to show that unreacted sperm fail to pick up the stain.

Table 4.1: Statistical comparison of stain intensity of acrosome reacted sperm when labelled with PNA, WGA and LCH.

Stain	Mean Intensity	SD	n	Significance
PNA	49.7	6.0	20	
WGA	18.7	1.9	18	p<0.05*
LCH	24.3	4.9	37	p<0.05*

Table 4.2: Statistical comparison of stain intensity of acrosome reacted sperm and image background when labelled with three different concentrations of PNA.

	Stain Concentration (µg L^{-1})	Mean Intensity	SD	n	Significance
Reacted Acrosomes	5	36.7	5.4	35.0	
	10	33.5	6.5	49.0	P<0.05*
	20	32.9	7.1	45.0	P>0.05
Background	5	13.1	2.5	10.0	
	10	20.9	4.6	10.0	P<0.05*
	20	35.6	2.8	10.0	P<0.05*

Table 4.3:Statistical comparison of stain intensity of acrosome reacted sperm when exposed to 10µM A23187 and 5µg L^{-1} PNA for three different incubation periods.

Incubation Period (Mins)	Mean Intensity	SD	n	Significance
10	39.5	11.3	99.0	
5	35.0	8.9	126.0	P<0.05*
20	37.9	7.2	130.0	P>0.05

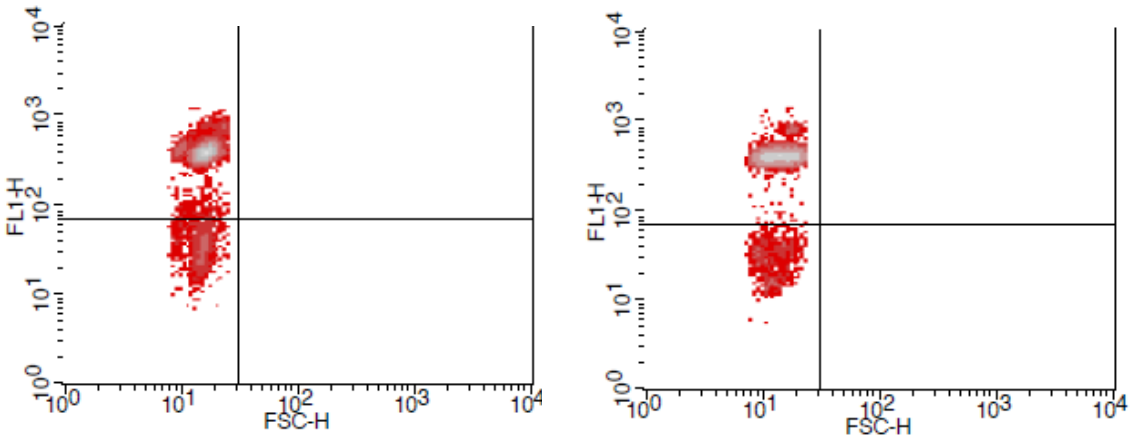


Figure 4.5: Cytograms of green fluorescence (FL1-H) of acrosome reacted sperm stained with 5µg L^{-1} PNA (left) and 10µg L^{-1} PNA (right).

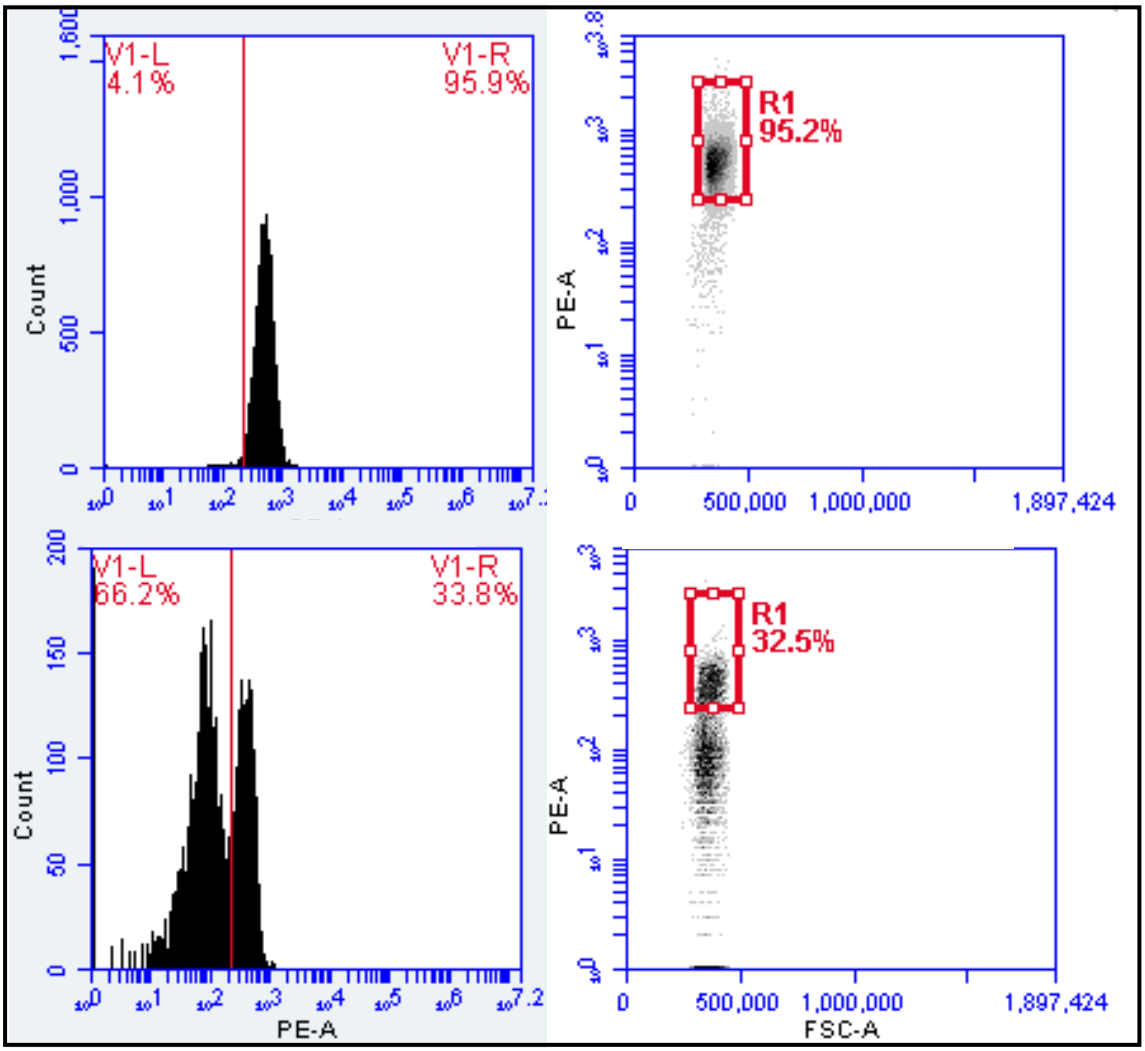


Figure 4.6**:** Histograms (left) and cytograms (right) of red fluorescence (FL2; PE-A) of fresh sperm (top) and aged sperm (bottom) exposed to 12nM TMRM.

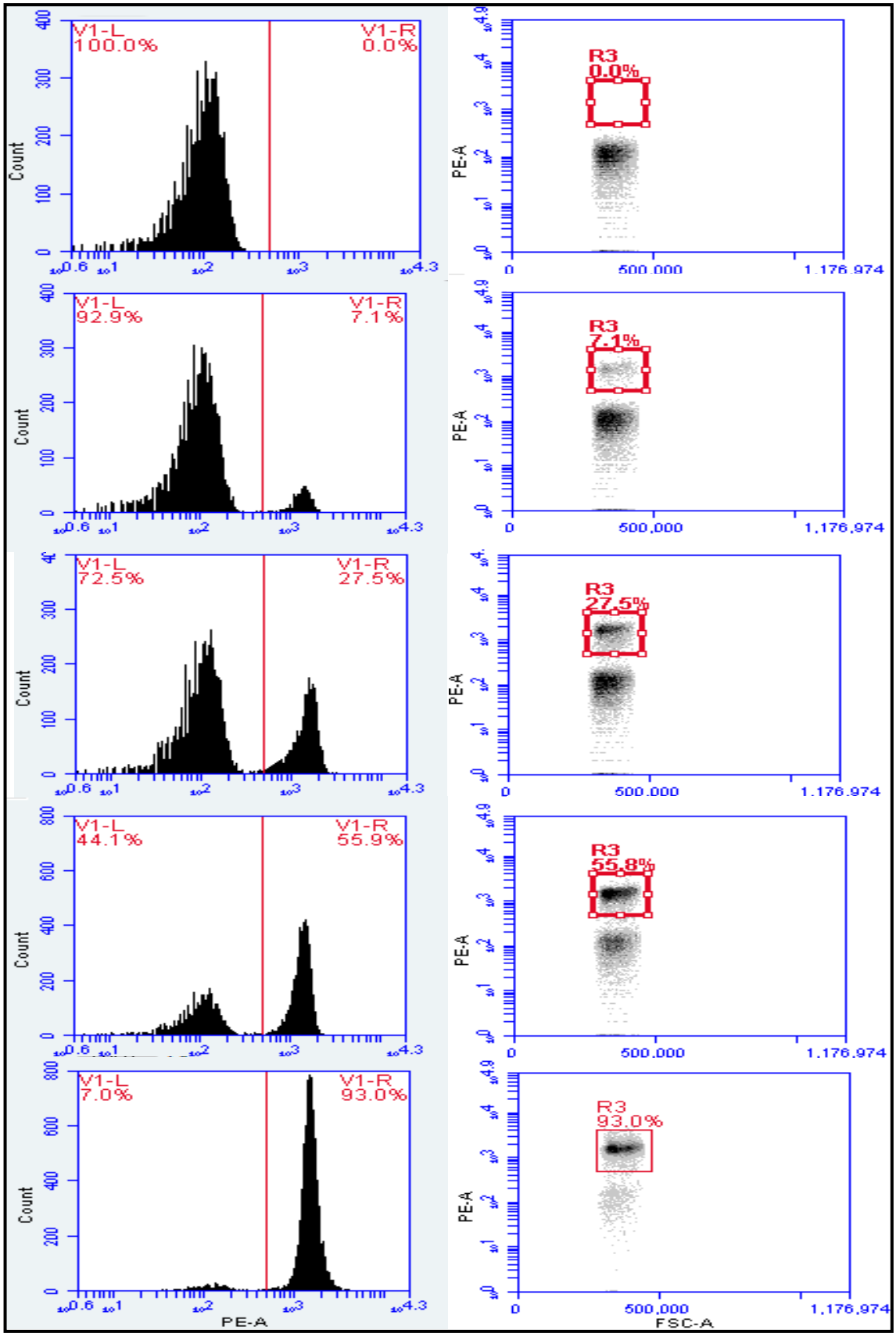


Figure 4.7: Histograms (left) and Cytograms (right) of red fluorescence (FL2; PE-A) for different ratios of live:dead sperm exposed to 12nm TMRM.

4.3.4 Flow cytometry

Galeolaria caespitosa sperm could successfully be identified from the FSW/A23187/FITC-PNA matrix and the FSW/TMRM matrix. Forward scatter (FSC) and side scatter (SSC) characteristics of the matrices were different to that of sperm (Figure 4.8). The matrices contained only a small number of particles in the gated sperm population (<0.2% of total events). Therefore, it is unlikely that non-sperm particles would bias our results. Two populations of sperm were observed, mature sperm accounted for approximately 70-80% of total events and only ~2-5% were identified in the second population. When examining the sample under the microscope a small number of immature sperm or spermatids were observed (Lu, Aitken and Lin, 2017) that were larger than mature sperm and would have greater FSC and SSC characteristics. Thus, this sub population was not included, and all further analysis was conducted on the mature sperm population as gated (Figure 4.8).

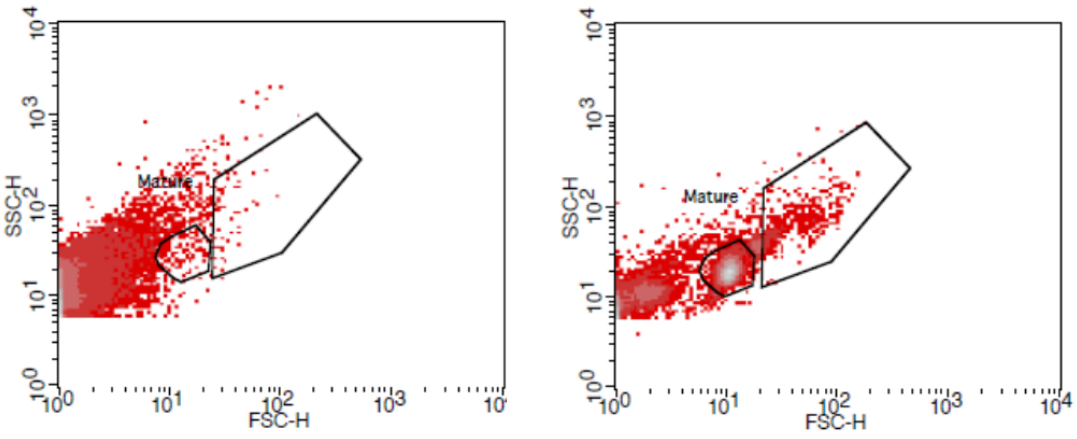


Figure 4.8: Cytogram of FSW/A23187/FITC-PNA matrix without *G. caespitosa* sperm (left) and with sperm (right).

4.3.5 Effects of Metals to the Acrosome Reaction

Flow cytometry could not differentiate between acrosome-reacted and unreacted sperm based on FSC and SSC characteristics alone. However, both microscopy and flow cytometric analysis successfully identified an increase in acrosome reacted sperm cells when exposed to A23187 via staining with FITC-PNA. FITC-PNA specifically bound to the acrosome region of reacted sperm, but not acrosome-intact sperm (Figure 4.9). Thus, it was possible to determine the effects of metals to acrosomal integrity. Pre-exposure to both Zn and Cu prevented sperm from undergoing the acrosome reaction when subsequently exposed to A23187 and presented characteristic concentration response relationships (Figures 4.10;4.11). Resulting EC10, 50 and 95 values for Zn were 71, 110 and 290 μg L^{-1} respectively. Resulting EC10, 50 and 95 for Cu were 310, 390 and 640 respectively (Table 4.4). However, Cd and Pb had no effect on the ability for sperm to undergo the acrosome reaction (Figures 4.12; 4.13).

4.3.6 Effects of Metals to Mitochondrial Membrane Potential

Flow cytometry could not differentiate between functional and non-functional mitochondria based on FSC and SSC characteristics alone. TMRM specifically labelled functional mitochondria in *G.caespitosa* sperm which was successfully identified using flow cytometry (Figure 4.14). Exposure of *G.caespitosa* sperm to Cu, Zn and Cd had a significant positive effect on TMRM uptake ($P<0.05$, Figures 4.15; 4.16; 4.17; Appendix 3). When exposed to elevated concentrations of these metals, >60% of sperm showed brighter fluorescence, outside of the

control gating. However, there was no conclusive effect on TMRM uptake when exposed to Pb (Figure 4.18, Appendix 3).

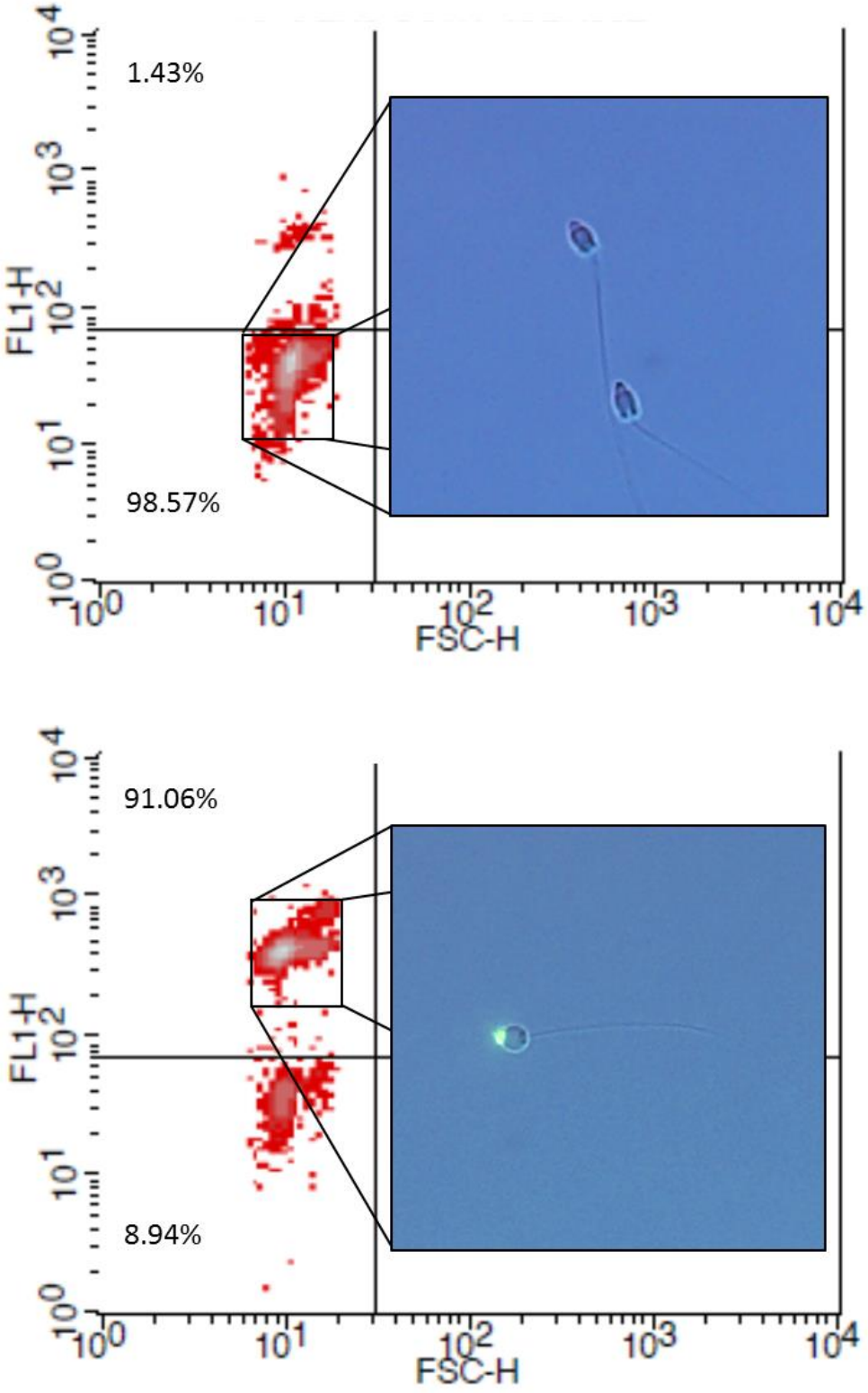


Figure 4.9: Cytogram and microscope images of green Fluorescence (FL1) for *G. caespitosa* sperm in FSW (top) and exposed to 10µM A23187 (bottom).

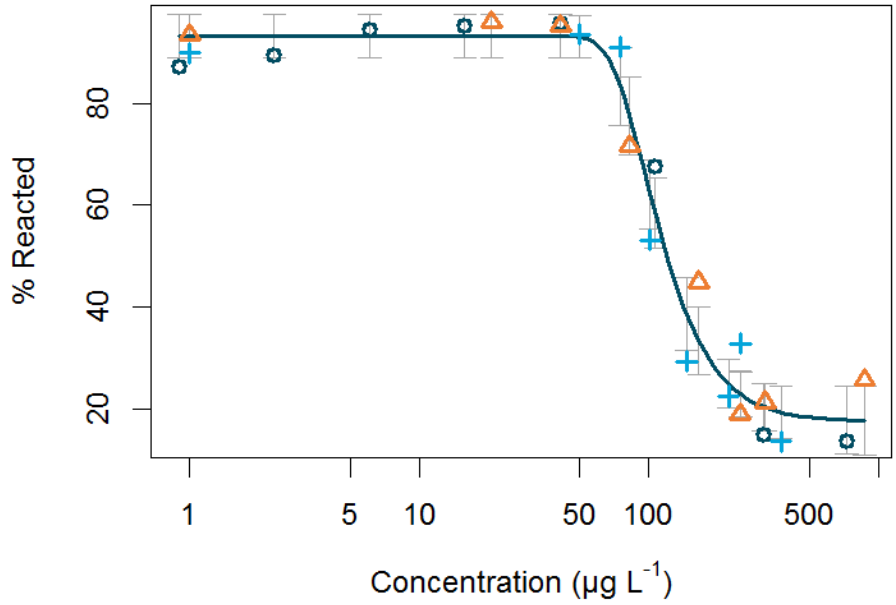


Figure 4.10: Concentration-response relationships for *G. caespitosa* sperm exposed to Zn, prior to initiating the acrosome reaction. Replicates are represented by the different symbols and colours. Error bars in the vertical direction represent confidence in the model.

