The effects of environmental contaminants and ocean acidification on reproductive success in marine invertebrates

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Abstract

Marine organisms are exposed to a range of environmental stressors, including anthropogenic pollutants and ocean acidification, which may have harmful effects. The reproductive processes of broadcast spawning marine invertebrates are considered to be particularly vulnerable to environmental insults. Predicting the potential impacts of ocean pollution and acidification on reproductive success is essential to anticipate the consequences of predicted marine change. This study evaluated the effects of exposure to the pharmaceutics diclofenac, ibuprofen and sildenafil citrate (Viagra), the metals cadmium and copper, and the endocrine disrupting compound nonylphenol (singly and under simulated ocean acidification scenarios; pH 8.1, 7.9 and 7.7) on sperm motility and fertilisation success of *Asterias rubens*, *Psammechinus miliaris* and *Arenicola marina*. Sperm motility was determined by Computer Assisted Sperm Analysis (CASA) and fertilisation success by the presence of embryonic cleavage at 60 minutes after fertilisation. Sperm motility (percentage of motile sperm and swimming speed) was reduced by nonylphenol concentrations $\geq 0.1\mu g/l$, diclofenac $\geq 1.0\mu g/l$, cadmium $1000\mu g/l$, copper $\geq 10\mu g/l$ and ocean acidification of pH 7.9 and 7.7. Exposure to $\geq 1.0\mu g/l$ of ibuprofen only affected *P. miliaris* sperm. Exposure of sperm of *A. rubens* and *P. miliaris* to sildenafil citrate at concentrations $\geq 18ng/l$ and $\geq 50ng/l$ respectively increased both percentage motility and swimming velocity. Sperm pre-incubated in test conditions prior to fertilisation showed significant reductions in fertilisation success in all single pollutant and ocean acidification scenarios with the exception of ibuprofen for which only *P. miliaris* was negatively affected. Pre-incubation with sildenafil citrate significantly increased fertilisation success of *A. rubens* and *P. miliaris* but not for *A. marina*. Curiously, the pre-incubated oocytes of *P. miliaris, A. rubens* and *A. marina* appeared remarkably robust to some of the pollutants at ambient pH. However, fertilisation was significantly inhibited when exposed to diclofenac, copper, cadmium and seawater pH 7.9 and 7.7 as single stressors. Fertilisation success of *P. miliaris* and *A. marina* was adversely affected by sperm-oocyte incubation of ≥ 20 minutes with $100\mu g/l$ and $100\mu g/l$ of ibuprofen respectively. Combined nonylphenol and elevated seawater pH significantly reduced sperm motility and fertilisation success. These results demonstrate the potential for marine invertebrates to be affected by single pollutants and suggest that these effects may be more severe under predicted ocean acidification conditions.

Dedication

This thesis is dedicated to the memory of my beloved Mom, Late Mrs Noriah Mat Noh.

Thank you for bringing me to this world; even though you are no longer here, you are always in my heart, love you Mom! Alfatihah.

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Chapter 1: Environmental Contaminants and Ocean Acidification

Abstract

Marine ecosystems face numerous environmental stressors; from organic and inorganic contaminants of human origin to major natural environmental stressors such as climate change (including ocean acidification and global warming). Many studies report the effects of contaminants and ocean acidification on marine organisms in isolation, with few studies providing a unified perspective. This chapter reviews the ecotoxicology of selected environmental contaminants – in relation to climate change where possible – including their occurrence, fate, and toxicological effects on aquatic organisms.

Effects on sperm motility and fertilisation success of selected marine invertebrates are examined in relation to organic and metal contaminants co-occurring with ocean acidification. Two keystone echinoderms (*Asterias rubens* and *Psammechinus miliaris*) and one keystone polychaete (*Arenicola marina*) were selected as test species. The activity of the pharmaceutical products diclofenac, ibuprofen, and sildenafil citrate, as emerging marine contaminants, the endocrine disruptor compound nonylphenol, and the metals copper and cadmium were assayed. Two predicted future ocean pH scenarios (pH 7.7, 7.9) were used to identify the ocean acidification effects. Synergistic or additive effects of these environmental stressors on sperm motility and fertilisation success were investigated.

This study furthers our understanding of the mechanisms that permit marine organisms to survive and reproduce in polluted habitats. This enables better prediction of the consequences of environmental stressors on the reproductive ecology of these keystone species and improves our capability to perform effective environmental risk assessments to prevent further ecological damage.

1.1 Pharmaceutical and Personal Care Products (PPCPs) as new environmental contaminants.

Pharmaceutical and Personal Care Products (PPCPs) are widely used in human and veterinary medicine and have become an emerging class of environmental contaminants (Fent et al., 2006). They are produced in large quantities and their increased presence within ecosystems is a cause for concern. This relatively new source of contaminant presents new challenges for the scientific community towards improving ecological and human risk assessments (Carlsson et al., 2006). Non-steroidal anti-inflammatory drugs (NSAID), antibiotics, lipid regulators, and neuroactive compounds are among the 3000 different substances usually used in human therapy and veterinary applications in the European Union (Fent et al., 2006).

The sale and consumption of PPCPs differs between countries. In Italy, tonnes of drugs are consumed annually (Calamari et al., 2003) while in Australia, England, and Germany, the figures are in the hundreds of tonnes (Huschek et al., 2004, Khan and Ongerth, 2004). After their consumption and therapeutic action the compounds are then excreted and finally end up in aquatic systems via different pathways such as domestic wastewater, disposal from hospitals, manufacturers' wastewater, landfill leachates, manure application to field, and direct application in aquaculture. Pharmaceutical compounds which are not fully degraded in wastewater treatment plants (WWTPs) are then released in treated effluents and result in contamination of surface water, groundwater, and drinking water (Sanderson et al., 2004, Kar and Roy, 2010). The routes of these PPCPs to aquatic ecosystems are well known; however, the behaviour and fate of PPCPs in the environment remains largely unknown. Awareness of these issues is increasing and there is an expanding number of studies assessing PPCPs in aquatic environments.

Clofibric was the first pharmaceutical compound reported and detected in treated wastewater in the USA. The concentration detected was 0.8µg/L (Garrison et al., 1976). In 1981, several PPCPs were detected in UK rivers with concentrations up to 1µg/L (Richardson and Bowron, 1985). Traces of ibuprofen and naproxen were also found in Canadian wastewater (Rogers et al., 1986). Advances in chemical analysis methods allowed polar compounds to be determined. Massive environmental analyses were performed in several countries and it was reported that these compounds were widely

spread in aquatic environments with 80-100 PPCPs detected in Europe and the USA from different medicinal classes (Parolini, 2009). Moderate concentrations of PPCPs were found in WWTP effluents (Halling-Sørensen et al., 1998, Ashton et al., 2004) and other dissimilar drugs were traced with concentrations between ng/L to low µg/L. Median level concentration of 76ng/L of propranalol, 3086ng/L of ibuprofen, 424ng/L of diclofenac, 133ng/L of mefenamic acid, 195ng/L of dextropropoxyphene and 70ng/L trimethoprime were reported by Ashton et al. (2004) after analysing samples taken from sewage treatment plant effluents in the UK. Lower concentrations and fewer PPCPs were detected in receiving stream. However, they were usually and often detected in surface waters.