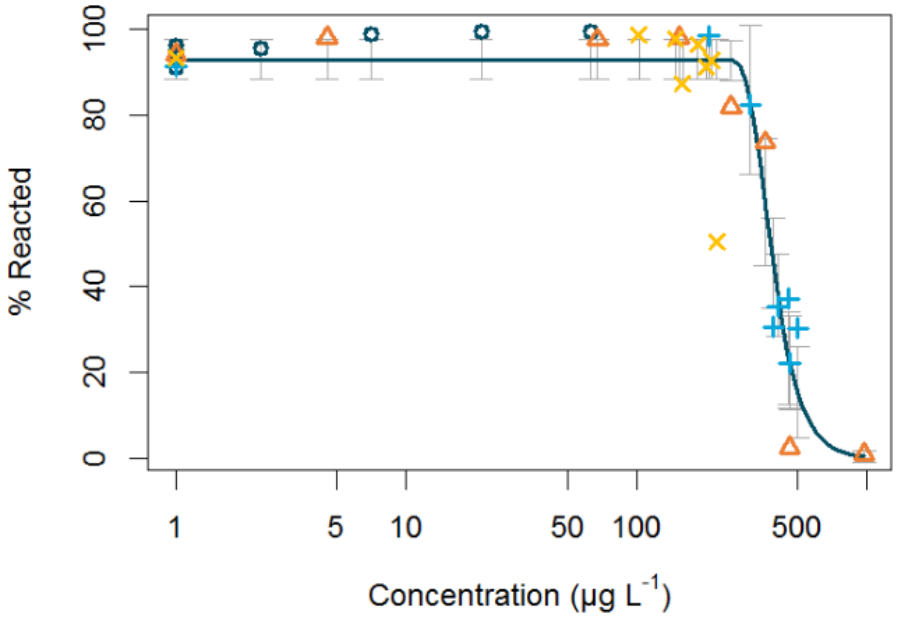


Figure 4.11: Concentration-response relationships for *G. caespitosa* sperm exposed to Cu, prior to initiating the acrosome reaction. Replicates are represented by the different symbols and colours. Error bars in the vertical direction represent confidence in the model.

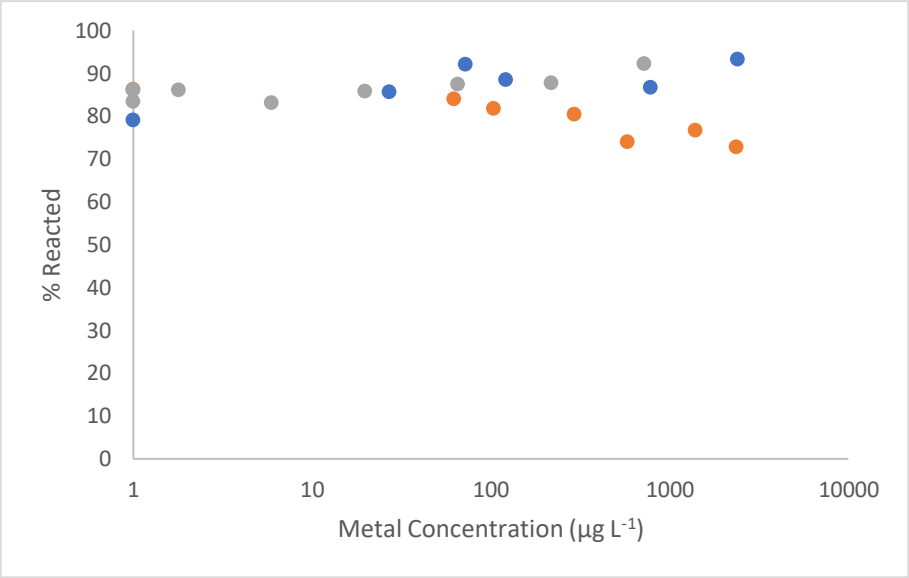


Figure 4.12: Concentration-response relationships for *G. caespitosa* sperm exposed to Lead, prior to initiating the acrosome reaction. Replicates are represented by the different colours.

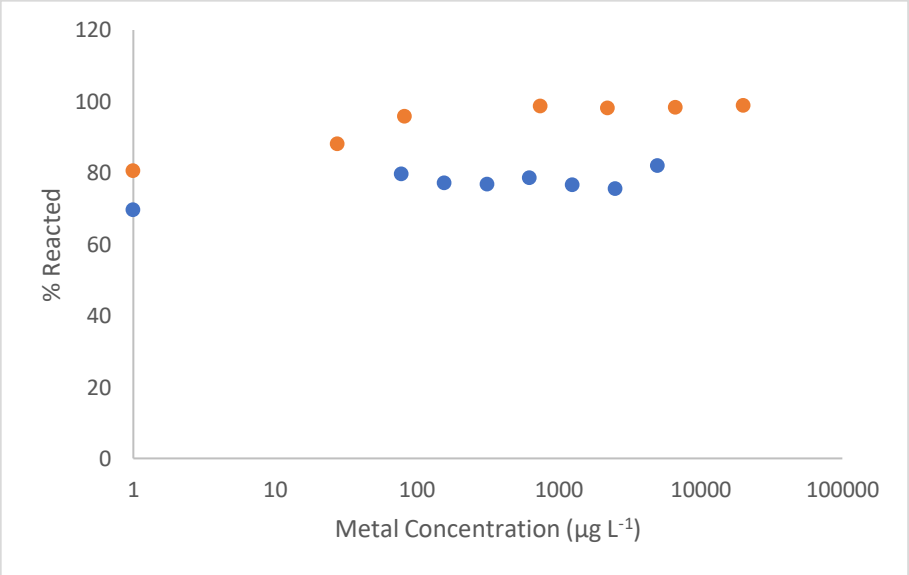


Figure 4.13: Concentration-response relationships for *G. caespitosa* sperm exposed to cadmium, prior to initiating the acrosome reaction. Replicates are represented by the different colours.

Table 4.4: EC10, EC50 and EC95 values and associated 95% confidence limits calculated based on acrosomal integrity for *G. caespitosa* sperm.

Metal	EC10*	EC50*	EC95*	Fert EC50	Sig.
Zn	72 (61-83)	110 (7-130)	290 (150-430)	260 (210-320)	P>0.05
Cu	310 (260-360)	390 (370-420)	640 (460-810)	20 (18-22)	P<0.05 *

*measured dissolved (0.45µm filtered)

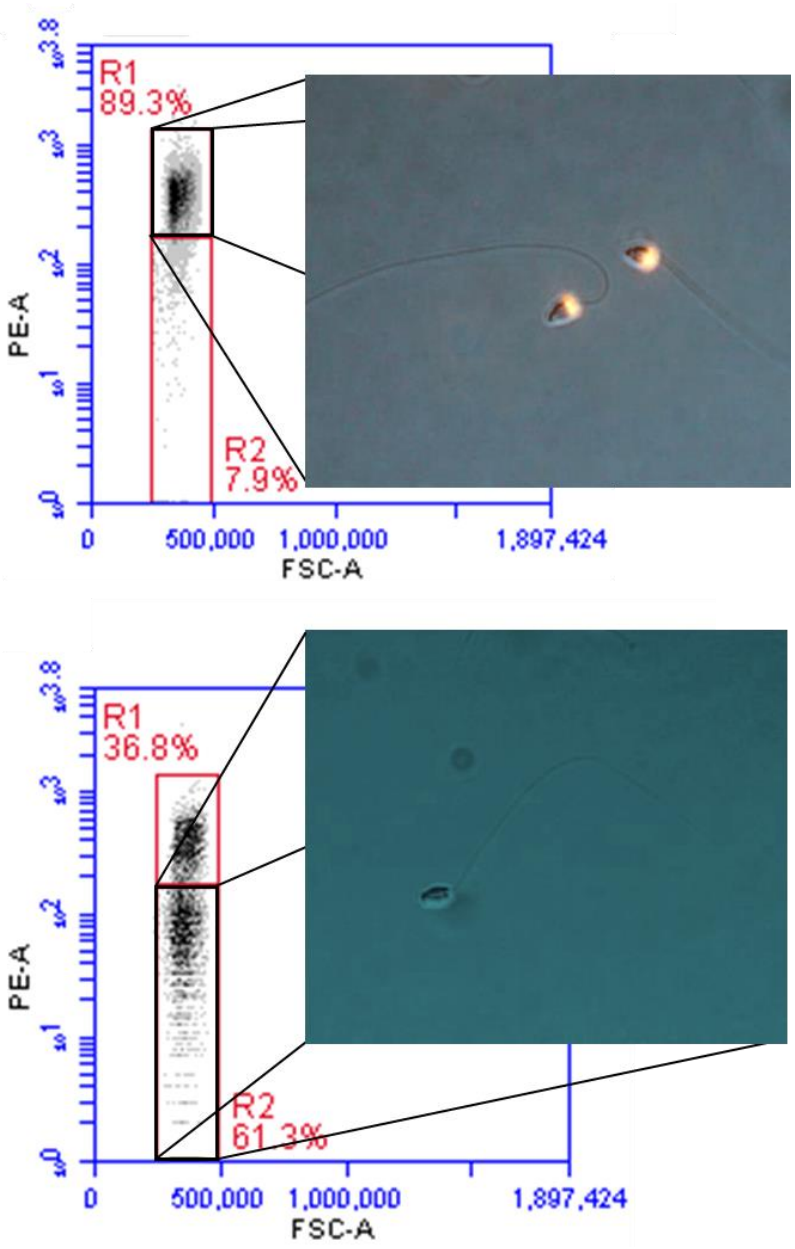


Figure 4.14: Cytogram and microscope images of red fluorescence (FL2; PE) for fresh *G. caespitosa* sperm (left) and aged sperm (right).

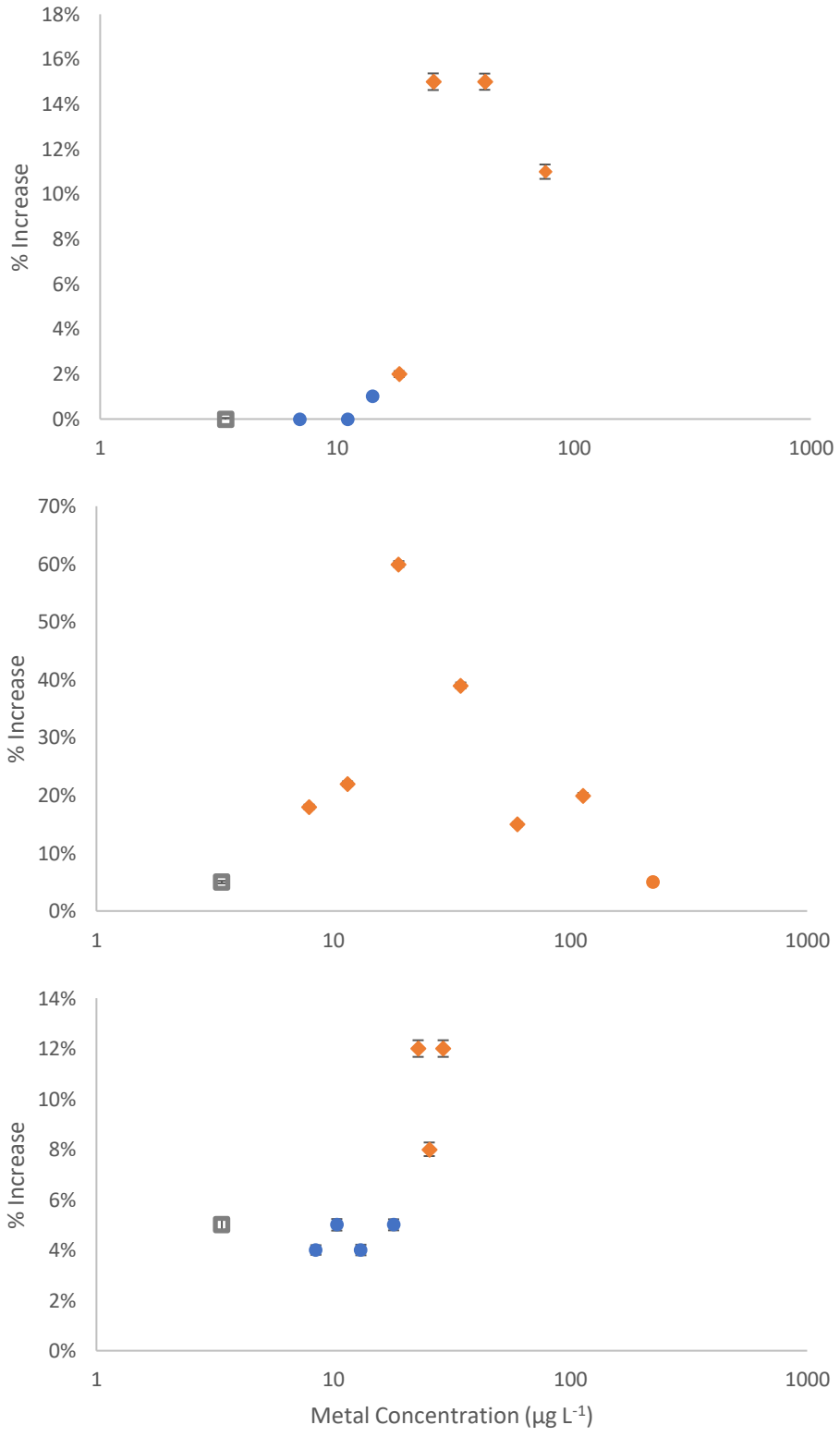


Figure 4.15: Percent of *G. caespitosa* sperm with increased TMRM fluorescence, gated outside of the FSW control, when exposed to Cu. Each replicate (top, middle, bottom) consisted of a FSW control and metal treatments with increasing concentration. Significant effects on the uptake of TMRM are represented by the orange diamonds. Error bars represent the standard error.

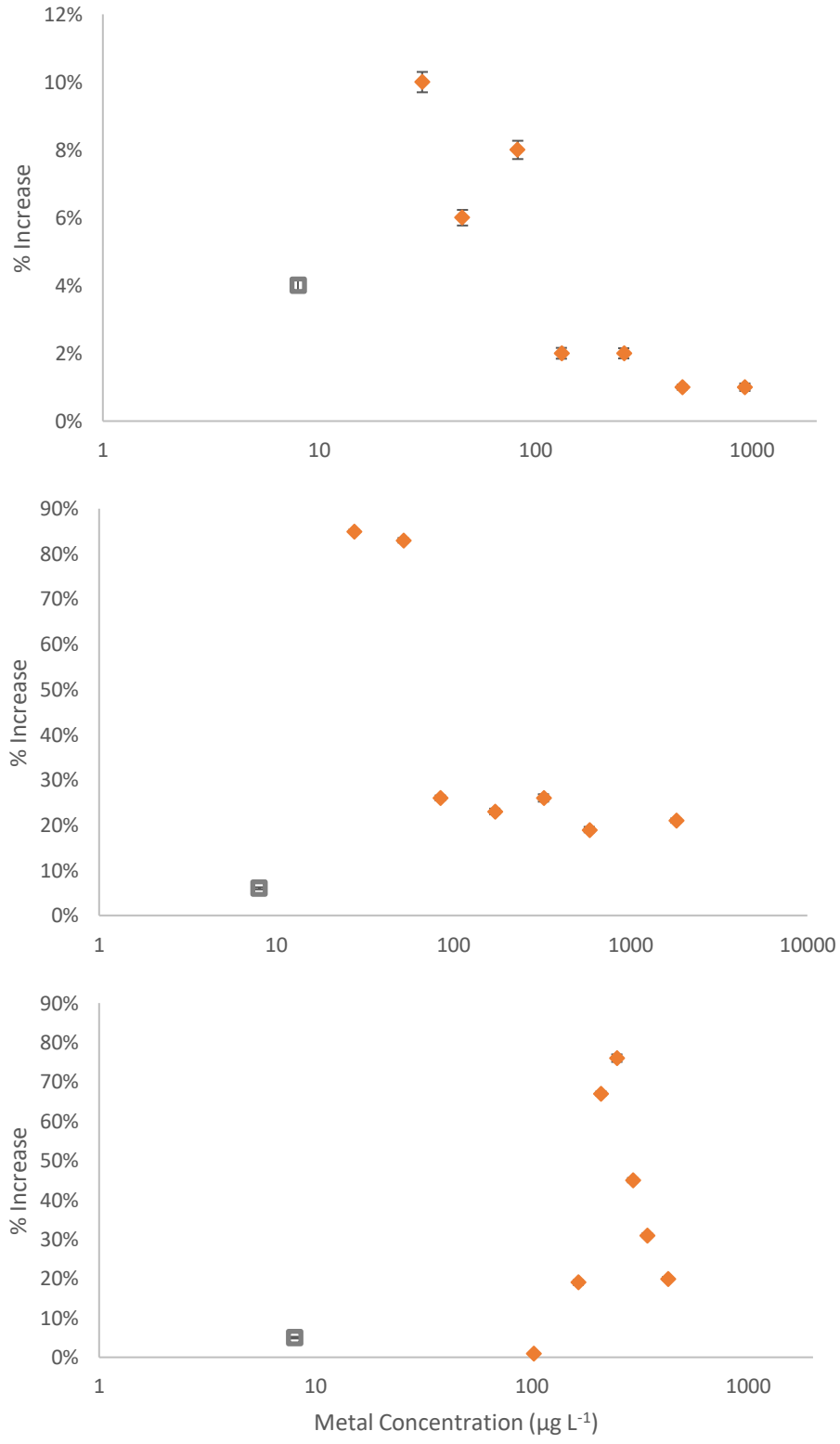


Figure 4.16: Percent of *G. caespitosa* sperm with increased TMRM fluorescence, gated outside of the FSW control, when exposed to Zn. Each replicate (top, middle, bottom) consisted of a FSW control and metal treatments with increasing concentration. Significant effects on the uptake of TMRM are represented by the orange diamonds. Error bars represent the standard error.

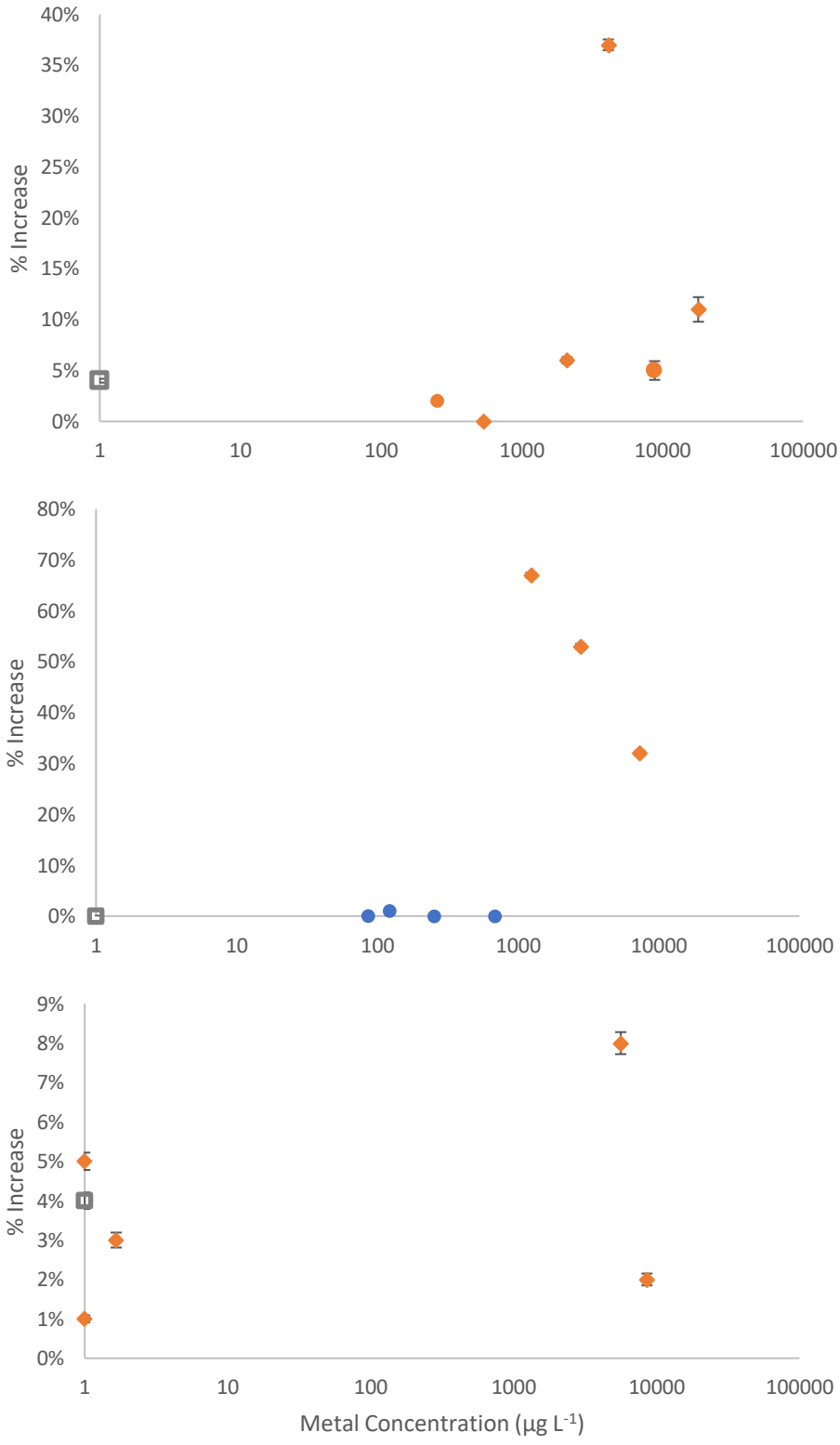


Figure 4.17: Percent of *G. caespitosa* sperm with increased TMRM fluorescence, gated outside of the FSW control, when exposed to Cd. Each replicate (top, middle, bottom) consisted of a FSW control and metal treatments with increasing concentration. Significant effects on the uptake of TMRM are represented by the orange diamonds. Error bars represent the standard error.

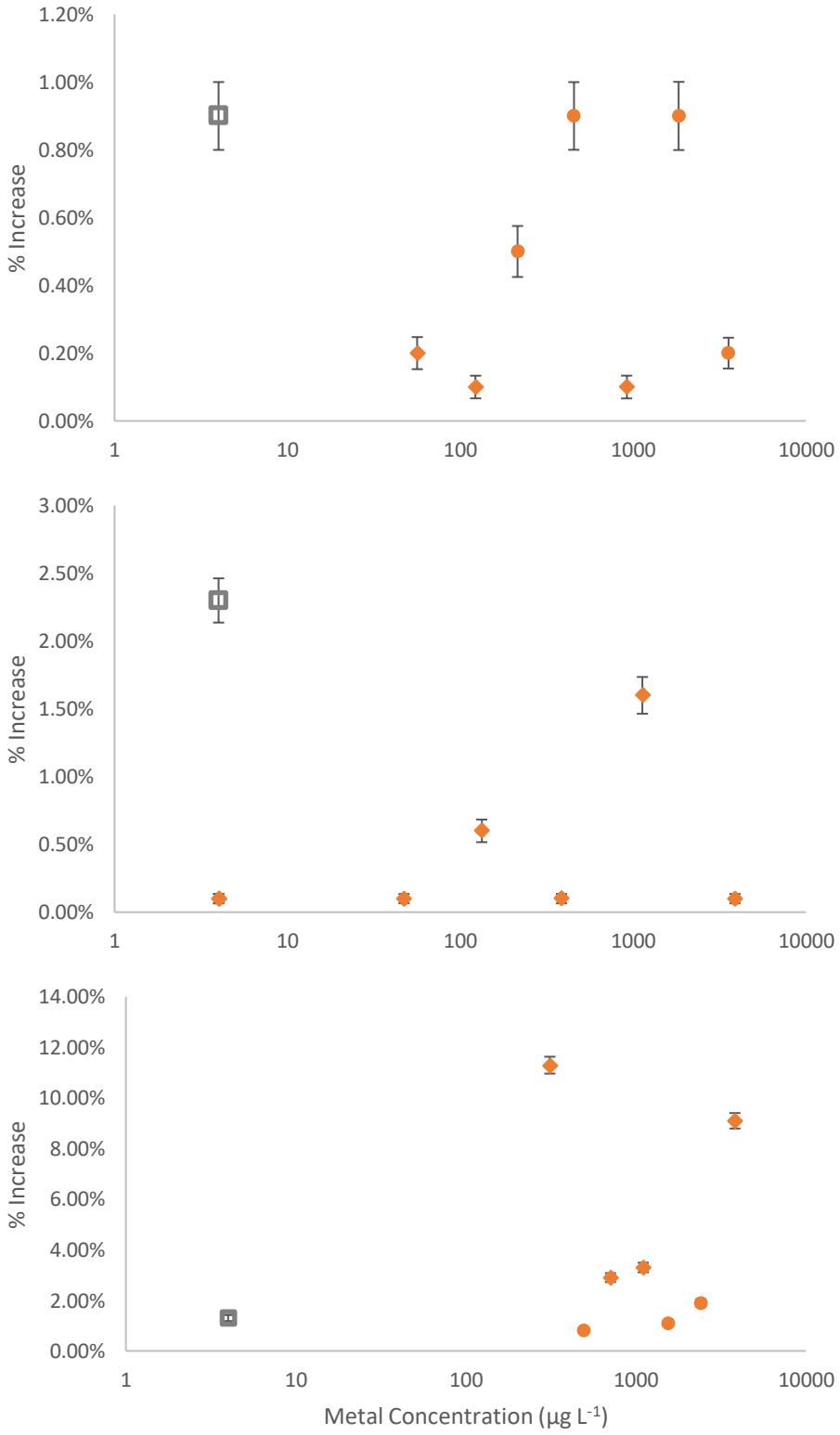


Figure 4.18: Percent of *G. caespitosa* sperm with increased TMRM fluorescence, gated outside of the FSW control, when exposed to Pb. Each replicate (top, middle, bottom) consisted of a FSW control and metal treatments with increasing concentration. Significant effects on the uptake of TMRM are represented by the orange diamonds. Error bars represent the standard error.

4.4 Discussion

4.4.1 Flow Cytometry

Flow cytometry is increasingly being recommended and adopted as a tool for sperm analysis in ecotoxicology (Adams, P. a. Hessian and Mladenov, 2003; Gravance *et al.*, 2003; Favret and Lynn, 2010; Jenkins *et al.*, 2015; Kekäläinen *et al.*, 2015; Gallo, Boni and Tosti, 2018; Peña *et al.*, 2018). In this study, a method using flow cytometry combined with FITC-PNA and TMRM staining was developed for evaluating acrosomal integrity and mitochondrial membrane potential of sperm from a marine invertebrate, *G. caespitosa*. Using these methods, we were successfully able to identify if sperm had the ability to undergo the acrosome reaction and whether they have functioning mitochondria after exposure to four metals (Cu, Zn, Pb and Cd). Overall, the method developed can offer an objective, rapid and accurate alternative to traditional microscopy methods for assessing morphological and functional changes in sperm (Kekäläinen *et al.*, 2015). Whilst this method was successful in identifying effects of metals to the acrosome and the mitochondria, many of the effects were positive and therefore cannot account for the effects of metals to fertilisation in *G. caespitosa*. Thus, the AR and ΔΨm may not be appropriate as stand-alone endpoints for metal toxicity.

Sperm toxicity tests using flow cytometry typically investigate sperm viability in combination with ΔΨm (Paniagua-Chávez *et al.*, 2006; Favret and Lynn, 2010; Akcha, Spagnol and Rouxel, 2012; Le Goïc *et al.*, 2013; Rolton *et al.*, 2015; Gallo, Boni and Tosti, 2018). This study demonstrates that use of just sperm

viability and ΔΨm may not provide an accurate representation of the effects of metals on sperm function. For example, exposure of *G. caespitosa* sperm to Zn would present as viable sperm with functioning mitochondria. However, these sperm do not have the capacity for successful fertilisation as they are unable to undergo the acrosome reaction. Similarly, for Cu, sperm would appear to be viable, with functioning mitochondria, but they are not motile (previous chapter). Before these endpoints (viability and ΔΨm) are adopted as routine tests, as recommended (Gallo, Boni and Tosti, 2018), a clear adverse outcome pathway of the toxicant needs to be proven. Thus, for flow cytometry to be adopted as a tool to assess the effects of metals to sperm (as a proxy for effects to fertilisation success) a better understanding is needed of how metals affect different cellular processes relevant to fertilisation.

4.4.2 The Acrosome Reaction

The results here show that dissolved metals (Zn and Cu) in seawater have the potential to prevent *G. caespitosa* sperm from undergoing the AR. At a concentration of 290 µg Zn L^{-1}, 95% of sperm were unable to undergo the AR. From the previous chapter, at 10^5 sperm mL^{-1}, a 95% reduction in viable sperm would cause a 50% effect on fertilisation success as the relationship between effects to sperm and effects to fertilisation is nonlinear. To help establish a causal relationship, we would expect the EC95 of sperm endpoints to be the same as the EC50 for fertilisation success at 10^5 sperm mL^{-1}. There was no significant difference between the EC95 for the AR and the EC50 for fertilisation success at this sperm density. Therefore, the effects of Zn to

fertilisation success can be explained by a prevention of the AR. Whilst elevated concentrations of Cu also prevented sperm from undergoing the AR, this occurred at much higher concentrations than effects on fertilisation success. Thus, other effects must be responsible for the effects to fertilisation.

Zhang *et al.*, (2010) studied the effects of metals on AR of the mud Crab (*Scylla serrata*) sperm using microscopy. They found the order of toxicity to be Cd > Zn > Cu. Interestingly, no effects of Cd to the acrosome reaction were observed in *G. caespitosa* sperm. However, it was observed that Zn was a greater inhibitor of the acrosome reaction than Cu. Effect concentration (EC50) estimates for S. serrata were 2.21 and 13.69µg L^{-1} for Zn and Cu respectively (nominal). Thus *S. serrata* sperm yielded much greater sensitivities to metal toxicity than *G. caespitosa*. Zhang et al., (2010) also compared their results to toxicity measured using larval development as an endpoint. Whilst the AR was a more sensitive endpoint than larval development, it is not possible to determine if the effects of these metals to the acrosome reaction account for the decreases in larval development in mud crabs via a sperm-limitation effect on fertilisation success, as seen with *G. caespitosa*; because crabs are internally fertilising, fertilisation success could not be measured in Zhang et al.'s study. Thus, begging the question as to whether the AR is an ecologically relevant endpoint for internally fertilising species as sperm would not be directly exposed to the toxicant.

Induction of the acrosome reaction requires extracellular Ca^{2+} (Collins and Epel, 1977), and it has been suggested that stimuli such as glycoproteins on the surface of the egg, or within egg jelly trigger an influx of Ca^{2+} (Sato and Osanai, 1990; Hoshi *et al.*, 1994). The requirement of calcium for the induction of the acrosome reaction can be supported here. The calcium ionophore A23187, which transports Ca^{2+} across cell membranes, was the most successful initiator of the acrosome reaction in control treatments (i.e. seawater treatments without added metals). Assuming, that an influx of Ca^{2+} is an essential requirement for the induction of the acrosome reaction, elevated concentrations of Zn and Cu may disrupt the transfer of Ca^{2+} into the cell. Ca^{2+} is transported across cell membranes through voltage-dependent and ligand-gated Ca^{2+} channels (Noh *et al.*, 2015). It has previously been suggested that Zn can replace Ca^{2+} in the binding sites of numerous transport proteins (Csermely *et al.*, 1989), suppress high-voltage dependent activated-Ca^{2+} channels (Bertolo, Bettger and Atkinson, 2001; Turan, 2003; Alvarez-Collazo *et al.*, 2012) and act as a competitive inhibitor for Ca^{2+} permeation (Bertolo, Bettger and Atkinson, 2001). High concentrations of Cu have also been shown to interfere with calcium channels and disrupt calcium homeostasis (Schulte, Miiller and Friedberg, 1995; Horning, Blakemore and Trombley, 2000). Thus, it is suggested that Zn and Cu prevent the influx of Ca^{2+} and subsequently the initiation of the AR in *G. caespitosa* sperm. However, toxic effects of Cu on fertilisation are observed at significantly lower concentrations than those on the AR.

4.4.2 Mitochondrial Membrane Potential

In the previous chapter, I hypothesized that Cu interferes with mitochondrial activity, thus preventing ATP production and hindering motility (and subsequently decreasing fertilisation success). However, in this chapter Zn, Cu and Cd all increased mitochondrial membrane potential at concentrations that correspond with effects on fertilisation success (Cu, Zn) or lower (Cd). Therefore, suggesting that these metals have a stimulatory effect on mitochondrial function at the concentrations tested. Increases in ΔΨm occurred at ~7µg Cu L^{-1}, 28µg Zn L^{-1} and 1,200µg Cd L^{-1}. For Cd and Zn an increase in ΔΨm may explain the increases in the sperm motility at these concentrations (previous chapter;(Paoli *et al.*, 2011)). Mitochondria actively accumulate Zn(Byczkowski and Sorenson, 1984) and Cd(Martel, Marion and Denizeau, 1990). At low concentrations, Zn and Cd have previously been found to stimulate mitochondrial respiration(Byczkowski and Sorenson, 1984) and activate mitochondrial function(Yamaguchi, Masatsugu and Shoji, 1982) . A marked elevation in ATP concentration and a corresponding increase in ATPase activity was observed in mitochondria of rat liver exposed to Zn (Yamaguchi, Masatsugu and Shoji, 1982). It has been suggested that Zn stimulates the electron transport system, and oxidative phosphorylation and as a result increases ATP concentration(Yamaguchi, Masatsugu and Shoji, 1982). The activation of mitochondrial respiration by Zn and Cd would explain the results in this chapter, as the increase in ΔΨm corresponds with increased sperm motility.

However, whilst ΔΨm increased with exposure to Cu, both motility and fertilisation success decrease. Copper is rapidly accumulated by the mitchondria and at low concentrations can increase mitochondrial respiration (Byczkowski and Sorenson, 1984), which may explain the increases in ΔΨm observed here and the low dose stimulation observed in motility (previous chapter). However, once taken up into the cell Cu can initiate the generation of Reactive Oxygen Species (ROS)(Aitken *et al.*, 2012). An increase in mitochondrial ROS generation does not involve a primary loss of mitochondrial membrane potential (Koppers *et al.*, 2008; Aitken *et al.*, 2012), which may explain why we do not see a decrease in mitochondrial membrane potential here. Reactive oxygen species production by the mitochondria, however, can cause sperm immobilization (Koppers *et al.*, 2008) and decreased axonemal function(Lamirande and Gagnon, 1992) which might then explain the decreases observed in sperm motility. Similarly, Cd and Pb can stimulate ROS production in mitochondria, however we did not see effects to motility at concentrations that exert a negative effect on fertilisation success. In this case, we hypothesize that Cd and Pb may either damage DNA, both directly (Acosta *et al.*, 2016) or through ROS production (Opuwari and Henkel, 2016), or disrupt egg activation.

4.5 Conclusion

This study developed a fast, reliable method for assessing acrosomal integrity and mitochondrial function using flow cytometry in combination with FITC-PNA and TMRM. Whilst flow cytometry shows promise as a useful tool in ecotoxicology, the effects of metals to AI and $\Delta\Psi m$ were not representative of the effects to fertilisation success. Only Zn was found to be an effective inhibitor of the acrosome reaction at concentrations that correspond with effects to fertilisation success. Future research should investigate sperm ROS production in response to metals.

CHAPTER 5

General Discussion: Marine Invertebrate Sperm as an Indicator of Metal Toxicity

5.1 Introduction

Many marine invertebrates reproduce through broadcast spawning, where sperm and eggs are released into the water column and are vulnerable to toxicants present in the environment. The potential impacts of toxicants on spawning success are often assessed through laboratory-based fertilisation tests. A review of the literature surrounding the effects of metals to fertilisation success in marine invertebrates indicated that sperm were sensitive to metal toxicity. Thus, marine invertebrate sperm show potential for the development of rapid endpoints that can be used to assess the effects of metals to fertilisation success. However, the ecological relevance, reliability and efficacy of such endpoints, in many cases has not yet been demonstrated. The primary aim of this research then, was to determine whether marine invertebrate sperm could be used as an indicator of metal toxicity to fertilisation success and, if so, to determine how toxicity data from sperm endpoints can be used to inform guideline derivation and ecological risk assessment.

To answer this question, I start by identifying the key events that lead to decreased fertilisation success in *G. caespitosa* when exposed to metals (Cu, Zn, Cd and Pb), determining effective and appropriate methods for the assessment of sperm function and viability and discussing how sperm toxicity data can be interpreted with regards to fertilisation success. This chapter will summarise the findings of this research and will present a framework that

provides more appropriate toxicity data for fertilisation success in marine invertebrates than the current approaches to toxicity testing.

5.2 Summary of Research

Metal toxicity is commonly assessed in marine invertebrates and algae using fertilisation success as an endpoint, which has a direct link to recruitment and effects at the population level (Shea, 2004; Hudspith, Reichelt-Brushett and Harrison, 2017). Standardised protocols for the assessment of toxicant effects to fertilisation success have been developed (USEPA, 1995; Simon and Laginestra, 1997; United States Environmental Protection Agency, 2002) and are used to evaluate toxicant effects to coastal marine waters globally. These methods use a pre-determined single sperm density that ensures 70-100% fertilisation success in a filtered seawater (FSW) control. However, using this approach does not provide any information regarding the mechanisms of toxicity and, theoretically, could underestimate toxicity if effects of a toxicant are to sperm. For sperm to be used as an indicator of effects on fertilisation success, it was necessary to determine whether the effects of each metal (Cu, Zn, Cd and Pb) were on sperm function(s), egg or larval viability, polyspermy blocks or a combination of the processes involved in fertilisation and early larval development. In Chapter 2, I identified the mechanism of metal toxicity to fertilisation success by conducting assays across multiple sperm densities and establishing a fertilisation model for varying concentrations of the toxicant. When sperm were exposed to dissolved metals the fertilisation models were shifted to the right, relative to a FSW control. Thus, greater

densities of sperm were required to achieve maximum fertilisation success when exposed to metals. No effects to egg/larval viability or polyspermy blocks were observed. Similar effects have been shown for other marine invertebrates, suggesting that sperm are generally sensitive to metal exposure. For example, effects of metals on sperm have been observed in the sea urchins *Anthocidaris crassispina* (Cd; Au *et al.*, 2001) and *Arbacia punctulata* (Cu, Zn, Mn, Hg; Young and Nelson, 1974), the mud crab *Scylla serrata* (Ag, Cd, Cu, Zn; Zhang *et al.*, 2010), the mussels *Mytilus trossulus* (Cu; Fitzpatrick *et al.*, 2008) and *Mytilus edulis* (Cu, Zn; Akberali, Earnshaw and Marriott, 1985) and the lugworm *Arenicola marina* (Cu; Campbell *et al.*, 2014). In contrast, unfertilised eggs of marine invertebrates appear to be relatively unaffected by metal exposure (Cam F Hollows, Johnston and Marshall, 2007; Fitzpatrick *et al.*, 2008; Gopalakrishnan, Thilagam and Raja, 2008).

By using the fertilisation model approach to toxicity testing it was possible to examine how the magnitude of the response varies among sperm densities. Threefold differences were observed in toxicity estimates for Cu and Zn at sperm densities that all achieved >80% fertilisation in controls (and could each have been used in tests, based on existing protocols). Thus, the resultant toxicity estimates were dependent upon sperm density, with lower effect concentrations found when tested at lower sperm densities. This study was the first study to determine toxicity data using the fertilisation model approach, and the first to confirm theoretical predictions and quantify the magnitude of the difference in toxicity estimates among tests conducted at different sperm

densities. The implications of this work are discussed later in this chapter (section 5..3.1).

As sperm of *G. caespitosa* and other marine invertebrates are sensitive to metal toxicity, ecotoxicological tests using endpoints based on sperm function as an indicator of the effects on fertilisation success could provide rapid alternatives to fertilisation assays. However, for these tests to be accepted for use in routine toxicity testing and water quality guideline derivation, ecological relevance needed to be established via a proven adverse outcome pathway (AOP; Groh *et al.*, 2015; Fay *et al.*, 2017; Knapen *et al.*, 2018). An AOP represents a series of key events that relate the initial toxic response in an organism (often at a molecular level), mechanistically, through to adverse outcomes at an ecologically relevant endpoint at the population level (e.g. reproduction, growth; Ankley *et al.*, 2010; Vinken *et al.*, 2017; see also figure 5.1).

The next step in Chapter 3 then, was to determine the key events that cause toxic effects on fertilisation success through sperm. As sperm motility is a process that is dependent upon the transfer of ions across the cell membrane, I hypothesised that an increase in external metal ion availability would disrupt ion homeostasis and subsequently sperm motility or velocity. Sperm of G.caespitosa were exposed to Cu, Zn, Cd and Pb and sperm swimming characteristics were analysed using CASA (Straightline velocity, Curvilinear Velocity and %Motility) and SAAS (collision rates). The data were analysed

semi-autonomously using macros that I developed using imageJ and its associated plugins. Interestingly, increases in the availability of Zn, Cd and Pb had stimulatory effects on sperm motility, at concentrations that elicit negative effects on fertilisation success. As such, sperm swimming was not an appropriate indicator of toxicity for these metals as increased motility ought to lead to more sperm-egg encounters in fertilisation assays and, at low sperm densities at least, greater rates of fertilisation (Styan, 1998). However, Cu inhibited sperm motility at concentrations that correspond with effects on fertilisation success. The effect of Cu on sperm was through effects on % motility and not velocity suggesting that exposure to elevated concentrations of Cu either prevents flagella movement or disrupts mitochondrial supply of ATP. This chapter also explored the relationship between effects on sperm and effects on fertilisation success and found the relationship to be nonlinear. Using fertilisation models for *G.caespitosa*, it was calculated that, at 10^5 sperm mL^{-1}, a 95% effect on sperm would only induce a 50% effect on fertilisation success, as there are still enough sperm to achieve 50% of maximum fertilisation. The EC95 for sperm motility and EC50 for fertilisation success were 19 and 20µg Cu L^{-1} respectively. There was no statistically significant difference between the two values. Whilst the effect of Cu on sperm motility can account for the effects of Cu on fertilisation success, this does not rule out effects of Cu to other fertilisation processes.