In German municipal WWTP effluents, about 32 of these compounds were detected with different therapeutic classes in rivers and streams (Ternes, 1998). Wiegel et al. (2004) reported and analysed these compounds in the Elbe River, Germany and its tributaries. He reported various antibiotic, carbamazepine, diclofenac, ibuprofen, and lipid regulators as present. Similar compounds were traced in the Po and Lambro rivers in Italy where drug concentrations were traced in the ng/L range (Calamari et al., 2003). Detectable concentrations of 95 micro-contaminants were reported from 139 samples taken from water downstream of urban areas and livestock production in the USA (Kolpin et al., 2002). They also reported that steroids, insect repellents, caffeine, triclosan, 4-nonylphenol, and other PPCPs were among of the main substances detected from 38 sampling sites. Further research by Kolpin et al. (2004) indicated that NSAIDs were commonly detected in water upstream and downstream of Iowa city, USA with concentrations up to µg/L. Since carbamazepine and its metabolites are frequently detected in surface waters with concentrations of more than 1µg/L, this drug has been described as an anthropogenic marker in aquatic environments (Clara et al., 2004).

Despite the increasing occurrence of PPCPs in aquatic ecosystem, there remains a lack of precise regulations for environmental risk assessment. Starting with the United States Food and Drug Administration (FDA), environmental assessment on all veterinary medicines was applied which required an ecological appraisal of the pharmaceutical mix in veterinary drugs (Boxall et al., 2003). Later, European Union (EU) Directive 92/18 EEC and corresponding "Note for Guidance" (EMEA, 1998) determined that all veterinary medicines were required to undergo and pass ecotoxicology testing to be registered. According to a draft guideline (Directive

2001/83/EC)(Stumpp et al., 2012), environmental risk assessment was applied to all human medicines before being authorised to market (EMEA, 2005). The FDA once again drafted guidelines requiring potential PPCPs expected to be found in aquatic systems with concentrations exceeding 1µg/L to undertake environmental risk assessment (FDA-CDER, 1998). This is because several studies found that aquatic species were threatened by pharmaceutical exposure.

Pharmaceuticals are designed to have specific modes of action, with several persevering and significantly affecting the environment. They can also affect animals with similar target organs, tissues, cells, and biomolecules (Fent et al., 2006). The mode of action for some drugs is not well understood and they sometimes possess different mechanisms of action. This makes specific toxicity analyses in lower animals difficult to complete and, at present, there is little information on the ecotoxicological effects of pharmaceuticals on living organisms.

The presence of these compounds in aquatic environments has affected aquatic organisms. Following the established guidelines, acute toxicity testing of pharmaceuticals are performed on aquatic organisms from different trophic levels. Examples include cnidarians (Quinn et al., 2008), algae (Yang et al., 2008), crustaceans (Haap et al., 2008), fish (Choi et al., 2008), and mussels (Canesi et al., 2007a). By comparing different trophic levels, researchers found that algae are the most sensitive to PPCPs, followed by the crustacean *Daphnia magna* and then fish (Webb, 2001). However, these tests do not deliberate enough on the diverse modes of action of the various drugs; therefore differences in toxicity across phyla are not easily explainable. In terms of the toxicity of pharmaceuticals, antidepressant drugs were reported as most toxic, then antibacterial, and antipsychotic drugs were less toxic. However, the range of reactions inside each of these classes was large (Webb, 2001).

Fent et al. (2006) reported that only 17% of pharmaceuticals demonstrated an acute toxicity lower than 100mg/L and drugs like acetylsalicylic acid, betaxolol, sotalol, bezafibrate, gemfibrozil, cimetidine, and ranitidine are among the 38% of drugs that showed LC_{50} values higher than 100mg/L. These drugs were classified as non-toxic to aquatic organisms based on EU Directive 93/67/EEC. Fent et al. (2006) also stated that the classification of toxicity was difficult with 45% of pharmaceutical drugs indicating a wide variability of acute toxicity values. Jones et al. (2002) warned of overlooking the

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risks to aquatic organisms of a few common drugs such as paracetamol, ibuprofen, amoxicillin, oxitetracylin, and methenamic acid. Despite these warnings, efforts to expand this crucial environmental topic and knowledge of the effects of pharmaceutical pollutants on the reproduction success of aquatic organisms remains limited.

The European Commission enacted the Water Framework Directive (WFD) in response to the occurrence of harmful contaminants in aquatic ecosystems. The main goal of this directive is to 'ensure that all aquatic ecosystems and with regard to their water needs, terrestrial ecosystems and wetlands meet 'good status' by 2015' (WFD 2000/60/EC). The aquatic areas covered by this directive include freshwater and transitional water (the estuarine and coastal area up to one nautical mile or 1.85km from shore). Implementation of the WFD benefits the environment, society, and the European economy. Since its enactment on 22 December 2000, the WFD has listed 33 substances as known pollutants in aquatic ecosystems. On December 2012 (WFD 2013/39/EU), 12 new substances were listed under the WFD 'watch list'. These require that all rivers, lakes, and coastal waters be monitored and emission of these substances to aquatic ecosystems be controlled across Europe.

1.1.1   Pharmaceuticals used in this study
1.1.1.1 Diclofenac

Diclofenac (DCF; 2-[(2,6-dichlorophenyl)amino] phenylacetic acid) is an important NSAID used to treat painful and inflammatory conditions. The estimated overall yearly global production is >1000 tonnes (Fent et al., 2006). Diclofenac has been commonly identified in aquatic ecosystems, with the main pathway being from dermal application (Heberer and Feldmann, 2005) as only 5-10% of the drug is absorbed by the skin and the remainder is discharged into water through washing (Letzel et al., 2009).

Diclofenac has a low removal rate during wastewater treatment. The concentrations traced in sewage treatment plant effluent from North and South America and Europe are at low μg/L (Deng et al., 2003, Lindqvist et al., 2005, Gómez et al., 2007). Surface water concentrations range from a few ng/L up to the μg/L level (Lindqvist et al., 2005, Gros et al., 2006) (Table 1.1). However, its ecotoxicological effects on non-target organisms are unexplained.