The effects of Zn, Cd and Pb to fertilisation success could not be explained by sperm motility, which led me to the next hypothesis that metals disrupt the

ability for sperm to undergo the acrosome reaction – a key process necessary for sperm to fuse with egg membranes during fertilisation. The acrosome reaction is dependent on the transfer of Ca^{2+} ions into the cell (Tosti and Ménézo, 2016) and thus may be disrupted by increased dissolved metals. I also hypothesised that the effects of Cu on sperm motility would be due to effects on the mitochondria, as mitochondria are the driving force for sperm motility and Cu has been reported to accumulate there. To identify how metals effect these functions, in Chapter 4, I developed methods for the assessment of the acrosome reaction (AR) and mitochondrial membrane potential ($\Delta\Psi m$) for *G. caespitosa* sperm using flow cytometry. Prior to using the AR and $\Delta\Psi m$ as endpoints in ecotoxicological tests the conditions for initiating and staining the AR and staining for $\Delta\Psi m$ in *G. caespitosa* had to be determined and optimised. The calcium ionophore A23187 was the most successful initiator of the AR, which was most suitably labelled using peanut agglutinin (PNA). Mitochondrial membrane potential was successfully labelled with tetramethylrhodamine, methyl esther (TMRM). Sperm were exposed to metals and subsequently exposed to A23187 to initiate the AR and labelled with PNA. For $\Delta\Psi m$ experiments, sperm were exposed to metals and labelled with TMRM. Exposure of sperm to Zn inhibited the AR at concentrations that correspond with effects on fertilisation success. Copper prevented sperm from undergoing the acrosome reaction, but at concentrations that exceed effects on fertilisation success. However, for Cd and Pb there was no effect on the ability of sperm to undergo the AR. Mitochondrial membrane potential increased (rather decreased) when exposed to elevated concentrations Cu, Zn

and Cd and no effect on ΔΨm was observed when sperm were exposed to Pb. Therefore, effects on mitochondria could not explain the decreases observed in motility from exposure to Cu. This led to the working hypothesis that increased metals may increase the production of reactive oxygen species (ROS) which can damage cellular ultrastructures within the sperm, with Cu particularly strong as an inducer of ROS (Aitken *et al.*, 2012) and thus leading to the loss of motility at relatively low Cu concentrations. This hypothesis remains untested and would require further investigation to determine whether increased ROS production, leading to loss of sperm motility, can account for the observed decrease in motility of G. caespitosa sperm.

Overall, sperm of *G. caespitosa* are sensitive to metal toxicity and could be used as a rapid indicator of effects on fertilisation success. This research aimed to establish AOPs for the Metals Cu, Zn, Cd and Pb. Interestingly, the pathways of toxicity were different for each metal (Table 5.1), thus there is no specific method that could be used to determine metal toxicity generally. Figure 5.2 represents the AOPs developed for fertilisation success in G.caespitosa when sperm were exposed to Zn and Cu.

The effects of Cu, Zn, Cd and Pb on sperm, have been summarised in Table 5.1. Exposure to elevated concentrations of Cu inhibits sperm motility, preventing sperm from locating and successfully fertilising an egg. This effect is likely through the production of ROS that damage ultracellular structures (Chapter 4). There were also effects of Cu on the ability for sperm to undergo the AR,

but these effects were at concentrations of Cu that exceed effects on fertilisation success. However, exposure to elevated concentrations of Zn prevented sperm from undergoing the AR, stopping sperm from fusing to the egg and transferring paternal genetic material. It is likely that Zn blocks Ca^{2+} channels, preventing the influx of Ca^{2+} essential for the AR (Figure 5.1). For Cd and Pb, we were not able to identify the mechanism of toxicity to fertilisation success. We observed increases of sperm motility (Cd and Pb) and mitochondrial activity (Cd), with no evidence of polyspermy. Neither Cd or Pb had inhibited the acrosome reaction in G.caespitosa sperm. Thus, suggesting that these metals may either damage DNA and subsequently preventing successful fertilisation, or interfere with sperm induced egg activation. Similarly, Cu and Zn may also affect other mechanisms that were not tested here. Whilst sperm motility can account for the observed effects on fertilisation success, there may be mixed effects of Cu occurring simultaneously (i.e reductions in motility accompanied by small reductions in AR). Correlations between effects on motility and effects on fertilisation success, do not rule out the effects of Cu on other sperm functions. Thus, while motility can be used to indicate the effects of Cu to fertilisation, the AOP framework may oversimplify the complexity of Cu interactions with fertilisation success. However, these linkages can provide a critical foundation for the use of predictive approaches, such as flow cytometry, in ecotoxicology and ecological risk assessment (Ankley et al., 2010; Connon, Geist and Werner, 2012).

Table 5.1a: A summary of the mechanisms of Cu, Zn, Cd and Pb toxicity to sperm.

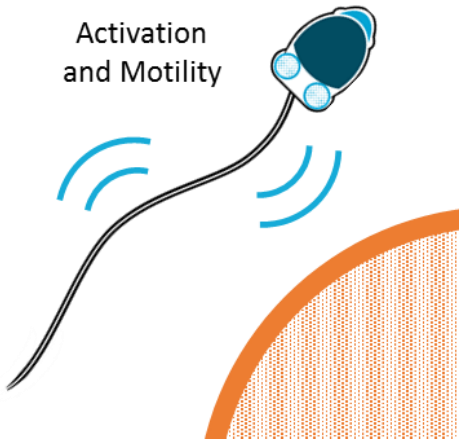
Metal	Mechanism of Toxicity	Description
Copper		Exposure to elevated concentrations of Cu inhibited sperm motility, preventing sperm from locating and successfully fertilising an egg. In this study decreased motility (%) occurred at concentrations that elicit negative effects on fertilisation success. When exposed to copper there were significant increases in mitochondrial activity, and so this does not explain the reductions in sperm motility. Reductions in motility are likely due to the ability of copper to induce oxidative stress, either through the direct formation of ROS, or by decreasing antioxidant levels. ROS within the sperm cell can then damage ultracellular structures and inhibit enzyme activity (ATPase).

Table 5.1b: A summary of the mechanisms of Cu, Zn, Cd and Pb toxicity to sperm.

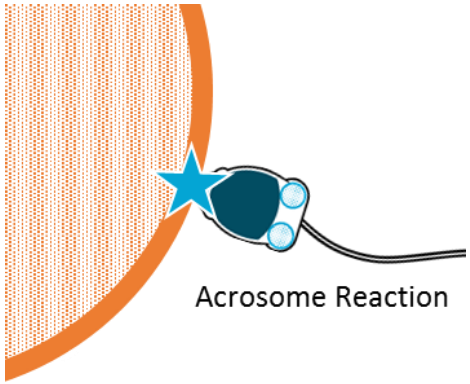
Metal	Mechanism of Toxicity	Description
Zinc		Zn prevented sperm from undergoing the AR, stopping sperm from fusing to the egg and transferring paternal genetic material. As Zn is a redox inert metal and does not participate in oxidation-reduction reactions, it is most likely that the mechanism of Zn toxicity is through ionic mimicry. Zn^{2+} can replace Ca^{2+} ions which can cause significant changes in biological process. It is likely that Zn blocks Ca^{2+} channels or replaces Ca^{2+} uptake into the cell This would then prevent the influx of Ca^{2+} that is essential for the AR.

Table 5.1c: A summary of the mechanisms of Cu, Zn, Cd and Pb toxicity to sperm

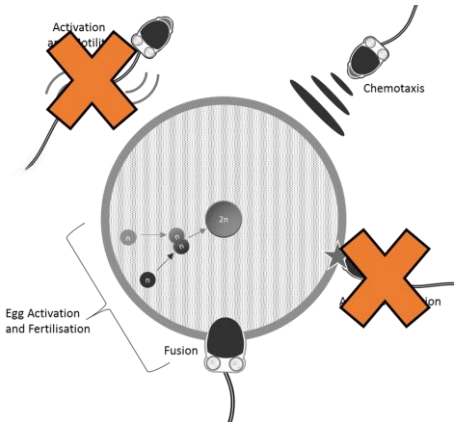
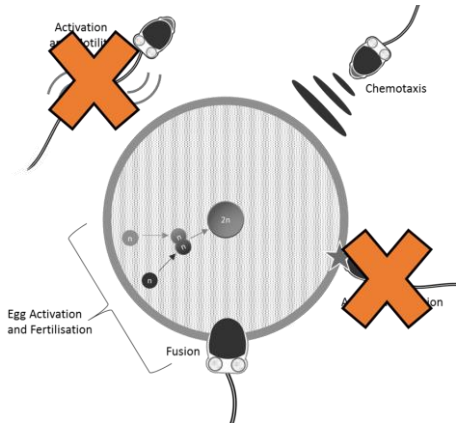
Metal	Mechanism of Toxicity	Description
Cadmium		This study did not successfully identify the mechanism of Cd toxicity to fertilisation success. Increases in sperm motility and mitochondrial activity were observed with no evidence of polyspermy. Cadmium did not inhibit the acrosome reaction in G.caespitosa sperm. Cadmium itself is unable to generate free radicals directly, however, it can indirectly result in the formation of ROS. ROS within the sperm cell can potentially damage DNA and subsequently prevent successful fertilisation, or disrupt the chemical processes involved in sperm induced egg activation. These hypotheses require further research.

Table 5.1d: A summary of the mechanisms of Cu, Zn, Cd and Pb toxicity to sperm

Metal	Mechanism of Toxicity	Description
Lead		This study did not successfully identify the mechanism of Pb toxicity to fertilisation success. Like Cd, ROS-induced damage by lead could be a cause of toxicity to fertilisation success. Lead can directly form ROS and deplete antioxidants in the cell causing oxidative stress. Whilst the effects of ROS generation did not manifest in sperm swimming or the acrosome reaction, the effects on sperm may be through other biological processes essential for fertilisation success such as the transfer of paternal genetic material, chemotaxis or sperm induced egg activation. Lead can also replace other ions like Ca^{2+}, Mg^{2+}, Fe^{2+} and Na+. The replacement of these ions with Pb could cause significant changes in various biological processes such as intra and inter-cellular signalling, protein folding, maturation, apoptosis, ionic transportation and enzyme regulation.

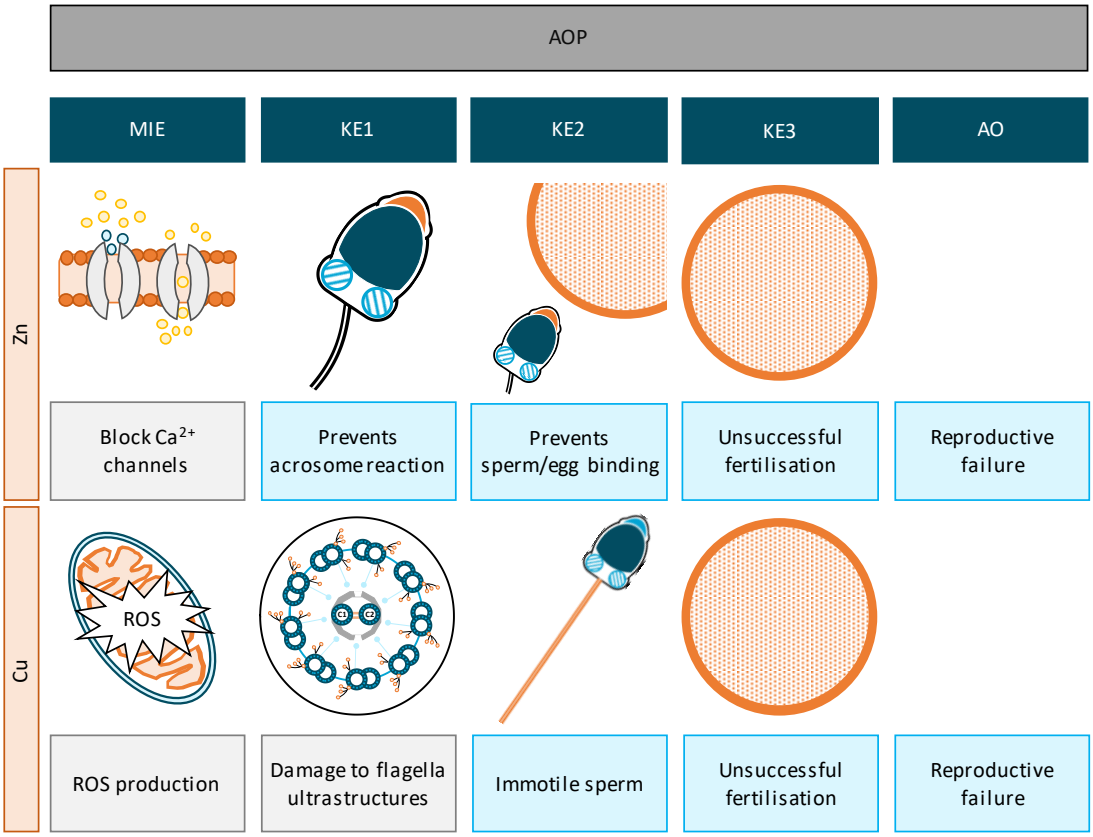


Figure 5.1: Adverse outcome pathways (AOP) for reproduction in *G. caespitosa* when sperm were exposed to Zn and Copper. The AOPs start with a molecular initiating event (MIE) that triggers key events (KE) that lead to an Adverse Outcome (AO). Events in grey are hypothetical (See chapter 4), Events in blue are measured and established in previous chapters (Chapters 2,3,4).

5.3 Implications of Research

The results of this research have implications for the assessment for metal toxicity to fertilisation success and are used to inform the following recommendations that should be considered for routine toxicity testing.

5.3.1 Importance of toxicity pathways in fertilisation assays

Fertilisation endpoints are often adopted as a tool for ecotoxicological assessment, with little regard for how a chemical is toxic to fertilisation processes. One of the key implications of this research is that the mechanism of toxicity is important and should be considered, particularly as the resultant toxicity data may be dependent upon this. Using fertilisation assays across multiple sperm densities provides this information and would give a more complete and ecologically relevant assessment of toxicity than standard tests. However, it is recognised that there may be situations in which this approach may be impractical in routine ecotoxicological testing, because of the time or costs involved, or because regulatory standards refer to simpler, standardised methodologies (Dinnel, Link and Stober, 1987; USEPA, 1995; Simon and Laginestra, 1997). Nonetheless, there are modifications to standard fertilisation tests that could be made based on this work.

Firstly, it is recommended that during a pre-test to determine an appropriate sperm density (sperm:egg ratio), one moderate level of the toxicant should be tested simultaneously across a range of sperm:egg ratios - similar to Lewis et al. (2008) who exposed the sperm of *Neries virens* to water-accommodated

fractions of crude oil across sperm densities ranging from 10^3 to 10^8 sperm mL^{-1}. This can provide some insight into the aspects of fertilisation that are likely to be affected, provided the concentration of the toxicant chosen provides some discernible impact on fertilisation (but not so much as to prevent it altogether).

Secondly, information about the likely mechanism of toxicity should help set the target fertilisation rate in FSW controls and the sperm density that will be used for subsequent toxicity tests. Where there is an indication that the toxicant affects sperm, a more sensitive test would be obtained by reducing the sperm density to one that achieves 50% fertilisation success in FSW controls; even lower fertilisation rates might be as, or even more sensitive, but a reasonable number of fertilisations are needed in controls to enable comparisons. Alternatively, if the main impact appears to be on egg/larval viability, then a greater sperm density more likely to achieve near 100% fertilisation in FSW controls would maximise the precision with which decreases in fertilisation can be measured. If a toxicant appears to affect polyspermy blocks, then a greater sperm density again will be needed to measure effects. Finally, if more than one of these effects are expected then a fertilisation assay approach will be needed, like the approach used in Chapter 2.

5.3.2 Importance of toxicity pathways for adoption of sperm endpoints

If sperm endpoints are to be used as an indicator of metal toxicity to fertilisation success, then these should be informed by a credible adverse outcome pathway. Predictive approaches, using sperm endpoints, are commonly adopted (Adams, Hessian and Mladenov, 2003; Gravance *et al.*, 2003; Favret and Lynn, 2010; Jenkins *et al.*, 2015; Kekäläinen *et al.*, 2015; Peña *et al.*, 2018) and sometimes recommended as rapid alternatives to fertilisation success (Gallo, Boni and Tosti, 2018) with little regard for the mechanisms of a toxicant. As such, these tests may not reliably represent the effects of the toxicant and can under - or over-estimate the effects to fertilisation success. A variety of tests have been developed to look at sperm functionality and viability including; computer assisted sperm analysis (CASA; Wilson-Leedy and Ingermann, 2007; Fabbrocini, Di Stasio and D'Adamo, 2010; Boryshpolets *et al.*, 2013; van der Horst, Bennett and Bishop, 2018), Sperm accumulation against surfaces (SAAS; Falkenberg, Havenhand and Styan, 2016), Flow cytometry (Binet *et al.*, 2014; Fallis *et al.*, 2014; Kekäläinen *et al.*, 2015; Peña *et al.*, 2018), Comet assays (Lewis and Galloway, 2008, 2009; Lacaze *et al.*, 2011; Barranger *et al.*, 2014) and microscopy (Pillai *et al.*, 1997; Zhang *et al.*, 2010; Lisa *et al.*, 2013). However, the effectiveness and relevance of these methods are dependent on the AOP of the toxicant. In this study, whilst CASA was an effective indicator of effects to fertilisation success for Cu, this was not the case for Zn, Cd and Pb. Zinc is an example here where sperm viability would not be a relevant endpoint as sperm were unable to undergo the acrosome reaction but they remained viable. In such cases, the toxicity of a chemical can

be misrepresented. Sperm endpoints can only be an effective indicator of effects to fertilisation success if a credible AOP has been established. Once an AOP has been identified, sperm endpoints can provide promising tools for routine ecotoxicological assessments as predictors of effects to fertilisation success.

5.3.3 Use of flow cytometry as a rapid ecotoxicological tool

Flow cytometric methods have the potential to provide rapid, sensitive and predictive tools for the assessment of sperm function. This research successfully developed methods for the analysis of acrosomal integrity and mitochondrial membrane potential in *G. caespitosa* sperm. Whilst the effects of Cu on fertilisation success could not be identified through staining for mitochondrial membrane potential and acrosomal integrity, we hypothesise that this might be identified in future work using flow cytometry and staining for ROS production (Figure 5.1; Chapter 4). As new stains are developed and the capacity to simultaneously assess multiple stains increases, the capabilities of flow cytometry continue to grow. It is not unreasonable to imagine a suite of stains used to identify various functional aspects of sperm that, when run concurrently, provide a holistic view of toxicant impacts to different sperm processes.

One major criticism of the AOP framework is the oversimplification of both the complexity of biological systems and the consequences of exposures to a toxicant (Vinken *et al.*, 2017). It is unlikely that sperm are exposed to a single

toxicant at any given time, more likely to multiple toxicants simultaneously (i.e. mixtures). Even if sperm were exposed to a single toxicant, this may induce toxicity by more than one mechanism (Knapen *et al.*, 2018) as demonstrated for Cu here. Flow cytometric methods, with multiple stain conditions, could provide insight into the various mechanisms of toxicity of both individual chemicals and mixtures. This makes flow cytometric analysis particularly applicable to toxicity identification evaluation (TIE). The TIE approach developed by the US Environmental Protection Agency (EPA; United States Environmental Protection Agency, 1992) aims to detect and identify the toxic agents of a mixture or effluent. The method combines chemical and physical techniques with the response of test organisms to identify the nature of the toxicants (Isidori *et al.*, 2003). With complex mixtures such as effluents, knowing the cause of toxicity is a key requirement for effective management. This information can ensure that targeted and cost-effective means are found to control the toxicity of an effluent. As an example; the smelter at Port Pirie, South Australia continues to discharge high quantities of Zn, Cd, Pb and other metals into the upper Spencer Gulf (Corbin and Wade, 2004). If sperm were exposed to the effluent at Port Pirie and were unable to undergo the acrosome reaction, but had no difference in ROS production, mitochondrial membrane potential and viability in comparison to a seawater control, we might infer that the cause of reproductive toxicity would be elevated concentrations of Zn and this could be managed accordingly.

5.3.4 Grounding sperm toxicity data to natural scenarios

Whilst flow cytometric methods are a promising tool for toxicity assessment, the data derived from sperm endpoints need to be directly related to effects on fertilisation success. Currently, the relationship between effects on sperm and effects on fertilisation success are not considered when deriving toxicity estimates from sperm endpoints (Favret and Lynn, 2010; Akcha, Spagnol and Rouxel, 2012; Volety *et al.*, 2016). Understanding that relationship requires a fertilisation model for the test species, which should be acquired during the pre-test for fertilisation assays. Using the fertilisation model developed for *G. caespitosa*, this research has highlighted that effects on sperm are nonlinear to effects on fertilisation success and are dependent upon sperm density. Thus, understanding this relationship is important for scaling toxicity estimates based on sperm to toxicity estimates for fertilisation success. For example, at 10^5 sperm mL^{-1}, a >95% effect on sperm was required to induce a 50% effect on fertilisation success. At greater sperm densities (e.g. 10^7 sperm mL^{-1}), > 99.9% of sperm would need to be affected to have the same 50% decrease on fertilisation. But at lower sperm densities (10^3 sperm mL^{-1}) the effect is closer to parity, with a 50% decrease in fertilisation potentially caused by a smaller (55%) decrease in the number of effective sperm. This then raises the question: what is the appropriate sperm density (or sperm:egg ratio) to base toxicity estimates on?

Theoretically, in populations where sperm abundance is not a limiting factor on fertilisation success, toxicant induced reductions in sperm may not

influence fertilisation as there will still be enough functioning sperm to achieve high levels of fertilisation (provided toxicants do not kill/inactivate 100% of sperm). Currently, *G. caespitosa* at Grange Beach, South Australia (Figure 5.2) are residing in an area in which dissolved metal contamination in seawater can be in excess of effect concentrations for fertilisation at low sperm densities (Gaylard, 2004). Maximum concentrations of Zn at Grange have been recorded to be up to 275 μg L^{-1} (Gaylard, 2004). Effect concentrations (10, 50%) for fertilisation success at 10^4 sperm mL^{-1} were 68 and 160 μg Zn L^{-1} (respectively). Thus, it is plausible that *G. caespitosa* in this area may have adapted to increase sperm densities either through highly synchronous spawning or by producing greater numbers of sperm (Lewis and Galloway, 2010). It may be that *G.caespitosa* are in such high-density populations that the number of individuals spawning produce sperm densities that exceed the ones tested here, and so effect concentrations would likely be much greater. Another explanation as to why *G. caespitosa* are able to reside in an area of metal contamination that exceed effects on fertilisation success, may be that they are reproductively affected at Grange, but are recruiting larvae from elsewhere. *Galeolaria caespitosa* can be found at almost all Adelaide metropolitan beaches, and thus the population at Grange may be part of an open population within the Adelaide region. However, the majority of metropolitan coastal waters within the Gulf St Vincent were contaminated with elevated concentrations of Zn (Gaylard, 2004), and so similar effects on reproduction would be expected. Thus, it is anticipated that sperm densities during *G. caespitosa* spawning, in the Adelaide region, are high. In such

scenarios, fertilisation tests using standard protocols, or high densities, may be appropriate. However, population dynamics in many broadcast spawning marine organisms are thought to be sperm limited (Levitan, 1998; Lewis, Pook and Galloway, 2008). In sperm limited environments the effect of the toxicant would become much more potent. In such environments, effect concentrations derived using predictive sperm endpoints would provide the most sensitive and precautionary toxicity data. However, if the population in question is not sperm limited toxicity data derived using sperm endpoints could be considered too conservative. When comparing the effect concentrations (50%) for Zn based on acrosomal integrity and fertilisation success at 10^6 sperm mL^{-1} we observe five-fold differences in toxicity. Thus, if natural spawning densities are high, sperm endpoints could significantly over-estimate the effect of a toxicant to fertilisation success. Overestimating the effects of a toxicant to fertilisation success could potentially result in unrealistic or unachievable water quality guidelines.

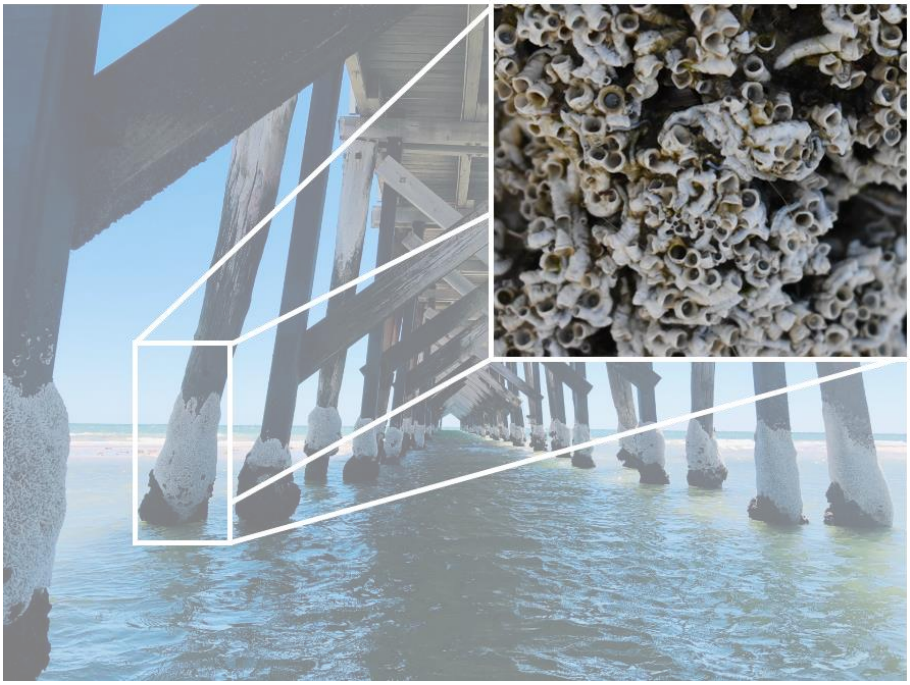


Figure 5.1: *Galeolaria caespitosa* on the Jetty Pilings at Grange Beach, South Australia.

Ideally, effect concentrations derived from sperm endpoints accompanied with an understanding of the fertilisation dynamics for a given species could be used in an ecological risk assessment process to determine the risk to reproduction based on local population or spawning densities. This way the likelihood that adverse ecological effects may occur as a result of exposure could be appropriately identified on a site-specific basis. Whilst site specific ecotoxicological data may be ideal, this is not typically the approach adopted in ecological risk assessment (ERA). Species sensitivity distributions (SSDs) are increasingly used in ecological risk assessment procedures (Solomon *et al.*, 1996; Steen *et al.*, 1999; Wheeler *et al.*, 2002) to determine a general toxicity threshold that is considered protective of ecosystem structure and functioning (Belanger *et al.*, 2017). SSDs are used to set benchmark or threshold criteria that aim to protect a given percentage of species (99%, 95%, 90%, 80%;

ANZECC and ARMCANZ, 2000). Thresholds (95%) are used to set guideline values that are deemed acceptable concentrations for marine community conservation (Belanger *et al.*, 2017). Toxicity tests using fertilisation as an endpoint are commonly incorporated into the development of SSDs for metals (Gadd and Hickey, 2016) where toxicity estimates could underestimate the potency of a toxicant. Toxicity information based on fertilisation success that is used in SSD's should require a better understanding of the natural spawning densities of marine invertebrates, as toxicity estimates are dependent upon this. For some marine invertebrates, such as the corals, *Montipora digitata* and *Goniastrea favulus,* where fertilisation rates in situ have been recorded at >80% (Babcock and Oliver, 1992; Miller and Mundy, 2005) sperm:egg ratios that mimic these success rates may be appropriate and as such toxicity data can be derived for these densities. However, for many species fertilisation success rates *in situ* are considerably lower and greatly variable (Coma and Lasker, 1997; Williams, Bentley and Hardege, 1997; Levitan, 1998). Where spawning densities are variable or unknown, and considering that the aim of an SSD is to ensure species protection, then sperm endpoints with a proven AOP could provide more sensitive and precautionary measures than fertilisation success when a toxicants impact is to sperm.

5.3.5 Assessing toxicity to fertilisation success in marine invertebrates

As discussed, this research has highlighted some limitations to the current approach to toxicity tests using fertilisation as an endpoint and provides recommendations for conducting routine ecotoxicological assessments. These

recommendations have been developed into a framework for the assessment of a toxicant to fertilisation success, that will ensure toxicity estimates are well informed, appropriate and representative of the effect (Figure 5.3). When assessing the effects of a toxicant to fertilisation success, the mechanism of toxicity should first be identified, as the resultant toxicity data are dependent upon this. This information should be used to inform fertilisation test methodologies. If the impact of a toxicant is to sperm, sperm endpoints could be used as an indicator of effects on fertilisation success provided a credible adverse outcome pathway can be proven. This should inform suitable test methodologies that are representative of the effect of the toxicant. Toxicity estimates from sperm endpoints should then be scaled to effects on fertilisation success based on known natural spawning densities. Where natural spawning densities are unknown, sperm endpoints and fertilisation dynamics models for a species, may provide conservative estimates of toxicity.

5.5 Limitations and Future Research

This research was solely based on the effects of metals to reproduction in *G. caespitosa*. *Galeolaria caespitosa* represents a model species for use in ecotoxicological studies as they are easily collected, amenable to laboratory holding and easy to spawn. Whilst the literature indicates that sperm of many species of marine invertebrates are often similarly sensitive to metal toxicity, the findings of this research obviously cannot be generalised to encompass the effects of metals on sperm of all marine invertebrates. The effects of metals to fertilisation in other marine invertebrates should be investigated further as

this will have consequences for the interpretation of metal toxicity data. As well as testing the relative toxicity among metals in other species (i.e. are EC50 for other species Cu<Zn<Pb<Cd), future work could also test for metal specific effects on different aspects of fertilisation (i.e. Cd and Pb and ROS production).

This research provides thorough estimates of toxicity to fertilisation in *G.caespitosa* for the metals Cu, Zn, Cd and Pb, but all in isolation. However, it is very unlikely that an organism will be exposed to these contaminants individually but more likely will be exposed to a number of contaminants simultaneously. In such cases, metals have the potential to have either synergistic or antagonistic effects (Preston *et al.*, 2000). Whilst the data reported here are useful for informing species sensitivity distributions and developing water quality guidelines for specific metals, they may not accurately represent the effects of metals in a real scenario, where the polluting activity releases a cocktail of metals (and possibly other contaminants) into the environment. The combined effects of metals on sperm function and fertilisation success is an area that needs further investigation.

Toxicity data derived from fertilisation assays are dependent upon sperm:egg ratios, where toxicity estimates can differ significantly between sperm densities. Whilst natural spawning densities are unknown, toxicity estimates could either underestimate or overestimate the potency of a toxicant. Ground

truthing natural spawning densities and understanding the fertilisation dynamics of toxicity test species would provide the most appropriate toxicity data.

Flow cytometric sperm analysis shows promise as a rapid, sensitive and accurate predictor of toxicant effects to fertilisation success. However, more research needs to be conducted to determine appropriate stain conditions for identifying multiple effects simultaneously.

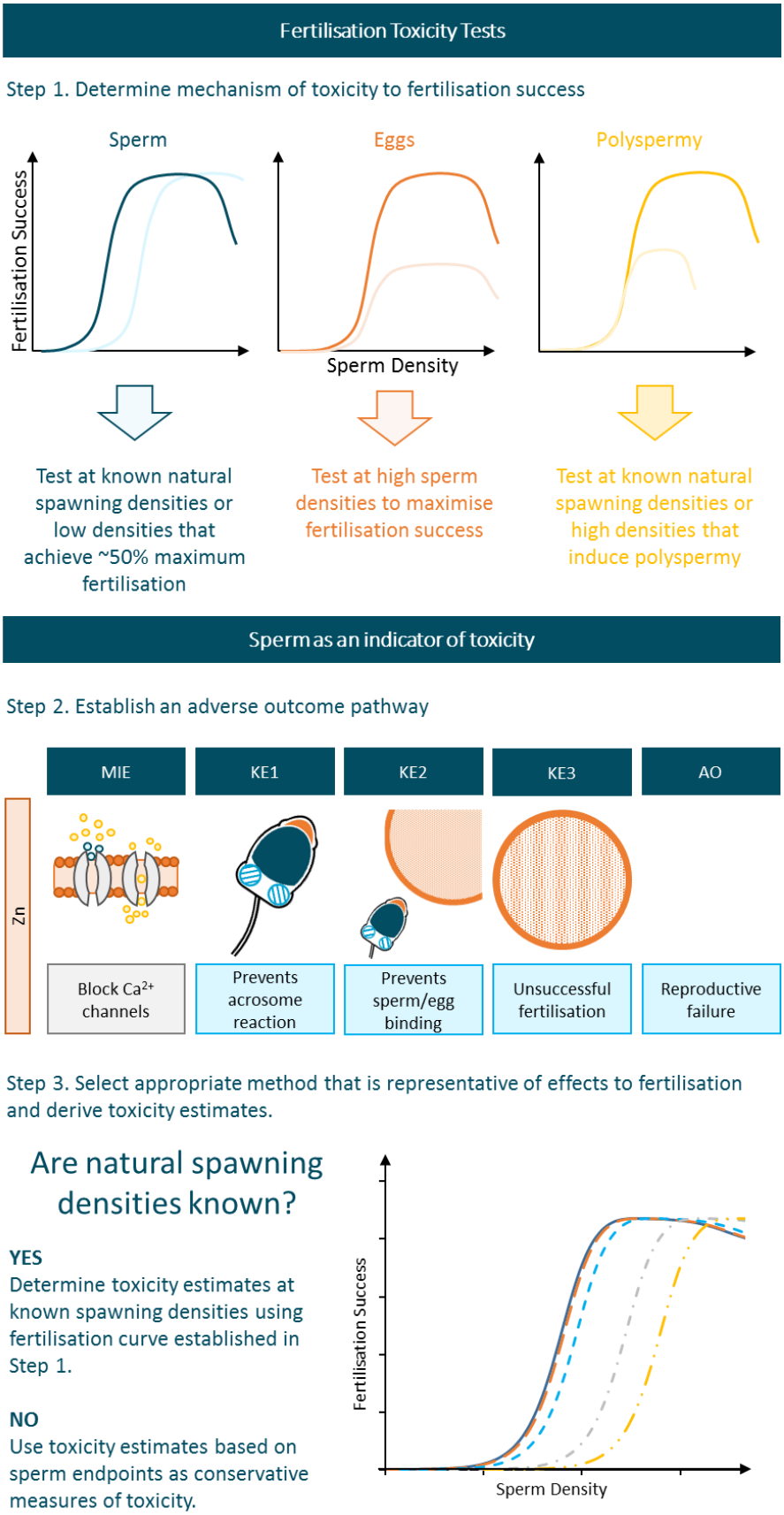


Figure 5.2 : A framework for the assessing the toxicity of a chemical to fertilisation success.

5.6 Concluding Remarks

Anthropogenic activities have led to increased metal contamination of coastal and marine environments over recent years, resulting in elevated concentrations that can be toxic to marine organisms. Fertilisation success has been widely used to assess metal toxicity as it can be directly related to effects on a population level and is an important method of evaluating the toxicity of contaminants to inform water quality guidelines and environmental management. This research shows that the primary cause of toxicity of metals to fertilisation success in one species of marine invertebrate (a common intertidal polychaete) is through effects on sperm. When the impact of a toxicant is to sperm, current protocols for fertilisation assays could underestimate toxicity. More appropriate toxicity estimates would be achieved by assessing toxicity using low sperm densities, for example, those which achieve only 50% fertilisation success. Alternatively, sperm endpoints could be used to provide sensitive (and conservative) toxicity estimates, provided an adverse outcome pathway can be established. For Cu and Zn, sperm motility and acrosomal integrity (respectively) can be used to assess toxicity as rapid indicators of effects to fertilisation success. However, For Cd and Pb, the effect on fertilisation success could not be appropriately represented by any of the sperm endpoints tested here. This research highlights the importance of understanding the mechanism of toxicity to fertilisation success and that this should inform future ecotoxicological assessments.

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Appendices

Appendix 1: Supplementary Information for Chapter 2

Table A1.1. Measured dissolved (<0.45 μm) metal concentrations in controls and treatments (μg L^{-1})

CU		ZN		PB		CD	
Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
1.7	5.6	19	19	23	240	1.7	54
1.2	9.3	17	160	<4	650	<1	2200
2.0	11	16	330	16	920	4.1	6140
1.2	11	44	930	<4	970	1.5	8280
1.3	13	41	1300	<4	1300	5.4	16000
1.3	15	19	1900	<4	2100	11.2	86900
1.3	26	54	2600	<4	19800	<1	188000
1.2	38	66	4000				

Table A1.2: Approximate sperm concentrations (2SF) and sperm to egg ratios used in fertilisation assay.

Sperm Concentration (Sperm mL^{-1})	Total Sperm (n)	Total Eggs (n)	Sperm: Egg Ratio
5,000,000	15,000,000	500	30,000:1
1,700,000	5,000,000	500	10,000:1
560,000	1,700,000	500	3,333:1
190,000	560,000	500	1,111:1
62,000	190,000	500	370:1
21,000	62,000	500	123:1
6,900	21,000	500	41:1
2,300	6,900	500	14:1
760	2,300	500	5:1
250	760	500	2:1
85	250	500	1:2
0	0	500	0:500

Supplementary information on the fertilisation kinetics model for fertilisation in marine invertebrates (Styan et al., 2008).

The model used to fit fertilisation curves to the fertilisation data across multiple sperm densities was that developed by Styan et al., 2008. This model is modified from a previous fertilisation kinetics model (Styan 1998) which predicts the likelihood of fertilisation given concentrations of allogametes and the amount of time they have to mix and fertilise. The previous fertilisation kinetics model (equation 16, Styan 1998) incorporates a parameter, 'Fertilisation efficiency' (F_e) which is a measure of the average gametic compatibility between eggs and sperm - ranging between 1 (sperm meeting an egg can always fertilise) to 0 (sperm never fertilise). The Styan et al. (2008) version of the fertilisation model adds another parameter B_e, block efficiency, which is defined as the probability that an egg, having been fertilised by a sperm will then successfully enable its polyspermy block and prevent subsequent fertilising sperm. Similarly, B_e ranges from 1 (blocks always activate) to 0 (blocks never activate). Based on Styan (1998) the probability of successful (monospermic) fertilisation is thus:

$$f(monospermic) = xe^{-x} + B_e(1 - e^{-x} - xe^{-x})e^{-b}$$

where x is a function of collision rates and the gametic compatibility between allogametes. b also takes into account the delay between fertilisation and the (successful) activation of blocks to polyspermy (see Styan 1998). Essentially, monospermic eggs are the eggs that are only ever hit once by a fertilising sperm (xe^{-x}) as well as the eggs that are hit twice or more ($1 - e^{-x} - xe^{-x}$) but in which the second

hit happens after polyspermy blocks would form (e^{-b}). The new version of the model allows for only a proportion, (B_e) of these latter eggs to successfully recognise the first fertilising sperm and so set up their block to polyspermy. When B_e is 1 the new model is the previous version of the fertilisation kinetics model (equation 16 in Styan 1998). Because the proportion of eggs that are only ever hit by one fertilising sperm can never exceed 37 % (Styan 1998), the effect of lower values of B_e is to decrease the maximum fertilisation success achievable.

x = the average number of potential fertilisers, estimated as:

$$x = F_e \frac{S_0}{E_0}(1 - e^{-\beta_0 E_0 \tau})$$

Where;

F_e = Fertilisation efficiency, estimated as:

$$F_e = \beta/\beta_0$$

β = the rate collision constant of sperm contacts with penetrable egg surface receptor sites

β_0 = the collision rate constant, estimated as:

$$\beta_0 = \sigma v$$

σ = the cross-sectional area of the egg (mm^2)

v = the sperm swimming speed ($mm\ sec^{-1}$)

S_0 = the starting ambient sperm concentration (sperm μL^{-1})

E_0 = the concentration of eggs (eggs μL^{-1})

τ = the time eggs are exposed to sperm (s)

b = mean number of extra fertilising sperm that will contact an egg in time period τ_b, estimated as:

$$x = F_e \frac{S_0}{E_0}(1 - e^{-\beta_0 E_0 \tau_b})$$

Appendix 2: Supplementary Information for Chapter 3

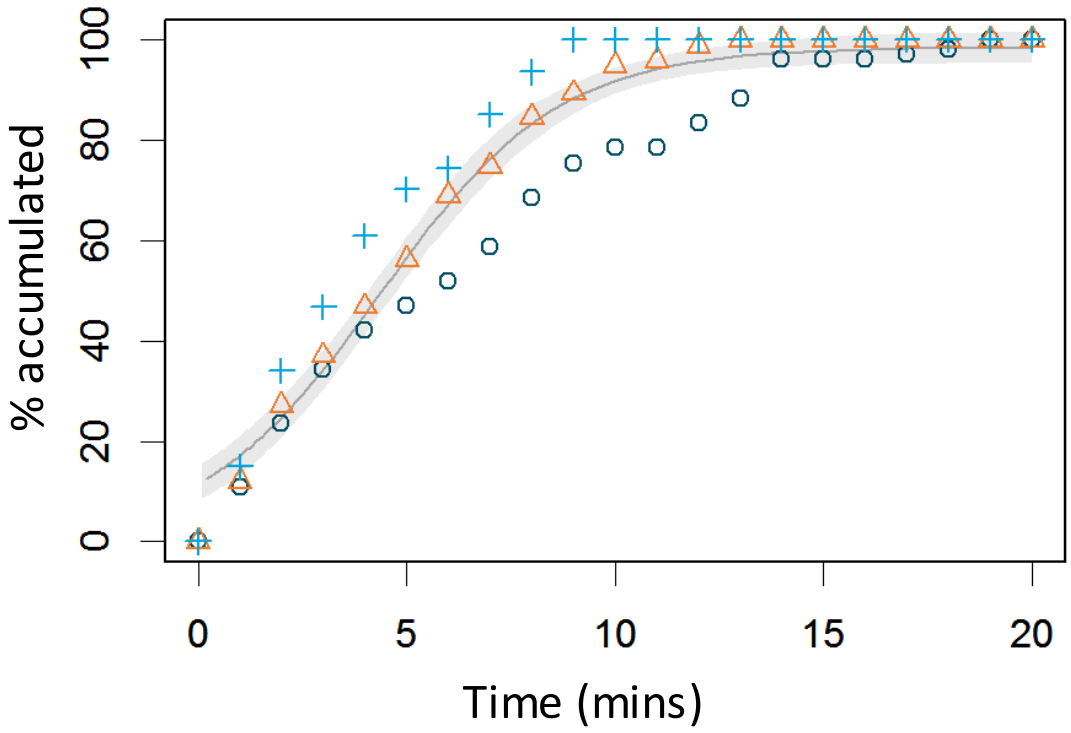
Table A2.1: Measured dissolved (<0.45 µm, bold) and nominal metal concentrations in controls and treatments (µg L-1) for CASA experiments. T= treatment, C = control.