The first case regarding diclofenac was reported with the decline of the Indian Gyps vulture population due to renal failure (Oaks et al., 2004). Until now, limited numbers of studies have reported effects on aquatic organisms. Several acute toxicity tests were recorded using *Vibrio fischeri* (Zhang et al., 2008), algae, *Daphnia magna* (Cleuvers, 2003, Ferrari et al., 2003), and zebrafish, (*Danio rerio*) (Dietrich and Prietz, 1999). From Effective Concentrations (EC_{50}) obtained, diclofenac demonstrated low acute toxicity. The test concentrations used were higher compared with the measured concentrations in the aquatic environment.

It is more important to assess the effects of chronic diclofenac exposure. A study on rainbow trout found that liver, kidney, and gills were affected by 1μg/L (Triebskorn et al., 2004). Exposure of Japanese medaka fish, *Oryzias latipes* showed cellular, genotoxic, and oestrogenic effects (Hong et al., 2007). Cytogenotoxicity was also found when diclofenac was exposed to haemocytes of the zebra mussel, *Dreissena polymorpha* (Parolini et al., 2009). Even though several studies showed the effects of diclofenac on aquatic organisms, until now no research has investigated the effects of diclofenac on invertebrate reproductive success.

Table 1.1 Concentration of diclofenac in aquatic environments.

Water type	Year	Measured maximum concentration (ng/L)	Source
Sewage treatment	1999	280	Mons et al. (2000)
Surface water	1999	20	Mons et al. (2000)
Surface water	2005-2006	84	Versteegh et al. (2007)
Drinking water	2005-2006	18	Versteegh et al. (2007)
Surface water	2006	0.6-843	Ankley et al. (2007)
Rhine water	2009	900	Ter Laak et al. (2010)
Sewage treatment	2010	460-3300	Pal et al. (2010)
Surface water	2010	21-41	Pal et al. (2010)
Surface water	2012	0.7-2.4	Gros et al. (2012)
Surface water	2013	4.1	Petrović et al. (2014)

1.1.1.2 Ibuprofen

Ibuprofen (IBU; ((+/-)-2-(p-isobutylphenyl) propionic acid with R and S isomers) is an NSAID, and is produced and sold in large quantities. The drug is considered to be fairly persistent (Bendz et al., 2005), biocumulative (Schwaiger et al., 2004, Maurer et al., 2007), water soluble, of low volatility (Breton and Boxall, 2003), and has a low tendency for absorption onto organic matter (Fent et al., 2006, Johnson et al., 2007). Ibuprofen can remain in the aquatic phase once discharged into the sewage systems. Ibuprofen has been traced in ng/L and µg/L ranges in river, estuaries, and wastewater treatment plant effluents of Europe and North America (Kolpin et al., 2002, Ashton et al., 2004, Thomas and Hilton, 2004, Wiegel et al., 2004).

However, there is a lack of ecotoxicological information regarding ibuprofen. Acute effect 96 hour EC_{50} of >100 mg/L and a No Observed Effect Concentration (NOEC) of 30 mg/L were reported in *Mysidopsis (Americamysis) bahia* (Barrows, 1986). A 48 hour EC_{50} toxicity (swimming immobility) for *Daphnia magna* ranged between 10-100mg/L (Cleuvers, 2003, Han et al., 2006, Heckmann et al., 2007). Pounds et al. (2004) found 17.1mg/L for a 96 hour Lowest Observed Effect Concentration ($LOEC_{50}$) for *Planorbis carinatus*. Low toxicity of ibuprofen was also reported in fish. Studies found 23.9mg/L for a 48 hour Lethal Concentration (LC_{50}) for embryos of *Danio rerio* (Kehrer and Nagel, 2006) and a 24 hour LC_{50} of 142mg/L for *Cirrhinus mrigala* (Saravanan et al., 2012). A 72 hour EC_{50} of 72.9mg/L was calculated for growth rate of the freshwater green algae *Desmodesmus subspicatus* (Cleuvers, 2004b).

In *Daphnia magna*, a chronic survival NOEC of 20-33mg/L was recorded after 21 days exposure (Han et al., 2006, Heckmann et al., 2007, Han et al., 2010) and 50mg/L for 10-12 days exposure. For reproduction and population growth, a 14 days exposure EC_{50} and survival NOEC of 13.4mg/L and 20mg/L were reported respectively (Heckmann et al., 2007). Chronic toxicity tests were also performed on fish. For *Oryzias latipes*, 144 days long-term exposure resulted in a NOEC for survival of 0.1µg/L (Han et al., 2010). No chronic effects was reported for the cnidarian *Hydra vulgaris* for seven days exposure up to 1mg/L (Pascoe et al., 2003). In another study a seven day 1mg/L exposure was sufficient to produce a 25% growth reduction of duckweed, *Lemna minor*, with as EC_{50} of 4mg/L. However, exposure to 10µg/L for seven days stimulated the growth of the cyanobacteria *Synechocystis* spp. (Pomati et al., 2004). From the reported

studies ibuprofen will likely affect aquatic biota although huge gaps still exist in examining the effects of this drug on marine organisms.

1.1.1.3 Sildenafil citrate (Viagra)

Sildenafil citrate, also known as Viagra, has the chemical formula $C_{22}H_{20}N_6O_4S$. Sildenafil citrate is a known phosphodiesterase-5 (PDE_5) inhibitor (PDE_5 blocker) and is used to treat erectile dysfunction (Althof et al., 2006). It has various beneficial erectogenic actions in the management of pulmonary hypertension, female sexual dysfunction, enhanced female genital blood flow, endometrial thickening, some gastrointestinal disorders and Raynaud's phenomenon (Grant and El-Fakahany, 2004). Since its introduction in 1998 until 2004, over 600,000 doctors have prescribed sildenafil citrate to over 16 million men (David et al., 2008). As a result of its huge success, the marketing strategy has extended to include younger men, so that sildenafil citrate is used increasingly by men of reproductive age (Aldridge and Measham, 1999, Smith and Romanelli, 2003).

Several studies have examined the effects of this drug on humans, unfortunately there are very limited ecotoxicology studies. The only study on aquatic organisms was on the teleost *Danio rerio* by Rocco et al. (2012). The fish were exposed to 26.25ng/L for up to 35 days to examine genotoxic effects. Results indicated that a significant loss of DNA integrity was observed after 35 days of exposure. The authors concluded that sildenafil citrate does not damage fish DNA in short-term exposures (up to 14 days) but damage did occur from 21 days onwards. There is huge knowledge gap regarding the toxicological effects of this drug on the aquatic ecosystems. The presence of this drug has been traced in sewage treatment plant effluents at ng/L levels. Proper environmental risk assessment needs to be done as the consumption and the production of this drug increases.

1.2   Nonylphenol

Nonylphenol is a term used to refer to a group of isomeric compounds each consisting of a nine-carbon alkyl chain attached to a phenol ring, with the chemical formula $C_{15}H_{24}O$. Major derivatives of nonylphenol are nonylphenol ethoxylates and nonylphenol acetic acid (also called nonylphenol ethoxycarboxylates).

Nonylphenol is a known endocrine disruptor and is capable of interfering in the endocrine system of various organisms. Nonylphenol is an end product by degradation of nonylphenol ethoxylates (Soares et al., 2008). It is hydrophobic, has low mobility, and is not soluble in water (Barber et al., 1988). The main source of nonylphenol in surface waters and sediments appears to be related to the release of effluent from sewage treatment works, proximity of industrialisation/urban areas, or related to anthropogenic activities (Hale et al., 2000). Table 1.2 presents the concentrations of nonylphenol reported in rivers, oceans, estuaries, and sediment in several countries.

Table 1.2 Concentration of nonylphenol reported in rivers, oceans, estuaries, and sediment in several countries.

Country	Sample Source	Concentration	References
USA	River water	<1µg/L	Naylor et al. (1992)
	River sediment	<300mg/kg	
Switzerland	River water	0.015-0.25µg/L	Ahel et al. (1994b)
	River sediment	3520mg/kg	
UK	River water	0.02-180µg/L	Blackburn (1995)
Canada	Lake and river water	0.01-0.92µg/L	Bennie et al. (1997)
	River sediment	0.17-72mg/kg	
UK	River water	23-53µg/L	Nico et al. (1998)
Italy	River water (1994)	158µg/L	Davì and Gnudi (1999)
	River water (1996)	8.8µg/L	
Canada & USA	Lake sediment	<37mg/kg	Bennett and Metcalfe (1998)
Portugal	River and ocean water	<10µg/L	Azevedo et al. (2001)
USA	River sediment	<1240mg/kg	Hale et al. (2000)
Germany	River water	0.0007-0.004µg/L	Bester et al. (2001)
	Estuaries	<0.03µg/L	
	Marine sediment	0.01-0.153mg/kg	
	Sea sediment	<0.01-0.055mg/kg	
Germany	River water	0.0006-0.135µg/L	Kuch and Ballschmiter (2001)
Japan	River water	0.051-1.08µg/L	Isobe et al. (2001)
	River sediment	0.5-13.0µg/L	

USA	River sediment	0.01-60mg/kg	Kannan et al. (2001)
Spain	River water	15µg/L	Petrovic et al. (2002b)
	River sediment	0.022-0.645mg/kg	
Spain	Ocean water	0.15-4.1µg/L	Petrovic et al. (2002a)
	Harbour sediments	0.008-1.05mg/kg	
Germany	River water	0.028-1.22µg/L	Fries and Püttmann (2003)
Holland	Estuary water	0.031-0.934µg/L	Jonkers et al. (2003)
	Estuary sediments	0.0004-1.08mg/kg	
USA	River water	0.1-0.5µg/L	Rice et al. (2003)
	River sediment	0.075-0.340mg/kg	
Canada	River water	<0.092µg/L	Sabik et al. (2003)
	River sediment	0.0403-0.293mg/kg	
Korea	River water	0.0232-0.1876µg/L	Li et al. (2004)
	River sediments	0.0254-0.932mg/kg	
UK	Surface water	0.011µg/L	Darbre et al. (2009)

Dodds and Lawson (1938) first discovered that alkylphenol could be oestrogenic. Later, Soto et al. (1991) found that nonylphenol could initiate proliferation in breast tumour cells by mimicking natural hormones. In the aquatic environment, the first case was the appearance of bisexual fish due to the presence of nonylphenol in the River Lea, southern England. Sumpter and Jobling (1995) measured vitellogenin concentrations of rainbow trout and nonylphenol concentrations in river water, mainly downstream of sewage treatment plants. As a result, it was suggested that one of the causes could be alkylphenol, especially nonylphenol from detergents used for cleaning wool and textile mills. Since then, there has been extensive study of the toxic effects of nonylphenol on aquatic organisms.

Studies regarding the effects of nonylphenol to endocrine systems of various organisms have been thoroughly assessed (White et al., 1994, Laws et al., 2000). Bacteria, algae, invertebrates, and fish are among the aquatic organisms affected by nonylphenol. For example, Jobling et al. (1996) found that nonylphenol was capable to inducing the production of female proteins in rainbow trout (*Oncorhynchus mykiss*) at 20.3mg/L (Jobling et al., 1996)) and medaka fish (*Oryzias latipes*) at 0.2mg/L (Tabata et

al., 2001). Kinnberg et al. (2000) reported that chronic exposure to ≥0.96mg/L for 4 weeks negatively affected testis morphology and male fertility of platyfish (*Xiphophorus maculates*). Nonylphenol was reported to disrupt liver development of tilapia (*Oreochromis spilurus*) at chronic exposure to 15µg/L and 30µg/L from larvae until fish were matured (Abdulla Bin-Dohaish, 2012). Further, exposure to sublethal concentrations in *Oreochromis mossambicus* showed that nonylphenol caused genetic damage (Balakrishnan et al., 2014). Many more effects of nonylphenol have been identified in fish such as reduced fertilisation, embryonic and larval toxicity, reduced growth and smoltification (Madsen et al., 1997, Ashfield et al., 1998a, Chaube et al., 2013).

Toxicity of nonylphenol to aquatic invertebrates has also been reported. A study using bivalve molluscs showed reduced respiration, absorption efficiency, reduced superoxide dismutase activity, and decreased re-burrowing and destabilisation of hemocyte lysosomal membranes (Matozzo and Marin, 2005, Canesi et al., 2007b). Cakal and Parlak (2007) reported malformations in the skeleton system of early life stages of the sea urchin, *Arbacia lixula* at concentration up to 18.74mg/L. The authors concluded that nonylphenol presents a major risk to normal development of this species. Several of the recorded toxicity values are included in Table 1.3.

Table 1.3 Toxicity values for nonylphenol and important observations regarding aquatic organisms.