	Cu			Zn				Pb			Cd		
T1	**1300**	**1500**	**2300**	**950**	**1000**	**990**	**710**	**1500**	**1200**	**1300**	**8700**	**15000**	**16000**
T2	440	500	750	320	340	330	240	760	600	650	2900	5100	5300
T3	150	170	250	110	110	110	79	380	300	330	960	1700	1800
T4	49	55	83	35	37	37	26	190	150	160	320	570	590
T5	16	18	28	12	12	12	9	96	75	82	110	190	200
T6	5	6	9	4	4	4	3	48	37	41	36	63	66
T7	2	2	3	1	1	1	1	24	19	20	12	21	22
C	**<10**	**<10**	**<10**	**<50**	**<50**	**<50**	**<50**	**<100**	**<100**	**<100**	**<50**	**<50**	**<50**

Table A2.2: Measured dissolved (<0.45 µm, bold) and nominal metal concentrations in controls and treatments (µg L-1) for SAAS experiments. T= treatment, C = control.

	Cu			Zn			Pb		Cd		
T1	**1400**	**1400**	**1400**	**4600**	**3500**	**44000**	**940**	**3400**	**570000**	**29000**	**550000**
T2	480	480	480	1500	1500	23000	390	2300	200000	13000	190000
T3	160	160	160	510	600	12000	160	1500	67000	6300	66000
T4	53	53	53	170	250	6400	69	1000	23000	2900	23000
T5	18	18	18	57	100	3300	29	690	7800	1300	7900
T6	6	6	6	19	43	1800	12	470	2700	630	2800
T7	2	2	2	6	18	**920**	5	**310**	**910**	**290**	**960**
C	**<10**	**<10**	**<10**	**<50**	**<50**	**<50**	**<100**	**<100**	**<50**	**<50**	**<50**

Figure A2.1: The rate of sperm accumulation against a surface for G. caespitosa sperm. Replicate 1 (Blue, +); Replicate 2 (Navy, o); Replicate 3 (Orange, Δ).



Sperm from *G. caespitosa* accumulated on the lower surface of the well-plates. Accumulation of live sperm was initially rapid and then after approximately 10 minutes began to plateau. Ninety percent of sperm had accumulated after 9 minutes.

Supplementary Information 1: Image J/CASA calibration.

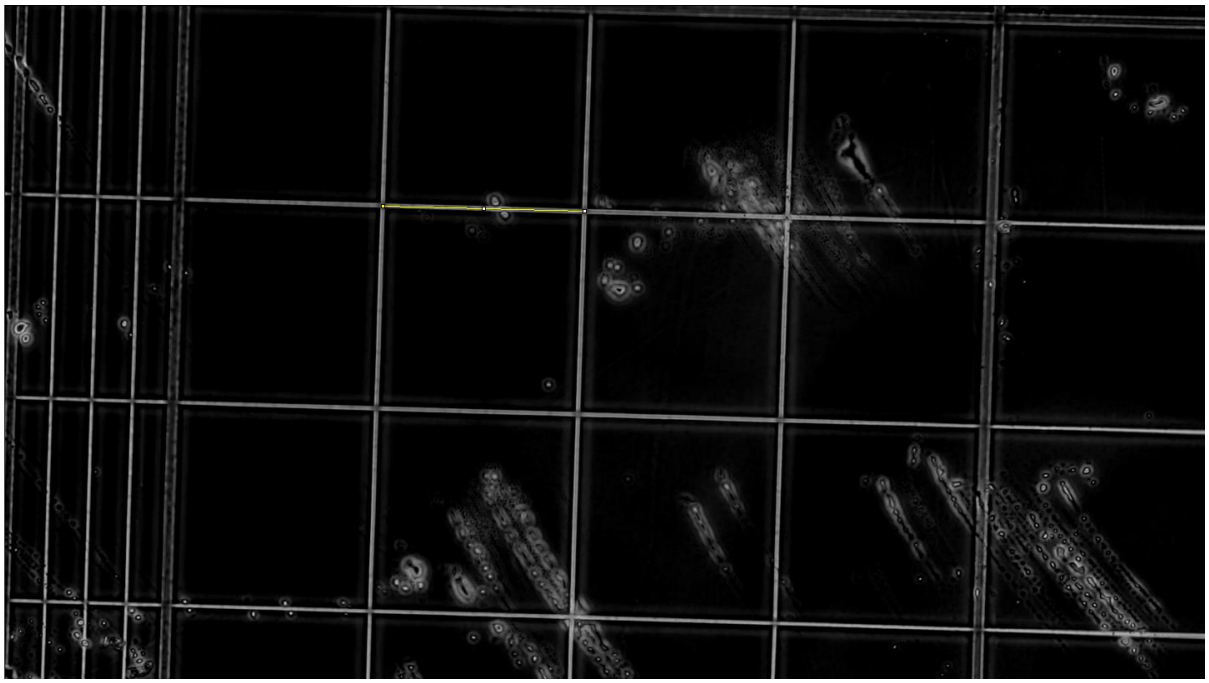


Figure A2.2: Haemocytometer at 200x magnification to determine pxiels/µm.

1920 x 1080, 0.5s, 50 frames.s-1

Length of line = 322.6 pixels = 250µm

1 pixel = 0.77 µm

1µm = 1.29 pixels

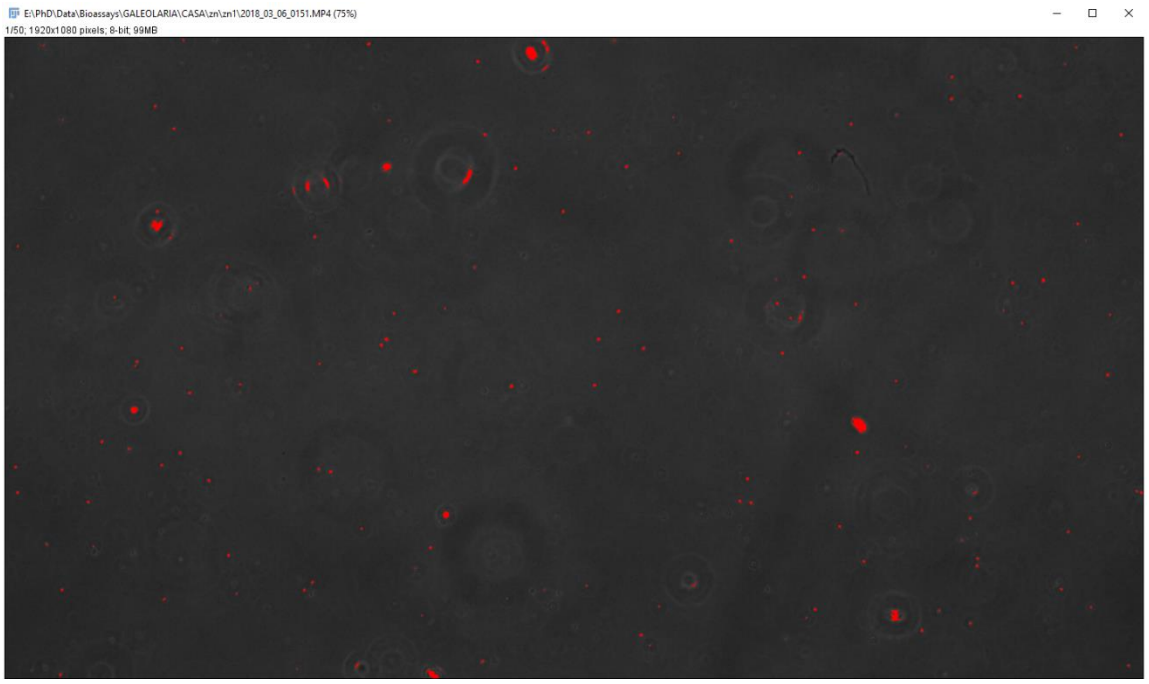


Figure A2.3: Image Thresholding

Threshold 64,255 Zn1: 2018_03_06_0151.MP4

Sperm Characteristics:

Assuming sperm heads are ~5 µm in length

Minimum sperm size = ~3 µm

Maximum sperm size = ~10 µm (allows for out of focus enlargement)

Min. Radius = 1.5 µm Max Radius = 5 µm

***Assuming sperm heads are circular:**

Area of sperm head = 7.07 – 78.5 µm

Area of sperm head = 9.1 – 101 pixels

Min Sperm Size = 9.1 pixels

Max Sperm Size = 101 pixels

Minimum Track Length = 5 frames

Maximum sperm velocity between frames

Max sperm swimming speed: 150µm s^{-1} (Kupriyanova and Havenhand, 2002)

= 150µm per 50 frames

=150 * 1.29 per 50 frames = 193.5 pixels per 50 frames

= 5.16 pixels per frame

Defining Particles as Sperm

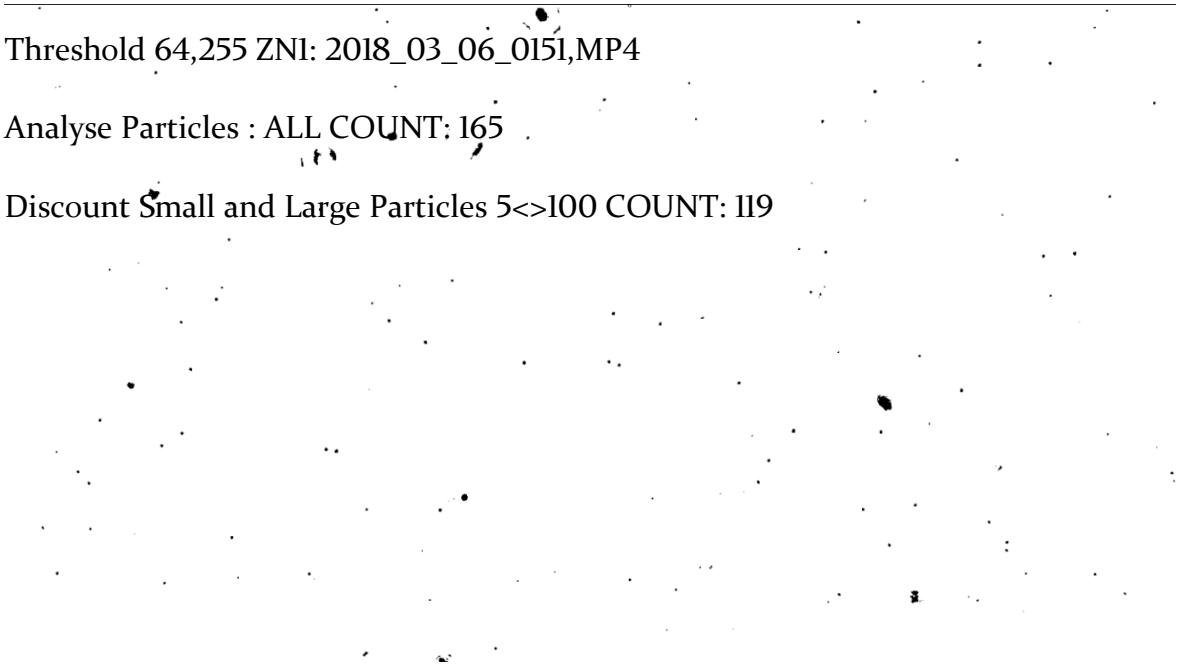
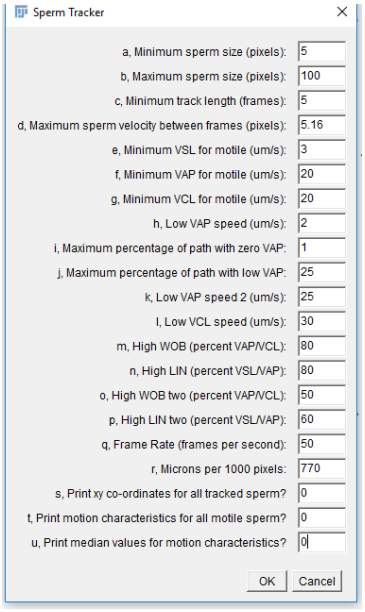


Figure A2.4: Image Particle Analysis

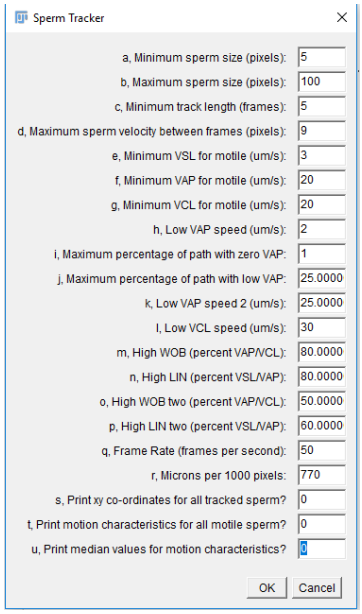
Testing Image J CASA Parameters

TEST 1



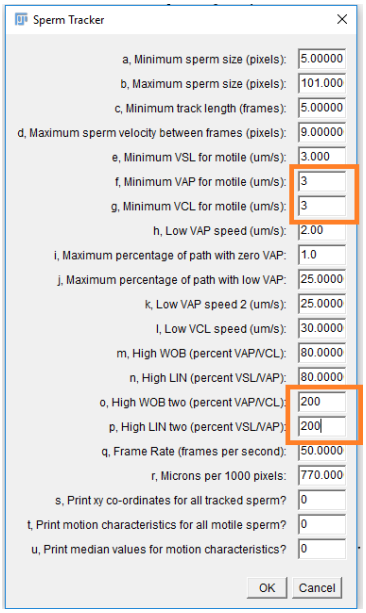
Result: No tracks identified.

TEST 2: Increased max velocity between frames to 9 pixels.



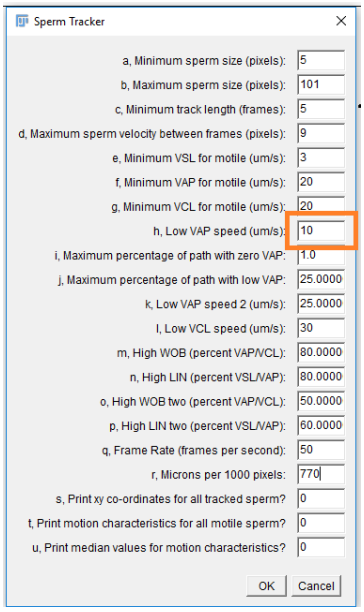
Result: Identified sperm paths, 18% motility. Some paths not included, some sperm counted twice

TEST 3: Reducing minimum VAP and VCL and increasing O,P to remove second filter for sperm curvature



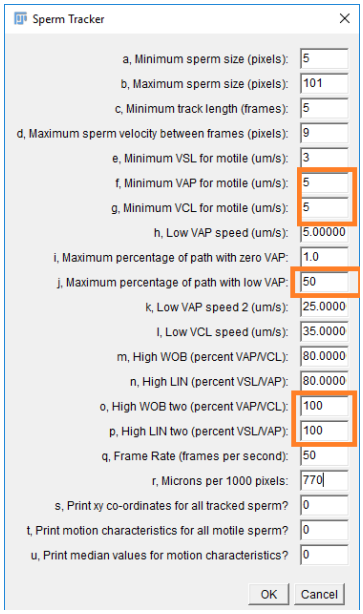
Result: 18% motile, some tracks still not included. Identical to test 1. >10 paths not counted.

TEST 4: Reducing low VAP Speed



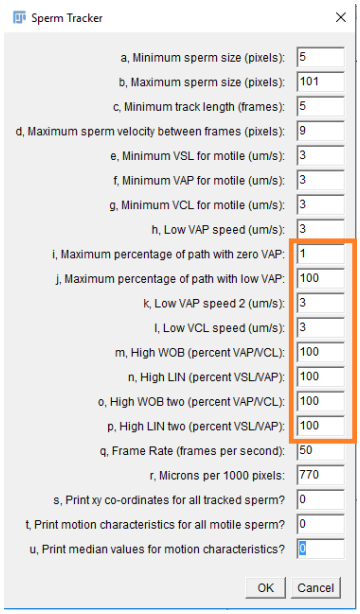
Result: IDENTICAL

TEST 5: reducing min VAP and VCL and removing second filter, and increasing max % of path with low VAP



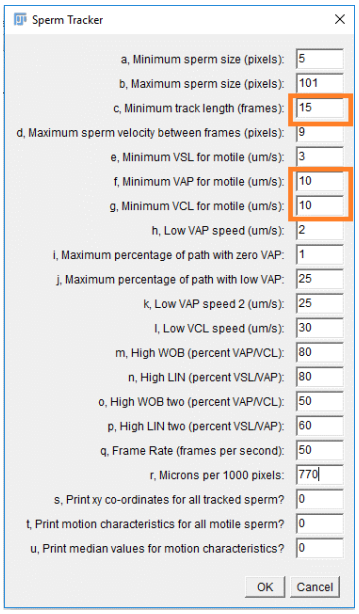
Result IDENTICAL

TEST6: Removing all filters (min speed low, max percentages high)



Result: 21% motility, includes some immotile sperm but does not include all motile sperm. Some tracks still uncounted.

TEST7: Increasing track length and setting min VCL/VAP to 10µm/s



Result: 29% Motility, small tracks discounted, only motile sperm counted. These setting were used for analysis of all images..

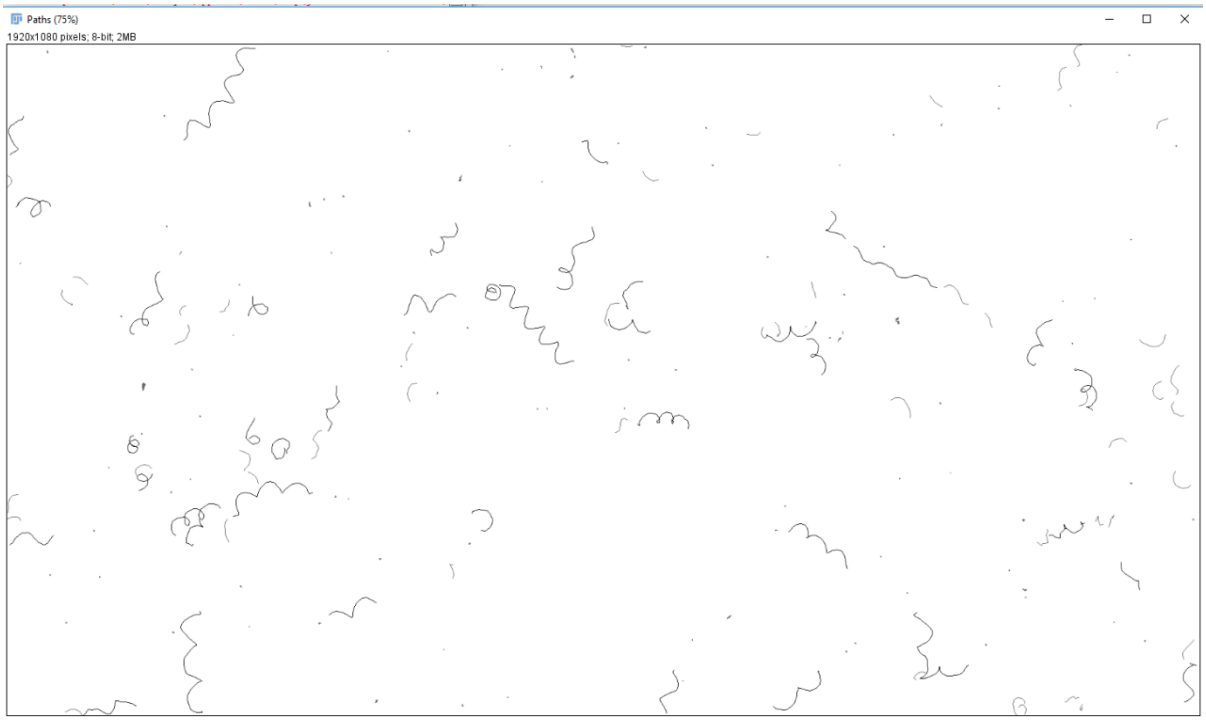


Figure A2.5: CASA test result (TEST 2). Short paths where sperm are passing through Z dimension not counted as motile.