Organism	Development Stage	Range of Toxic Concentrations	References
Daphnia magna	Adult	EC_{50}, 300µg/L NOEC, 24µg/L	(Comber et al., 1993)
Algae	Adult	EC_{50}, 25-750µg/L	(Naylor, 1995)
Invertebrates	Adult	LC_{50}, 20-90µg/L	(Naylor, 1995)
Fish	Adult	LC_{50},130-1400µg/L	(Naylor, 1995)

Fishes, bivalves, crustaceans	Adult	EC_{50}, 17-195µg/L	(Lussier et al., 2000)
Pseudokirchneriella subcapitata (algae)	Adult	EC_{50}, 500-530µg/L	(Graff et al., 2003)
Littoral zooplankton	Adult	NOEC, 1µg/L	(O'Halloran et al., 1999)
Fundulus heteroclitus (killifish)	Embryos	EC_{50}, 5400µg/L	(Kelly and Di Giulio, 2000)
	Newly hatched larvae	EC_{50}, 210µg/L	
	2-week old larvae	EC_{50}, 210µg/L	
	4-week old larvae	EC_{50}, 260µg/L	

- EC_{50}- Effective concentration of the nonylphenol that produces a response in 50% of the population development by 50%
- LC_{50}- Lethal concentration that reduces the population by 50%
- NOEC-Non-observed effect concentration

There are numerous studies on acute and chronic toxicity effects using different methods and tests on many species to assess nonylphenol toxicity to aquatic organisms. However, the number of studies addressing the effects of this substance on the gametes of marine invertebrates, especially to broadcast spawners are relatively small.

1.3   Metals
1.3.1 Copper

Copper is an essential trace nutrient required in small amounts (most likely 5-20µg/g) by humans, other mammals, fish, and shellfish for carbohydrate metabolism and the functioning of more than 30 enzymes. The highest concentrations are found in decapod crustaceans, gastropods, and cephalopods in which the respiratory pigment haemocyanin contains copper (Tipping, 2009, Tessier et al., 2011). Copper is a widely distributed contaminant in aquatic environments especially at coastal and estuarine sites close to outputs of anthropogenic activities including road runoff, antifouling coatings, and sewage as well as in areas with a copper mining heritage (Bryan and Langston, 1992, Schiff et al., 2004). In coastal and estuarine waters total dissolved copper concentration were detected at ranges between 0.004µM (Jones and Bolam, 2007) and 1.61µM (Bryan and Gibbs, 1983) in highly polluted environments. The 76/464/EEC

directive environmental quality standard (EQS) for copper is stated at 5μg/L. Table 1.4 show historic trends in concentrations (nM) of some metals in the ocean (Swarzenki et al., 2000).

Table 1.4 Historic trends in concentrations (nM) of some metals in the ocean waters (Nozaki, 1993, Swarzanki et al., 2000).

Element	1963	1975	1983	1992	1995
Fe	180	36	0.7	0.6	0.02-1
Cu	50	8	2	2	0.5-4.5
Ag	3	0.3	0.03	0.02	0.001-0.023
Au	0.02	0.02	0.06	0.00015	0.00005-0.00015
Pb	0.2	0.2	0.005	0.005	0.03-0.15
Bi	0.1	0.1	0.05	0.00015	0.00002-0.0025

Although numerous metals are necessary for biological functions, excessive levels may cause an organisms' antioxidant defences to fail and induce oxidative damage of cellular components such as lipid, protein and DNA via the production of reactive oxygen species (ROS). Interruption of ion transport especially Na^+, K^+ and Ca^{2+} (Stagg and Shuttleworth, 1982) and impaired osmoregulatory capacity (Blanchard and Grosell, 2006) have been reported in fish when exposed to sublethal copper concentrations. Bryan (1976b) reported that several aquatic organisms were affected by 1-10μg/L of copper. Young bay scallops (*Argopecten irradians*) and soft clams (*Spisula solidissima*) were significantly affected when exposed to 2μg/L (Nelson et.al., 1988). Copper was reported to reduce fertilisation success of the coral *Goniastrea aspera* - exposure of gametes to 20μg/L resulted in a 41% reduction in fertilisation success (Reichelt-Brushett and Harrison, 1999). Copper also reduced fertilisation success of the sea urchin *Paracentrotus lividus* (Warnau et al., 1996, Arizza et al., 2010) and Japanese oyster *Crassostrea gigas* (Geffard et al., 2001). A similar result was obtained when the polychaete *Galeolaria caespitosa* was copper exposed (Cam et al., 2007). Further, copper exposure during early development of the polychaete *Hydroides elegans* lead to morphologic abnormalities in embryos and larvae and reduced fertilisation success (Gopalakrishnan et al., 2006). Despite these studies, there are still knowledge gaps on the impacts of copper exposure to sperm motility and the eventual impact of this on fertilisation success.

1.3.2 Cadmium

Unlike copper, cadmium is not an essential element for any organism. The presence of cadmium in aquatic environments normally results from natural and anthropogenic sources. The sea adopts cadmium mobilised from the crust through riverine and atmospheric input. OSPAR (2002) reported cadmium content was detected at 5-20ng/L in open seas, 80-250ng/L in French and Norwegian coastal zones, and 10-100ng/L in European rivers. In addition, cadmium was recorded in sediments from rivers and lakes at 5mg/kg and 0.03-1mg/kg in marine sediments.

Cadmium is comparatively soluble compared to other metal contaminants, can be accumulated by marine organisms, and may lead to poisoning (Pascal et al., 2010a). AMAP (2002) reported that exposure of lake trout to different cadmium concentrations affected foraging behaviour and resulted in lower success prey capture and behavioural changes. Exposure to sublethal concentrations in polychates (*Perinereis nuntia*) delayed sexual maturity, reduced fertilisation and zygote hatching, and increased vitellogenin expression. The authors concluded that cadmium has strong feminisation effects on this species (Zheng et al., 2010). Early developmental stages of the polychaete *Hydroides elegans* are very sensitive to cadmium (Gopalakrishnan et al., 2008); however, cadmium exposure did not affect fertilisation success in the coral *Goniastrea aspera*. A robust understanding of the effects of cadmium on invertebrate sperm is, however, still lacking.

1.4 Ocean acidification

Burning fossil fuels releases carbon dioxide (CO_2) into the Earth's atmosphere, causing seawater warming and changing seawater chemistry as the oceans absorb approximately 30% of anthropogenic CO_2 (Cialis and Sabine, 2013). However, beyond a certain level of atmospheric CO_2, the oceans can no longer act as carbon sinks without changing its carbonate chemistry (Feely et al., 2004).

The ocean's pH is slightly alkaline; around 8.1-8.2 pH in surface seawater. Excess CO_2 dissolves in surface seawater and forms carbonic acid (H_2CO_3) which dissociates in seawater and releases hydrogen ions and bicarbonates ions HCO_3^-. The same process occurs where bicarbonate ions dissociate and release hydrogen ions to

form carbonate ions (CO_3^{2-}) (see equation 1). Increasing hydrogen ion concentrations in seawater decreases the seawater pH and the oceans become more acidic - a process called ocean acidification (Cooley, 2012).

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \; H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \quad [equation\ 1]$$
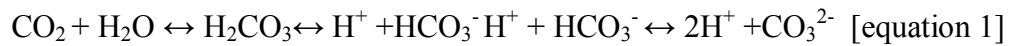
Carbonate ions are essential for marine calcifiers to form skeletons or shells. However, increased carbonic acid in the oceans decreases carbonate ion availability. Marine calcifiers use the minerals calcite and/or aragonite to build shells and skeletons. Bicarbonate ions will associate with calcium ions forming calcium bicarbonate (equation 2) in the seawater, which is subsequently used to form calcium carbonate (equation 3) (Sabine et al., 2004, Dickson et al., 2007). Ocean acidification reduces the saturation states of carbonate ions and the calcium carbonate ($CaCO_3$) biominerals (calcite and aragonite) (Table 1.5).

$$Ca^{2+} + 2HCO_3^- \rightarrow Ca(HCO_3)_2 \quad [equation\ 2]$$
$$Ca(HCO_3)_2 \rightarrow CaCO_3 + HCO_3 + H^+ \quad [equation\ 3]$$
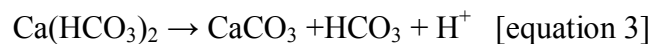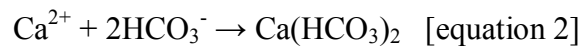
As atmospheric CO_2 continues to increase, surface seawater pH is predicted to reduce from a level of 8.1-8.2 (current pH) by 0.3-0.5 units (pH 7.6-7.9) by the year 2100 and is estimated to decline by between 0.7-0.77 (pH 7.33-7.5) by 2300 (Portner and Farrel, 2008, Buck and Folger, 2009, Ross et al., 2011, Bopp et al., 2013).

Table 1.5 Average changes to ocean chemistry and pH based on ocean carbon models and surface ocean measurements (Roleda and Hurd, 2012).

Parameter	Symbol	Unit	Preindustrial	Present	2 x preindustrial	3 x preindustrial	4 x preindustrial
pCO_2 in seawater	pCO_2	µatm	280	380	560	840	1120
Carbonic acid	H_2CO_3	µmol/kg	9	13	19	28	38
Bicarbonate ion	HCO_3^-	µmol/kg	1768	1867	1976	2070	2123
Carbonate ion	CO_3^{2-}	µmol/kg	225	185	141	103	81
Mean surface pH			8.18	8.07	7.92	7077	7.61
pH change relative to preindustrial				-0.11	-0.26	-0.41	-0.53
Calcite saturation	$\Omega_{calcite}$		5.3	4.4	3.3	2.4	1.9
Aragonite saturation	$\Omega_{aragonite}$		3.4	2.8	2.1	1.6	1.2

Ω = Saturation state

Ocean acidification has led to changes to the carbon chemistry of seawater. Predicted changes for future seawater carbon chemistry are closely related to increases in carbon dioxide in the atmosphere, i.e. ocean acidification is emissions-dependent (Bopp et al., 2013). Thus in the 5th Assessment report (AR5) of the Intergovernmental Panel on Climate Change (IPCC), (IPCC, 2013) they developed an 'emissions scenario' as 'Representative Concentration Pathways' (RCPs) (Figure 1.1). Under the lowest IPCC emissions (RCP 2.6), atmospheric carbon dioxide concentration will peak at ~443 ppm in 2050 before declining to ~421 ppm by 2100 corresponding to a reduction of global mean surface ocean pH of ~0.1 units. Under the low emissions scenario (RCP 4.5) and moderate emissions scenario (RCP 6.0), atmospheric carbon dioxide concentration will reach ~538 ppm and ~670 ppm respectively and the mean global reduction of surface ocean pH would be at least 0.15 and 0.2 units respectively. Under the current emissions trajectory (which is the highest emission scenario (RCP 8.5)), atmospheric carbon dioxide concentration will reach ~936 ppm by the end of this century with a decline of the mean surface ocean pH by at least 0.3 units. The equates to an increase of 25% of H^+ for lower changes of pH and 170% of H^+ for higher changes pH in addition to the 25% increase that has already happened since the industrial revolution.
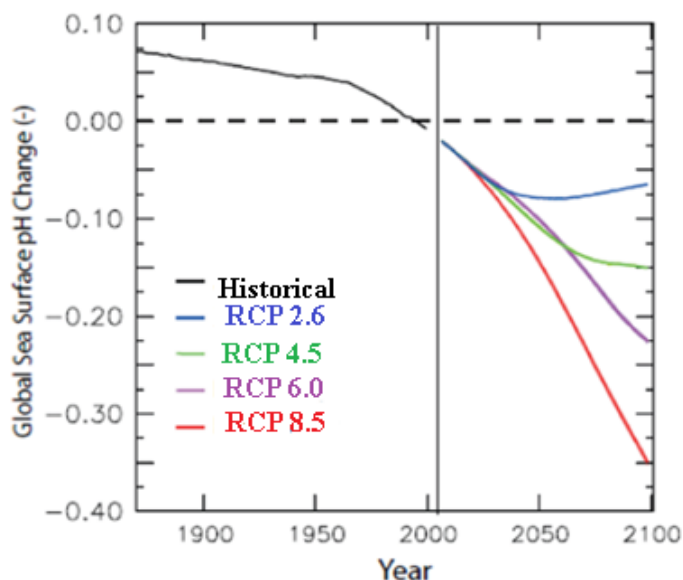
Figure 1.1 Historical and projected changes in global surface ocean pH over 1970-2100 for the four IPCC AR5 scenarios. Model means from the Climate model Intercomparison Project (Bopp et al., 2013).