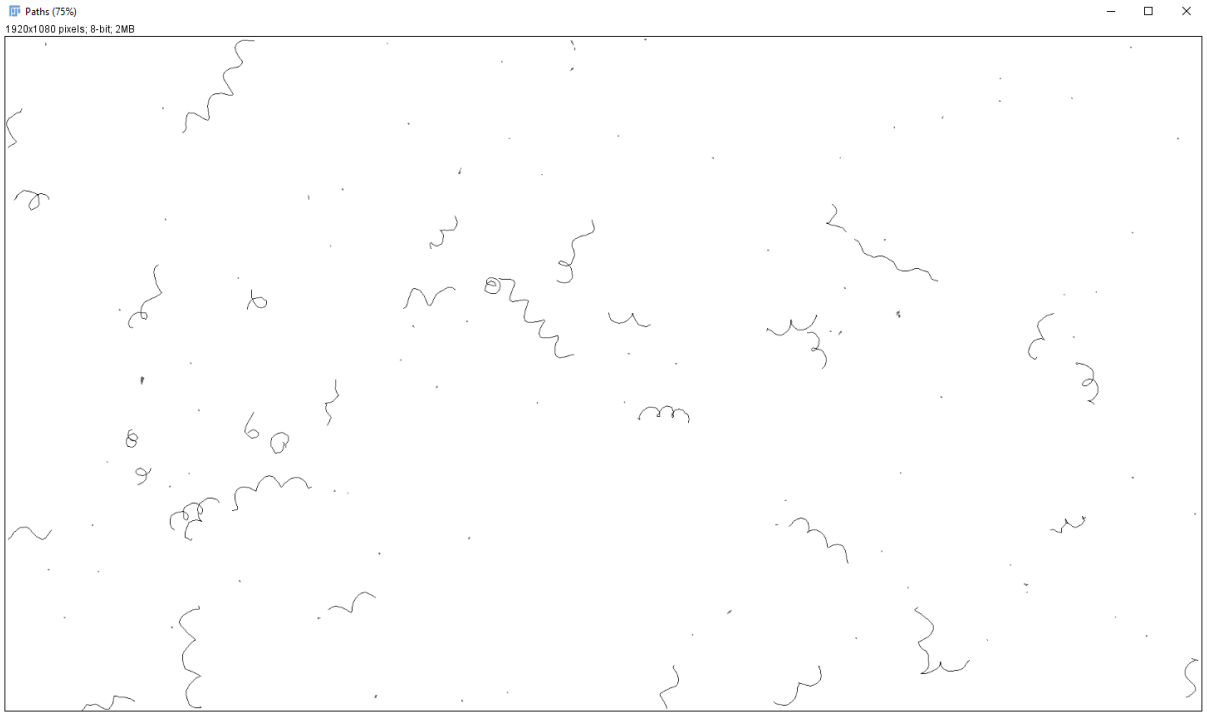


Figure A2.6: CASA test results (TEST 7). Short paths where sperm are passing through z dimension discounted from analysis.

Table A2.3: CASA calibration Test results

TEST	%MOT	VCL	VAP	VSL	LIN	WOB	PROG	BCF	n	n mot
1	0.00	NaN	NaN	NaN	NaN	NaN	NaN	NaN	170	0
2	0.18	219.21	145.09	110.06	0.76	0.66	762.64	9.81	224	40
3	0.18	219.21	145.09	110.06	0.76	0.66	762.64	9.81	224	40
4	0.18	219.21	145.09	110.06	0.76	0.66	762.64	9.81	224	40
5	0.18	219.21	145.09	110.06	0.76	0.66	762.64	9.81	224	40
6	0.21	193.46	122.80	93.10	0.76	0.63	643.78	11.01	224	48
7	0.29	218.65	141.37	113.83	0.81	0.65	840.37	10.67	123	36

Results of ANOVA and Tukey Test for sperm motility (%) when exposed to elevated concentrations of Cadmium for each replicate.

Replicate 1:

Source of Variation	Sum of Squares	d.f	Variance	F	P	
Between Group	141065.3084	5	28213.06	23.0574		0
Within Groups	647286	529	1223.603			
Total	788351.3084	534				

Tukey HSD Post-hoc Test...
1 vs 2: Diff=0.0000, 95%CI=-13.7835 to 13.7835, p=1.0000
1 vs 3: Diff=-4.0000, 95%CI=-18.0744 to 10.0744, p=0.9652
1 vs 4: Diff=-9.0000, 95%CI=-23.6296 to 5.6296, p=0.4934
1 vs 5: Diff=25.0000, 95%CI=10.7813 to 39.2187, p=0.0000
1 vs 6: Diff=48.0000, 95%CI=29.1442 to 66.8558, p=0.0000

Replicate 2:

Source of Variation	Sum of Squares	d.f	Variance	F	P	
Between Group	81381.5385	4	20345.38	10.8144		0
Within Groups	430822	229	1881.319			
Total	512203.5385	233				

Tukey HSD Post-hoc Test...
1 vs 2: Diff=11.0000, 95%CI=-10.7008 to 32.7008, p=0.6325
1 vs 3: Diff=23.0000, 95%CI=-0.2147 to 46.2147, p=0.0535
1 vs 4: Diff=45.0000, 95%CI=16.0446 to 73.9554, p=0.0003
1 vs 5: Diff=47.0000, 95%CI=24.6119 to 69.3881, p=0.0000

Replicate 3:

Source of Variation	Sum of Squares	d.f	Variance	F	P	
Between Group	98554.2688	6	16425.71	8.7043		0
Within Groups	464220	246	1887.073			
Total	562774.2688	252				

1 vs 2: Diff=-2.0000, 95%CI=-31.3470 to 27.3470, p=1.0000
1 vs 3: Diff=-2.0000, 95%CI=-31.6320 to 27.6320, p=1.0000
1 vs 4: Diff=9.0000, 95%CI=-24.1371 to 42.1371, p=0.9841
1 vs 5: Diff=24.0000, 95%CI=-5.6320 to 53.6320, p=0.1999
1 vs 6: Diff=54.0000, 95%CI=27.0191 to 80.9809, p=0.0000
1 vs 7: Diff=11.0000, 95%CI=-15.6596 to 37.6596, p=0.8833

Results of ANOVA and Tukey test for Sperm Accumulation (n) when exposed to elevated concentrations of Cadmium for each replicate.

Replicate 1:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	100268.6	7	14324.09	26	0
Within Groups	8928.3	16	558.0188		
Total	109197	23			

1 vs 2: Diff=-9.0000, 95%CI=-75.7795 to 57.7795, p=0.9997
1 vs 3: Diff=-67.0000, 95%CI=-133.7795 to -0.2205, p=0.0489
1 vs 4: Diff=-14.0000, 95%CI=-80.7795 to 52.7795, p=0.9947
1 vs 5: Diff=84.0000, 95%CI=17.2205 to 150.7795, p=0.0090
1 vs 6: Diff=117.0000, 95%CI=50.2205 to 183.7795, p=0.0004
1 vs 7: Diff=99.0000, 95%CI=32.2205 to 165.7795, p=0.0020
1 vs 8: Diff=-41.0000, 95%CI=-107.7795 to 25.7795, p=0.4399

Replicate 2:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	69832	5	13966.4	24	0
Within Groups	6941.16	12	578.43		
Total	76773.16	17			

1 vs 2: Diff=-26.0000, 95%CI=-91.9610 to 39.9610, p=0.7675
1 vs 3: Diff=-6.0000, 95%CI=-71.9610 to 59.9610, p=0.9995
1 vs 4: Diff=-77.0000, 95%CI=-142.9610 to -11.0390, p=0.0194
1 vs 5: Diff=22.0000, 95%CI=-43.9610 to 87.9610, p=0.8640
1 vs 6: Diff=-163.0000, 95%CI=-228.9610 to -97.0390, p=0.0000

Replicate 3:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	22000	7	3142.929	6	0.0019
Within Groups	8758.38	16	547.3988		
Total	30758.88	23			

1 vs 2: Diff=1.0000, 95%CI=-65.1410 to 67.1410, p=1.0000
1 vs 3: Diff=4.0000, 95%CI=-62.1410 to 70.1410, p=1.0000
1 vs 4: Diff=21.0000, 95%CI=-45.1410 to 87.1410, p=0.9478
1 vs 5: Diff=91.0000, 95%CI=24.8590 to 157.1410, p=0.0041
1 vs 6: Diff=35.0000, 95%CI=-31.1410 to 101.1410, p=0.6097
1 vs 7: Diff=47.0000, 95%CI=-19.1410 to 113.1410, p=0.2784
1 vs 8: Diff=59.0000, 95%CI=-7.1410 to 125.1410, p=0.0990

Results of ANOVA and Tukey test for sperm motility (%) when exposed to elevated concentrations of Zinc for each replicate.

Replicate 1:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	174082.2653	6	29013.7109	12.208	0
Within Groups	1578074	664	2376.6175		
Total	1752156.265	670			

1 vs 2: Diff=0.0000, 95%CI=-17.7242 to 17.7242, p=1.0000
1 vs 3: Diff=30.0000, 95%CI=10.9942 to 49.0058, p=0.0001
1 vs 4: Diff=40.0000, 95%CI=17.6705 to 62.3295, p=0.0000
1 vs 5: Diff=30.0000, 95%CI=11.3047 to 48.6953, p=0.0001
1 vs 6: Diff=30.0000, 95%CI=8.2201 to 51.7799, p=0.0010
1 vs 7: Diff=0.0000, 95%CI=-24.0039 to 24.0039, p=1.0000

Replicate 2:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	126522.4755	7	18074.6394	13.496	0
Within Groups	482134	360	1339.2611		
Total	608656.4755	367			

1 vs 2: Diff=-4.0000, 95%CI=-22.8112 to 14.8112, p=0.9981
1 vs 3: Diff=8.0000, 95%CI=-11.2724 to 27.2724, p=0.9110
1 vs 4: Diff=41.0000, 95%CI=19.0462 to 62.9538, p=0.0000
1 vs 5: Diff=52.0000, 95%CI=29.1526 to 74.8474, p=0.0000
1 vs 6: Diff=17.0000, 95%CI=-9.1424 to 43.1424, p=0.4955
1 vs 7: Diff=26.0000, 95%CI=-3.6139 to 55.6139, p=0.1333
1 vs 8: Diff=17.0000, 95%CI=-5.6529 to 39.6529, p=0.3034

Replicate 3:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	64495.1602	5	12899.032	9.1417	0
Within Groups	246926	175	1411.0057		
Total	311421.1602	180			

1 vs 2: Diff=8.0000, 95%CI=-14.4186 to 30.4186, p=0.9079
1 vs 3: Diff=12.0000, 95%CI=-13.3107 to 37.3107, p=0.7472
1 vs 4: Diff=64.0000, 95%CI=31.3418 to 96.6582, p=0.0000
1 vs 5: Diff=18.0000, 95%CI=-10.6025 to 46.6025, p=0.4600
1 vs 6: Diff=47.0000, 95%CI=15.0977 to 78.9023, p=0.0005

Replicate 4:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	61284.2028	6	10214.0338	4.8152	0.0001
Within Groups	434848	205	2121.2098		
Total	496132.2028	211			

1 vs 2: Diff=-4.0000, 95%CI=-37.4562 to 29.4562, p=0.9998
1 vs 3: Diff=13.0000, 95%CI=-16.7496 to 42.7496, p=0.8509
1 vs 4: Diff=12.0000, 95%CI=-23.5712 to 47.5712, p=0.9525
1 vs 5: Diff=16.0000, 95%CI=-24.2866 to 56.2866, p=0.9001
1 vs 6: Diff=-27.0000, 95%CI=-67.2866 to 13.2866, p=0.4207
1 vs 7: Diff=-29.0000, 95%CI=-58.5821 to 0.5821, p=0.0587

Results of ANOVA and Tukey Test for Sperm Accumulation (n) when exposed to elevated concentrations of Zinc for each replicate.

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	959.625	7	137.0893	6	0.0021
Within Groups	390	16	24.435		
Total	1350.585	23			

1 vs 2: Diff=-2.0000, 95%CI=-15.9741 to 11.9741, p=0.9995
1 vs 3: Diff=-3.0000, 95%CI=-16.9741 to 10.9741, p=0.9939
1 vs 4: Diff=-12.0000, 95%CI=-25.9741 to 1.9741, p=0.1213
1 vs 5: Diff=2.0000, 95%CI=-11.9741 to 15.9741, p=0.9995
1 vs 6: Diff=-17.0000, 95%CI=-30.9741 to -3.0259, p=0.0119
1 vs 7: Diff=-10.0000, 95%CI=-23.9741 to 3.9741, p=0.2713
1 vs 8: Diff=-11.0000, 95%CI=-24.9741 to 2.9741, p=0.1841

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	39638.625	7	5662.661	7	0.0006
Within Groups	12857.52	16	803.595		
Total	52496.145	23			

1 vs 2: Diff=-6.0000, 95%CI=-86.1378 to 74.1378, p=1.0000
1 vs 3: Diff=59.0000, 95%CI=-21.1378 to 139.1378, p=0.2435
1 vs 4: Diff=-3.0000, 95%CI=-83.1378 to 77.1378, p=1.0000
1 vs 5: Diff=23.0000, 95%CI=-57.1378 to 103.1378, p=0.9689
1 vs 6: Diff=-33.0000, 95%CI=-113.1378 to 47.1378, p=0.8330
1 vs 7: Diff=-74.0000, 95%CI=-154.1378 to 6.1378, p=0.0815
1 vs 8: Diff=-61.0000, 95%CI=-141.1378 to 19.1378, p=0.2128

Source of Variation	Sum of Squares	d.f	Variance	F	P	
Between Group	84292.5	7	12041.79	16	0	
Within Groups	12151.2	16	759.45			
Total	96443.7	23				

1 vs 2: Diff=-92.0000, 95%CI=-169.9055 to -14.0945, p=0.0151
1 vs 3: Diff=-32.0000, 95%CI=-109.9055 to 45.9055, p=0.8347
1 vs 4: Diff=-62.0000, 95%CI=-139.9055 to 15.9055, p=0.1753
1 vs 5: Diff=-118.0000, 95%CI=-195.9055 to -40.0945, p=0.0016
1 vs 6: Diff=-153.0000, 95%CI=-230.9055 to -75.0945, p=0.0001
1 vs 7: Diff=-167.0000, 95%CI=-244.9055 to -89.0945, p=0.0000
1 vs 8: Diff=-166.0000, 95%CI=-243.9055 to -88.0945, p=0.0001

Results of ANOVA and Tukey test for sperm motility (%) when exposed to elevated concentrations of Lead for each replicate.

Replicate 1:

Source of Variation	Sum of Squares	d.f	Variance	F	P	
Between Group	21601.1567	5	4320.2313	4.5743	0	
Within Groups	422176	447	944.4653			
Total	443777.1567	452				

1 vs 2: Diff=6.0000, 95%CI=-8.8198 to 20.8198, p=0.8562
1 vs 3: Diff=8.0000, 95%CI=-5.7828 to 21.7828, p=0.5586
1 vs 4: Diff=1.0000, 95%CI=-14.3228 to 16.3228, p=0.9999
1 vs 5: Diff=-11.0000, 95%CI=-25.4103 to 3.4103, p=0.2472
1 vs 6: Diff=-7.0000, 95%CI=-21.0701 to 7.0701, p=0.7127

Replicate 2:

Source of Variation	Sum of Squares	d.f	Variance	F	P	
Between Group	30593.7055	6	5098.9509	3.3623	0.003	
Within Groups	679404	448	1516.5268			
Total	709997.7055	454				

1 vs 2: Diff=4.0000, 95%CI=-15.2178 to 23.2178, p=0.9963
1 vs 3: Diff=8.0000, 95%CI=-12.6274 to 28.6274, p=0.9126
1 vs 4: Diff=11.0000, 95%CI=-8.2673 to 30.2673, p=0.6228
1 vs 5: Diff=26.0000, 95%CI=5.3726 to 46.6274, p=0.0039
1 vs 6: Diff=9.0000, 95%CI=-12.9632 to 30.9632, p=0.8889
1 vs 7: Diff=20.0000, 95%CI=-1.6039 to 41.6039, p=0.0906

Replicate 3:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	41004	6	6834	3.3654	0.0029
Within Groups	1005178	495	2030.6626		
Total	1046182	501			

1 vs 2: Diff=0.0000, 95%CI=-20.0384 to 20.0384, p=1.0000
1 vs 3: Diff=-8.0000, 95%CI=-28.6786 to 12.6786, p=0.9137
1 vs 4: Diff=-9.0000, 95%CI=-32.1518 to 14.1518, p=0.9119
1 vs 5: Diff=-5.0000, 95%CI=-27.1069 to 17.1069, p=0.9942
1 vs 6: Diff=21.0000, 95%CI=-0.7744 to 42.7744, p=0.0670
1 vs 7: Diff=7.0000, 95%CI=-13.3783 to 27.3783, p=0.9501

Results of ANOVA and Tukey Test for Sperm Accumulation (n) when exposed to elevated concentrations of Lead for each replicate.

Replicate 1:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	7726.5	7	1103.786	22	0
Within Groups	805.08	16	50.3175		
Total	8531.58	23			

1 vs 2: Diff=-49.0000, 95%CI=-69.0529 to -28.9471, p=0.0000
1 vs 3: Diff=-49.0000, 95%CI=-69.0529 to -28.9471, p=0.0000
1 vs 4: Diff=-52.0000, 95%CI=-72.0529 to -31.9471, p=0.0000
1 vs 5: Diff=-45.0000, 95%CI=-65.0529 to -24.9471, p=0.0000
1 vs 6: Diff=-61.0000, 95%CI=-81.0529 to -40.9471, p=0.0000
1 vs 7: Diff=-52.0000, 95%CI=-72.0529 to -31.9471, p=0.0000
1 vs 8: Diff=-58.0000, 95%CI=-78.0529 to -37.9471, p=0.0000

Replicate 2:

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	70728	7	10104	9	0.0002
Within Groups	18116.88	16	1132.305		
Total	88844.88	23			

1 vs 2: Diff=120.0000, 95%CI=24.8737 to 215.1263, p=0.0088

1 vs 3: Diff=45.0000, 95%CI=-50.1263 to 140.1263, p=0.7231
1 vs 4: Diff=167.0000, 95%CI=71.8737 to 262.1263, p=0.0004
1 vs 5: Diff=148.0000, 95%CI=52.8737 to 243.1263, p=0.0012
1 vs 6: Diff=110.0000, 95%CI=14.8737 to 205.1263, p=0.0178
1 vs 7: Diff=113.0000, 95%CI=17.8737 to 208.1263, p=0.0144
1 vs 8: Diff=41.0000, 95%CI=-54.1263 to 136.1263, p=0.8009

Results of ANOVA and Tukey Test for Sperm velocities when exposed to elevated concentrations of Cd for each replicate.

REP = 1.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	3833.082	5	766.616	1.021	.411
	Within Groups	63091.737	84	751.092		
	Total	66924.819	89			
VSL	Between Groups	8784.006	5	1756.801	1.385	.238
	Within Groups	106578.879	84	1268.796		
	Total	115362.885	89			

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	3.00	3.55	14.11682	11.43993	.819	-19.2483	47.4819
		47.85	12.24278	13.25066	.939	-26.4034	50.8889
		175.64	4.84218	21.06721	1.000	-56.6013	66.2856
		2365.93	20.52586	9.47317	.264	-7.1030	48.1548
		8683.51	15.94207	10.04675	.609	-13.3597	45.2439
		8683.51	11.09989	20.20402	.994	-47.8260	70.0258
VSL	3.00	3.55	24.11178	14.86870	.587	-19.2534	67.4770
		47.85	23.47732	17.22213	.749	-26.7518	73.7064
		175.64	39.24509	27.38144	.707	-40.6141	119.1043
		2365.93	24.69789	12.31245	.348	-11.2119	60.6077
		8683.51	33.24094	13.05795	.123	-4.8432	71.3250
		2365.93	1.22057	14.74815	1.000	-41.7931	44.2342
		8683.51	9.76362	15.37601	.988	-35.0812	54.6084

REP = 2.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	17462.570	4	4365.643	3.199	.018
	Within Groups	100972.323	74	1364.491		
	Total	118434.893	78			
VSL	Between Groups	51061.553	4	12765.388	13.614	.000
	Within Groups	69388.893	74	937.688		
	Total	120450.446	78			

Post Hoc Tests

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	3.00	502.43	24.49451	15.53737	.517	-18.9507	67.9397
		1568.93	5.12111	15.08029	.997	-37.0460	47.2883
		4899.30	4.76146	15.53737	.998	-38.6838	48.2067
		15299.02	35.85582	13.60812	.074	-2.1949	73.9065
VSL	3.00	502.43	67.72621*	12.88016	.000	31.7110	103.7414
		1568.93	55.58015*	12.50125	.000	20.6244	90.5359
		4899.30	82.57066*	12.88016	.000	46.5554	118.5859
		15299.02	76.75397*	11.28085	.000	45.2107	108.2972

*. The mean difference is significant at the 0.05 level.

REP = 3.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	25633.329	6	4272.221	2.693	.020
	Within Groups	120572.683	76	1586.483		
	Total	146206.012	82			
VSL	Between Groups	47713.473	6	7952.245	5.552	.000
	Within Groups	108864.056	76	1432.422		
	Total	156577.529	82			

Post Hoc Tests

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	19.08	66.41	-23.68307	22.21649	.936	-90.9706	43.6045
		198.80	-9.51696	20.99261	.999	-73.0977	54.0638
		595.09	-5.80349	20.99261	1.000	-69.3842	57.7773
		1781.38	-27.99861	17.27174	.669	-80.3099	24.3127
		5332.51	-10.07405	15.08154	.994	-55.7518	35.6037
		15962.67	30.01893	17.27174	.593	-22.2924	82.3302
VSL	19.08	66.41	-27.92404	21.11024	.839	-91.8611	36.0130
		198.80	-22.29462	19.94730	.921	-82.7094	38.1202
		595.09	-10.09161	19.94730	.999	-70.5064	50.3232
		1781.38	-27.90677	16.41171	.618	-77.6133	21.7997
		5332.51	20.55283	14.33057	.782	-22.8505	63.9561
		15962.67	38.58694	16.41171	.234	-11.1196	88.2934

*. The mean difference is significant at the 0.05 level.

Results of ANOVA and Tukey Test for Sperm velocities when exposed to elevated concentrations of Pb for each replicate.