However, the predicted decline in global surface ocean pH will not be uniform, instead it will vary regionally (Figure 1.2). Atmospheric carbon dioxide solubility and basin-scale circulation will determine patterns of future change according to latitudinal differences. The aragonite saturation horizon, i.e. the depth of ocean that is saturated with dissolved aragonite (the more soluble form of calcium carbonate) is predicted to rise by year 2100 from a few thousands meters to just a few hundred metres in many temperate and tropical oceans (Orr et al., 2005). In this condition, temperature effects carbon chemistry. Any addition of atmospheric carbon dioxide to the ocean at higher latitude regions will result in a larger decline of ocean surface pH.
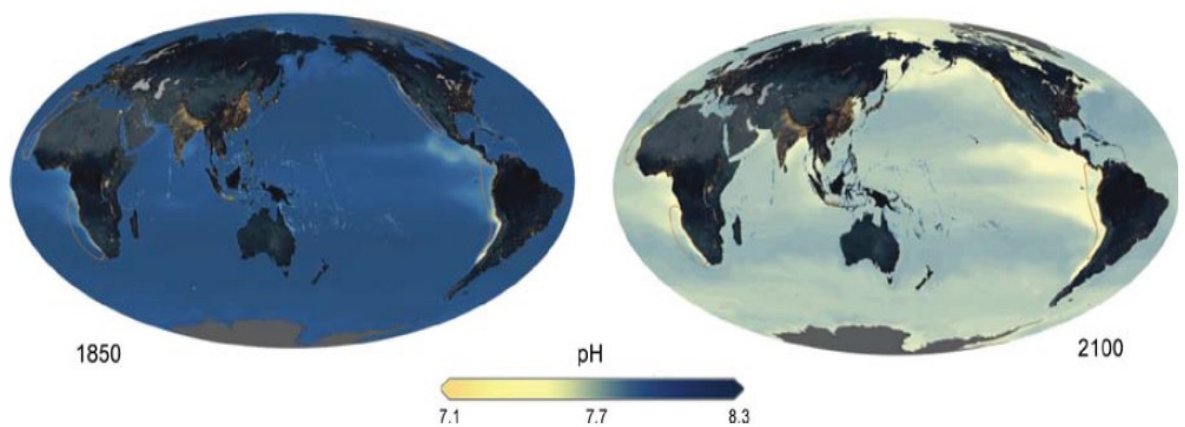
Figure 1.2 Model-derived maps of historical (1850) and projected (2100) ocean surface pH with the latter based on the IPCC RCP 8.5 emission trajectory (IGBP, 2013).

In the Southern Ocean, 450ppm of atmospheric carbon dioxide is sufficient to make this ocean region under-saturated with respect to aragonite (McNeil and matear, 2008). Moreover, much of the surface waters of the Arctic Ocean are projected to become under-saturated for aragonite throughout the year within the next 50 years under most emissions scenarios (Figure 1.3) (Steinacher et al., 2009, Denman et al., 2011a, Bopp et al., 2013).
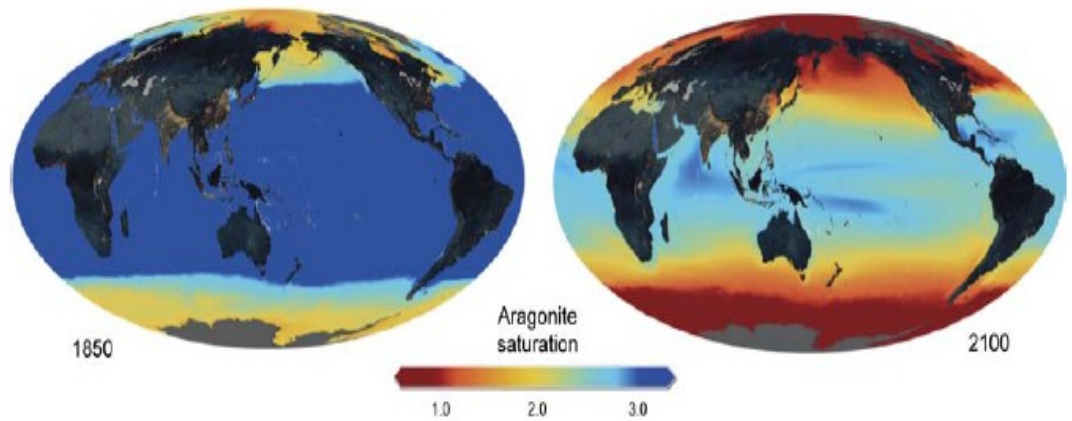
Figure 1.3 Model-derived maps of historical (1850) and projected (2100) aragonite saturation state, with the latter based on the IPCC RCP 8.5 emission trajectory (IGBP, 2013).

1.4.1 Potential effects of ocean acidification on marine organisms

Many marine organisms such as corals, crustaceans, molluscs, and certain algae are sensitive and vulnerable to small changes in seawater pH. These species are mainly marine calcifiers. Marine calcifiers use minerals present in seawater to build their shells or skeletons, as the oceans are supposed to be supersaturated with aragonite, calcium, and high-magnesium calcite. Elevated levels of CO_2 in seawater produces carbonic acid which decreases the level of mineral saturation. If seawater becomes under saturated, the shells and skeletons of marine calcifiers could dissolve and endanger the species.

Calcifying species may struggle to exist above a certain depth known as the 'saturation horizon'. Ocean acidification is expected to shift the saturation horizon up to 50-200 meter closer to the surface from its position during the 1800s (Doney, 2006). The Southern and Arctic Oceans are naturally colder and more acidic than the other ocean basins. It has been predicted that by end of this century species with aragonite shells (weaker mineral forms of calcium carbonate) cannot survive in these oceans (EurOCEAN, 2007). This is because aragonite is more soluble than calcite in acidic water. In early larvae stages, several marine organisms start to synthesise the skeleton by depositing amorphous calcium carbonate crystals. These larval calcareous structures are 30 times more soluble compared to adult deposited structures (Table 1.6).

Table 1.6. Calcium carbonate polymorphs deposited by marine larvae (Ross et al., 2011).

Organism	Skeleton type*	Mineralogy
Echinoderms	Endoskeleton	Amorphous calcium carbonate, high-magnesium calcite
Molluscs	Exoskeleton	Amorphous calcium carbonate, aragonite, calcite^, high-, or low- magnesium calcite
Crustaceans	Exoskeleton	Amorphous calcium carbonate, high-magnesium calcite

*Primary skeleton type but not limited to

^Usually after settlement

Ocean acidification will also affect pre-larval life stages, particularly the gametes (Kurihara, 2008a) (Kurihara et al., 2007, Kurihara, 2008a, Parker et al., 2009, Byrne et al., 2009, Dupont et al., 2010a). This will likely impact the fertilisation and cleavage processes with possible reductions in embryo quality and fitness. These fitness compromises may have 'carry-over' consequences on larval development, dispersal, settlement and survival (Figure 1.4). This may ultimately result in population decreases (Figure 1.5).