REP = 1.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	30118.275	5	6023.655	3.050	.018
	Within Groups	96776.386	49	1975.028		
	Total	126894.661	54			
VSL	Between Groups	16481.318	5	3296.264	1.081	.383
	Within Groups	149398.138	49	3048.942		
	Total	165879.456	54			

a. REP = 1.00

Post Hoc Tests

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	8.40	29.26	-39.71599	19.59677	.343	-97.8287	18.3968
		56.58	-32.12852	18.14308	.493	-85.9305	21.6734
		109.40	-26.07490	21.59459	.831	-90.1120	37.9622
		790.93	-25.83520	26.70589	.926	-105.0295	53.3591
		1529.38	48.68213	26.70589	.461	-30.5122	127.8765
VSL	8.40	29.26	9.57528	24.34851	.999	-62.6284	81.7790
		56.58	-17.06455	22.54234	.973	-83.9122	49.7831
		109.40	12.98453	26.83075	.997	-66.5800	92.5491
		790.93	-36.60141	33.18142	.878	-134.9984	61.7956
		1529.38	30.27796	33.18142	.941	-68.1191	128.6750

*. The mean difference is significant at the 0.05 level.

a. REP = 1.00

REP = 2.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	30737.553	6	5122.926	2.540	.025
	Within Groups	201718.749	100	2017.187		
	Total	232456.302	106			
VSL	Between Groups	41650.794	6	6941.799	4.301	.001
	Within Groups	161382.641	100	1613.826		
	Total	203033.434	106			

a. REP = 2.00

Post Hoc Tests

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	8.40	28.64	-1.13853	19.90561	1.000	-61.0387	58.7616
		53.35	-45.99509	20.18209	.265	-106.7272	14.7371
		99.37	-42.98378	18.78850	.260	-99.5223	13.5548
		344.80	-44.21047	18.33570	.205	-99.3865	10.9655
		642.27	-30.00558	20.86933	.780	-92.8058	32.7947
		1196.38	-25.17607	19.25634	.847	-83.1224	32.7703
VSL	8.40	28.64	2.49248	17.80454	1.000	-51.0851	56.0701
		53.35	-45.77841	18.05184	.158	-100.1002	8.5434
		99.37	-49.99063	16.80535	.055	-100.5615	.5802
		344.80	-31.55274	16.40034	.470	-80.9048	17.7993
		642.27	-43.62736	18.66654	.237	-99.7989	12.5442
		1196.38	-52.31131*	17.22380	.046	-104.1414	-.4813

*. The mean difference is significant at the 0.05 level.

a. REP = 2.00

REP = 3.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	8751.546	6	1458.591	1.329	.252
	Within Groups	102058.036	93	1097.398		
	Total	110809.582	99			
VSL	Between Groups	6367.185	6	1061.198	.463	.834
	Within Groups	213150.689	93	2291.943		
	Total	219517.874	99			

a. REP = 3.00

Post Hoc Tests

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	8.40	35.03	10.01149	11.38218	.975	-24.2927	44.3157
		64.01	12.30286	14.07627	.976	-30.1209	54.7266
		213.76	-1.81945	16.74653	1.000	-52.2910	48.6521
		390.62	9.08512	14.07627	.995	-33.3386	51.5089
		713.82	6.00960	10.24024	.997	-24.8529	36.8721
		1304.42	27.04053	10.76274	.167	-5.3967	59.4778
VSL	8.40	35.03	6.21301	16.44921	1.000	-43.3624	55.7885
		64.01	19.13558	20.34264	.965	-42.1741	80.4452
		213.76	2.05089	24.20163	1.000	-70.8892	74.9909
		390.62	-11.74282	20.34264	.997	-73.0525	49.5668
		713.82	-.02868	14.79891	1.000	-44.6304	44.5730
		1304.42	13.41802	15.55401	.977	-33.4594	60.2955

Results of ANOVA and Tukey Test for Sperm velocities when exposed to elevated concentrations of Zn for each replicate.

REP = 1.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	85381.252	6	14230.209	13.720	.000
	Within Groups	327745.339	316	1037.169		
	Total	413126.592	322			
VSL	Between Groups	69749.001	6	11624.834	10.210	.000
	Within Groups	359794.545	316	1138.590		
	Total	429543.546	322			

a. REP = 1.00

Post Hoc Tests

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	10.00	39.06	7.80314	7.16641	.931	-13.4628	29.0691
		117.19	2.55180	6.63798	1.000	-17.1460	22.2496
		351.56	45.66572*	7.13286	.000	24.4994	66.8321
		1054.69	29.20198*	6.55881	.000	9.7391	48.6649
		3164.07	27.17339*	7.49027	.006	4.9464	49.4004
		9492.22	37.03111*	9.89721	.004	7.6617	66.4005
VSL	10.00	39.06	4.12154	7.50863	.998	-18.1599	26.4030
		117.19	16.71016	6.95496	.201	-3.9283	37.3486
		351.56	37.50165*	7.47348	.000	15.3245	59.6788
		1054.69	35.87789*	6.87202	.000	15.4856	56.2702
		3164.07	33.06997*	7.84796	.001	9.7816	56.3583
		9492.22	42.78872*	10.36984	.001	12.0168	73.5606

*. The mean difference is significant at the 0.05 level.

a. REP = 1.00

REP = 2.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	19778.709	7	2825.530	2.725	.015
	Within Groups	74667.879	72	1037.054		
	Total	94446.588	79			
VSL	Between Groups	33002.584	7	4714.655	6.303	.000
	Within Groups	53859.016	72	748.042		
	Total	86861.600	79			

a. REP = 2.00

Post Hoc Tests

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	10.00	13.87	23.97791	26.94323	.986	-60.1337	108.0895
		41.62	-4.21009	17.63849	1.000	-59.2741	50.8539
		124.87	3.81572	16.10166	1.000	-46.4506	54.0820
		374.61	-7.77763	15.95461	1.000	-57.5849	42.0296
		1123.82	5.40221	19.50008	1.000	-55.4733	66.2777
		3371.45	42.88574	19.50008	.365	-17.9898	103.7613
		10114.36	30.65981	17.96215	.683	-25.4146	86.7342
VSL	10.00	13.87	-20.73199	22.88295	.985	-92.1682	50.7042
		41.62	-16.77188	14.98041	.950	-63.5378	29.9941
		124.87	-2.18721	13.67518	1.000	-44.8785	40.5041
		374.61	3.03561	13.55029	1.000	-39.2658	45.3370
		1123.82	6.25837	16.56146	1.000	-45.4433	57.9601
		3371.45	38.59203	16.56146	.292	-13.1097	90.2937
		10114.36	51.15428*	15.25530	.027	3.5302	98.7784

*. The mean difference is significant at the 0.05 level.

a. REP = 2.00

REP = 3.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	118846.768	5	23769.354	14.466	.000
	Within Groups	65723.584	40	1643.090		
	Total	184570.352	45			
VSL	Between Groups	62160.704	5	12432.141	16.537	.000
	Within Groups	30071.493	40	751.787		
	Total	92232.197	45			

a. REP = 3.00

Post Hoc Tests

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	10.00	40.64	8.19697	24.35853	.999	-64.6892	81.0831
		121.91	10.89343	25.40668	.998	-65.1291	86.9159
		365.72	21.85053	23.66737	.938	-48.9675	92.6686
		1097.17	56.84458	26.16526	.273	-21.4477	135.1369
		9874.56	141.47668*	24.35853	.000	68.5905	214.3629
VSL	10.00	40.64	42.48614	16.47661	.126	-6.8156	91.7879
		121.91	29.60210	17.18560	.526	-21.8211	81.0253
		365.72	59.25753*	16.00910	.008	11.3547	107.1603
		1097.17	93.35967*	17.69872	.000	40.4011	146.3182
		9874.56	119.77681*	16.47661	.000	70.4751	169.0785

*. The mean difference is significant at the 0.05 level.

a. REP = 3.00

REP = 4.00

ANOVA[a]

		Sum of Squares	df	Mean Square	F	Sig.
VCL	Between Groups	30286.206	6	5047.701	2.930	.013
	Within Groups	122327.852	71	1722.927		
	Total	152614.058	77			
VSL	Between Groups	86996.999	6	14499.500	11.140	.000
	Within Groups	92411.061	71	1301.564		
	Total	179408.060	77			

a. REP = 4.00

Post Hoc Tests

Multiple Comparisons[a]

Tukey HSD

Dependent Variable	(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
VCL	10.00	10.10	20.88706	16.54211	.866	-29.3095	71.0836
		29.25	38.44271	13.27624	.071	-1.8437	78.7291
		87.74	9.93039	15.65011	.995	-37.5594	57.4202
		789.62	37.40261	17.11096	.316	-14.5201	89.3253
		2368.87	54.29706	31.02922	.586	-39.8602	148.4543
		7106.60	68.68706*	21.11715	.028	4.6077	132.7665
VSL	10.00	10.10	50.82389*	14.37773	.012	7.1951	94.4527
		29.25	74.80240*	11.53917	.000	39.7871	109.8177
		87.74	61.60934*	13.60244	.000	20.3331	102.8855
		789.62	85.88308*	14.87215	.000	40.7540	131.0122
		2368.87	108.10497*	26.96933	.003	26.2673	189.9426
		7106.60	104.15908*	18.35417	.000	48.4639	159.8543

Appendix 3: Supplementary Information for Chapter 4

Table A3.1 Measured dissolved (<0.45 µm) metal concentrations in controls and treatments (µg L^{-1}) for acrosomal integrity experiments.

Metal	Control	1	2	3	4	5	6	7
Cu	<10	5	68	154	258	363	465	977
	<10	207	312	393	411	460	464	502
	<10	103	148	159	185	201	212	223
	<10	<10	<10	<10	<10	<10	<10	<10
Zn	<50	<50	<50	94	107	125	316	728
	<50	<50	165	180	250	322	872	1087
	<50	<50	101	139	146	223	251	377
Cd	<10	22	78	781	2445	7135	20697	
	<10	90	343	681	1532	2519	2828	5627
	<10	258	526	1053	2056	4126	8493	16401
Pb	<100	<100	121	784	2396			
	<100	<100	104	133	294	583	1392	2357
	<100	<100	<100	<100	<100	<100	217	720

Table A3.2: Measured dissolved (<0.45 µm) metal concentrations in controls and treatments (µg L^{-1}) for mitochondrial membrane potential experiments.

Metal	Control	1	2	3	4	5	6	7
Cu	3.38	7	11	14	18	25	42	75
		8	11	19	35	60	113	223
		8	10	13	18	23	25	29
Zn	8	30	46	83	132	256	477	931
		28	52	84	172	323	586	1811
		102	164	209	248	293	342	426
Cd	1	250	535	1009	2098	4124	8801	18022
		86	122	255	682	1237	2793	7343
		1	2	1	2	1	5624	8582
Pb	2.38	3	2	3	7	4	7	6
		4	5	4	13	4	18	2
		24	46	4	3	4	2	4

Results of ANOVA and Tukey Test for Mitochondrial Membrane Potential (ΔΨm) when exposed to elevated concentrations of Cu for each replicate.

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	3259551.8	8	407444%	844%	0%
Within Groups	42041912.8	87123	483%		
Total	15321464.6	87131			

1 vs 2: Diff=0.0000, 95%CI=-0.9815 to 0.9815, p=1.0000
1 vs 3: Diff=0.0000, 95%CI=-0.9795 to 0.9795, p=1.0000
1 vs 4: Diff=1.0000, 95%CI=0.0224 to 1.9776, p=0.0403
1 vs 5: Diff=2.0000, 95%CI=1.0211 to 2.9789, p=0.0000
1 vs 6: Diff=15.0000, 95%CI=14.0233 to 15.9767, p=0.0000
1 vs 7: Diff=15.0000, 95%CI=14.0244 to 15.9756, p=0.0000
1 vs 8: Diff=11.0000, 95%CI=10.0245 to 11.9755, p=0.0000

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	24313117.3	8	3039139.67	2302.52	0.00
Within Groups	112619994	85324	1319.91		
Total	136933111	85332			

1 vs 2: Diff=13.0000, 95%CI=11.3733 to 14.6267, p=0.0000
1 vs 3: Diff=17.0000, 95%CI=15.3739 to 18.6261, p=0.0000
1 vs 4: Diff=55.0000, 95%CI=53.3511 to 56.6489, p=0.0000
1 vs 5: Diff=34.0000, 95%CI=32.3586 to 35.6414, p=0.0000
1 vs 6: Diff=10.0000, 95%CI=8.3675 to 11.6325, p=0.0000
1 vs 7: Diff=15.0000, 95%CI=13.3755 to 16.6245, p=0.0000
1 vs 8: Diff=0.0000, 95%CI=-1.6256 to 1.6256, p=1.0000

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	1077810.2	8	134726.28	235.7526	0
Within Groups	49578723.1	86756	571.4731		
Total	50656533.3	86764			

1 vs 2: Diff=-1.0000, 95%CI=-2.0683 to 0.0683, p=0.0879
1 vs 3: Diff=0.0000, 95%CI=-1.0657 to 1.0657, p=1.0000
1 vs 4: Diff=-1.0000, 95%CI=-2.0664 to 0.0664, p=0.0867
1 vs 5: Diff=0.0000, 95%CI=-1.0641 to 1.0641, p=1.0000
1 vs 6: Diff=7.0000, 95%CI=5.9363 to 8.0637, p=0.0000
1 vs 7: Diff=3.0000, 95%CI=1.9376 to 4.0624, p=0.0000
1 vs 8: Diff=7.0000, 95%CI=5.9368 to 8.0632, p=0.0000

Results of ANOVA and Tukey Test for Mitochondrial Membrane Potential (ΔΨm) when exposed to elevated concentrations of Zn for each replicate.

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	724685.27	8	90585.66	236.78	0.00
Within Groups	27210071.5	71125	382.57		
Total	27934756.8	71133			

1 vs 2: Diff=6.0000, 95%CI=5.1269 to 6.8731, p=0.0000
1 vs 3: Diff=2.0000, 95%CI=0.9134 to 3.0866, p=0.0000
1 vs 4: Diff=4.0000, 95%CI=3.0912 to 4.9088, p=0.0000
1 vs 5: Diff=-2.0000, 95%CI=-3.4760 to -0.5240, p=0.0008
1 vs 6: Diff=-2.0000, 95%CI=-2.9104 to -1.0896, p=0.0000
1 vs 7: Diff=-3.0000, 95%CI=-3.8782 to -2.1218, p=0.0000
1 vs 8: Diff=-3.0000, 95%CI=-3.8736 to -2.1264, p=0.0000

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	51967916.1	8	6495989.51	4841.45	0.00
Within Groups	72953350.6	54372	1341.74		
Total	124921267	54380			

1 vs 2: Diff=79.0000, 95%CI=77.3665 to 80.6335, p=0.0000
1 vs 3: Diff=77.0000, 95%CI=75.0927 to 78.9073, p=0.0000
1 vs 4: Diff=20.0000, 95%CI=18.1932 to 21.8068, p=0.0000
1 vs 5: Diff=17.0000, 95%CI=14.9474 to 19.0526, p=0.0000
1 vs 6: Diff=20.0000, 95%CI=17.5609 to 22.4391, p=0.0000
1 vs 7: Diff=13.0000, 95%CI=10.9113 to 15.0887, p=0.0000
1 vs 8: Diff=15.0000, 95%CI=13.2948 to 16.7052, p=0.0000

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	39323336.3	8	4915417.04	3843.83	0.00
Within Groups	90908526.2	71090	1278.78		
Total	130231863	71098			

1 vs 2: Diff=-4.0000, 95%CI=-5.5960 to -2.4040, p=0.0000
1 vs 3: Diff=14.0000, 95%CI=12.0074 to 15.9926, p=0.0000
1 vs 4: Diff=62.0000, 95%CI=60.3384 to 63.6616, p=0.0000
1 vs 5: Diff=71.0000, 95%CI=68.3014 to 73.6986, p=0.0000
1 vs 6: Diff=40.0000, 95%CI=38.3355 to 41.6645, p=0.0000
1 vs 7: Diff=26.0000, 95%CI=24.3943 to 27.6057, p=0.0000
1 vs 8: Diff=15.0000, 95%CI=13.4028 to 16.5972, p=0.0000

Results of ANOVA and Tukey Test for Mitochondrial Membrane Potential (ΔΨm) when exposed to elevated concentrations of Cd for each replicate.

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	724685.27	8	90585.66	236.78	0.00
Within Groups	27210071.5	71125	382.57		
Total	27934756.8	71133			

1 vs 2: Diff=-2.0000, 95%CI=-3.1048 to -0.8952, p=0.0000
1 vs 3: Diff=-4.0000, 95%CI=-5.1055 to -2.8945, p=0.0000
1 vs 5: Diff=2.0000, 95%CI=0.7377 to 3.2623, p=0.0001
1 vs 6: Diff=33.0000, 95%CI=31.8545 to 34.1455, p=0.0000
1 vs 7: Diff=1.0000, 95%CI=-2.3276 to 4.3276, p=0.9851
1 vs 8: Diff=7.0000, 95%CI=3.9634 to 10.0366, p=0.0000

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	52098987.6	8	6512373.45	7678.91	0.00
Within Groups	72167807.4	85095	848.09		
Total	124266795	85103			

1 vs 2: Diff=0.0000, 95%CI=-1.3104 to 1.3104, p=1.0000
1 vs 3: Diff=1.0000, 95%CI=-0.3108 to 2.3108, p=0.3033
1 vs 4: Diff=0.0000, 95%CI=-1.3095 to 1.3095, p=1.0000
1 vs 5: Diff=0.0000, 95%CI=-1.3093 to 1.3093, p=1.0000
1 vs 6: Diff=67.0000, 95%CI=65.6827 to 68.3173, p=0.0000
1 vs 7: Diff=53.0000, 95%CI=51.6818 to 54.3182, p=0.0000
1 vs 8: Diff=32.0000, 95%CI=30.6787 to 33.3213, p=0.0000

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	355461.391	7	50780.20	152.84	0.00
Within Groups	25111484.1	75582	332.24		
Total	25466945.5	75589			

1 vs 2: Diff=0.0000, 95%CI=-0.7991 to 0.7991, p=1.0000
1 vs 3: Diff=-1.0000, 95%CI=-1.8005 to -0.1995, p=0.0037
1 vs 4: Diff=-3.0000, 95%CI=-3.7991 to -2.2009, p=0.0000
1 vs 6: Diff=1.0000, 95%CI=0.1963 to 1.8037, p=0.0040
1 vs 7: Diff=4.0000, 95%CI=3.1938 to 4.8062, p=0.0000
1 vs 8: Diff=-2.0000, 95%CI=-2.8047 to -1.1953, p=0.0000

Results of ANOVA and Tukey Test for Mitochondrial Membrane Potential (ΔΨm) when exposed to elevated concentrations of Pb for each replicate.

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	17929.0692	7	2561.2956	53.5411	0
Within Groups	3430887.652	71719	47.84		
Total	344816.7212	71726			

1 vs 2: Diff=-1.0000, 95%CI=-1.3083 to -0.6917, p=0.0000
1 vs 3: Diff=0.0000, 95%CI=-0.3150 to 0.3150, p=1.0000
1 vs 4: Diff=-1.0000, 95%CI=-1.3147 to -0.6853, p=0.0000
1 vs 5: Diff=0.0000, 95%CI=-0.3139 to 0.3139, p=1.0000
1 vs 6: Diff=0.0000, 95%CI=-0.3152 to 0.3152, p=1.0000
1 vs 7: Diff=-1.0000, 95%CI=-1.3139 to -0.6861, p=0.0000
1 vs 8: Diff=-1.0000, 95%CI=-1.3142 to -0.6858, p=0.0000

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	43579.0244	7	6225.5749	104.3182	0
Within Groups	4063164.157	68084	59.68		
Total	4106743.181	68091			

1 vs 2: Diff=-2.2000, 95%CI=-2.5608 to -1.8392, p=0.0000
1 vs 3: Diff=-0.7000, 95%CI=-1.0588 to -0.3412, p=0.0000
1 vs 4: Diff=-2.2000, 95%CI=-2.5603 to -1.8397, p=0.0000
1 vs 5: Diff=-1.7000, 95%CI=-2.0589 to -1.3411, p=0.0000
1 vs 6: Diff=-2.2000, 95%CI=-2.5586 to -1.8414, p=0.0000
1 vs 7: Diff=-2.2000, 95%CI=-2.5584 to -1.8416, p=0.0000
1 vs 8: Diff=-2.2000, 95%CI=-2.5598 to -1.8402, p=0.0000

Source of Variation	Sum of Squares	d.f	Variance	F	P
Between Group	998192.6291	7	142598.947	389.132	0
Within Groups	26231504.53	71582	366.45		
Total	27229697.16	71589			

1 vs 2: Diff=7.8000, 95%CI=6.9321 to 8.6679, p=0.0000
1 vs 3: Diff=0.6000, 95%CI=-0.2648 to 1.4648, p=0.4135
1 vs 4: Diff=-0.2000, 95%CI=-1.0675 to 0.6675, p=0.9970
1 vs 5: Diff=2.0000, 95%CI=1.1333 to 2.8667, p=0.0000
1 vs 6: Diff=1.6000, 95%CI=0.7352 to 2.4648, p=0.0000
1 vs 7: Diff=-0.5000, 95%CI=-1.3651 to 0.3651, p=0.6534
1 vs 8: Diff=10.0000, 95%CI=9.1364 to 10.8636, p=0.0000