Figure 1.4 Potential ocean acidification impacts on marine life stages (Kurihara, 2008a).
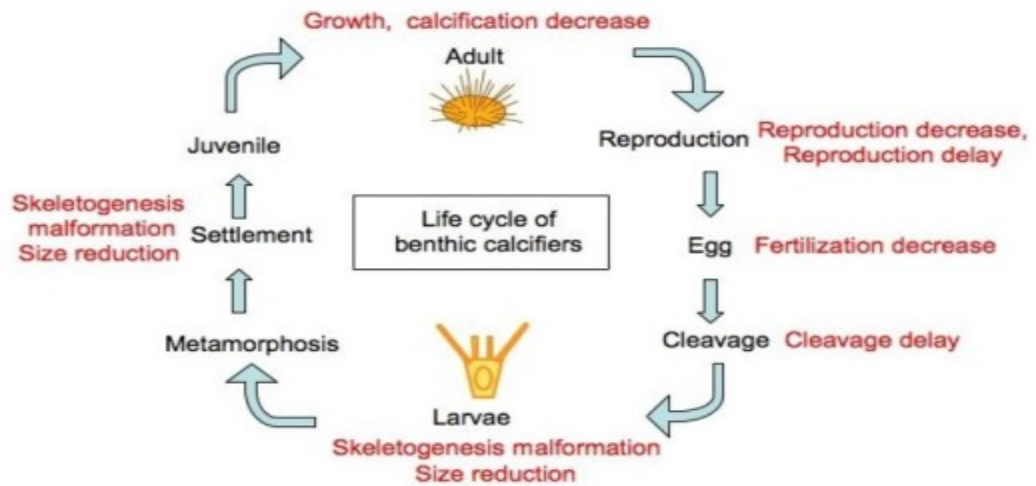
Figure 1.5. Summary of the 'carry-over' consequences on marine organisms (Kurihara, 2008a).

Numerous studies have examined the effects of ocean acidification on early life stages of marine benthic invertebrates. For example, Kurihara et al. (2007) found the fertilisation rates and embryo development of the oyster *Crassostrea gigas* were compromised when gametes were exposed to 2268 μatm pCO_2 with malformed shells in more than 80% of the D-shaped larvae grown in elevated-CO_2 seawater. The effects of acidified water were more pronounced in early life stages of benthic calcifiers relative to adults. Since most of these species possess planktonic larval stages, high mortality rates might affect the adult population size (Green et al., 2004). Ocean acidification also affected the sea urchins *Hemicentrotus pulcherrimus* and *Echinometra mathaei* by reducing fertilisation success, developmental rates, larval size, and spicule skeletogenesis (Kurihara and Shirayama, 2004a).

In the sea urchin *Hemicentrotus erythrogramma* elevated pCO_2 reduced sperm swimming speed by 11.7%, percentage sperm motility by 16.3% and fertilisation success by 24.9% (Havenhand et al., 2008b). In contrast, no significant effects were found on fertilisation success, cleavage, and gastrulation using the same species from a different geographical location when exposed to reduced pH (pH 7.6-8.25) but with the extra factor of exposure to different temperatures (19-26°C) (Byrne et al., 2009, Byrne et al., 2010a, Byrne et al., 2010b). It is believed that the temperature can buffer the effect of elevated seawater pCO_2. Similar results were obtained for sperm swimming speed and fertilisation success with no significant reduction observed for *Crassostrea gigas* from either Japan or Sweden (Kurihara et al., 2007, Havenhand and Schlegel, 2009).

Byrne et al. (2010a) found no reduction in fertilisation of *Heliocidaris erythogramma, H. tubercula, Tripneustes gratilla, Centrostephanus rodgersil* and *Patiriella regularis* when exposed to elevated CO_2, increasing temperature, and interaction between elevated CO_2 and temperature. Synergism between elevated CO_2 and the range of sperm concentrations did not show any reduction in fertilisation success of these species (Byrne et al., 2010b, Ericson et al., 2010b, Reuter et al., 2011a). Sperm concentration becomes a crucial factor for marine organisms when other conditions are suboptimal (Parker et al., 2013). Exposure to elevated CO_2 seawater (pH 7.9) did not affect the fertilisation success *Strongylocentrotus franciscanis* (Ericson et al., 2010b). Many reports indicated the effects of ocean acidification on the health and physiology of marine invertebrates. 63% of echinoderms and 51.6% molluscs were used to test ocean acidification which had a negative impact on them (Lewis et al., 2016).

1.4.2 Potential interaction ocean acidification with contaminants

Ocean acidification has the potential to alter behavioural and speciation characteristics of many marine contaminants and thus may alter their bioavailability and toxicity. Many environmental chemicals form complexes with the ions and organic materials in seawater and these complexes can be pH sensitive (Martin and Whiteley, 2016). Metals can be found in high concentration along coastal and estuarine environments and are some of the most common coastal contaminants (Bryan and Longston, 1992). Depending on metal type, ocean acidification is expected to alter the bioavailability of waterborne metals by either increasing or decreasing their free ion concentration (Millero et al., 2009). Bioavailability alteration will potentially affect the uptake and bioaccumulation kinetics of these metals if exposed to marine organisms.

There is evidence reported that lethal and sub-lethal toxicity effects of low level metal contamination are significantly increased when organisms are exposed to metals under ocean acidification conditions. Roberts et al. (2013) looked at the metal fluxes from contaminated sediment under predicted ocean acidification conditions and evaluated toxicity impacts of this combination to the infaunal crustacean *Corophium volutator*. No significant changes were found in metals fluxes, instead mortality and DNA damage was observed under high exposure of carbon dioxide. DNA damage was detected 1.7 fold in the clean area sediment at 1,140μatm pCO$_2$, however, 2.7 fold DNA damage was observed in the contaminated sediment at just 750μatm pCO$_2$. In a different

study using the tubeworm *Pomatoceros lamarckii*, the larvae of this species had lower survival under future ocean scenarios when there was also a low level of copper present in the seawater (Lewis et al., 2013).

Ocean acidification can also change the physiological consequences of metals for organisms in a species-specific manner, that may subsequently affect their fitness and survival. Ocean acidification was reported to cause an increase of accumulation of copper and cadmium in the mollusc gill and led to increases in adenosine monophosphate (AMP) and reductions in glycogen, ATP and ADP. An increase in AMP indicates an increase in energy deficiency as a result of a combination of exposure to copper and the elevated partial pressure of carbon dioxide (Gotze et al., 2014).

A more acidic ocean will also likely affect organic degradation due to an alteration of metal speciation and nutrient bioavailability. One example is iron. Iron is one of the metal cofactors that are crucial in microbial pathways in organic pollutants and oil degradation (Bugg, 2003). The reduction of iron bioavailability caused by a reduction in seawater pH will promote potentially harmful effects on degradation of organic pollutants and oils. Ocean acidification has also been recorded to cause declines in oxygen levels in the ocean (Diaz and Rosenberg, 2008, Hofmann and Schellnhuber, 2009, Rabalais et al., 2014). Processes for microbial hydrocarbon degradation require oxygen. Depletion of the oxygen level will reduce microbial activities and consequently cause a decline in degradation as anaerobic degradation has a lower degradation rate under ordinary circumstances (Erses et al., 2008).

Absorption or binding of colloidal organic matter with organic pollutants in the ocean will form organic-metal-mineral complexes and Jurado et al. (2005) reported more than 50% were formed in this complex in the ocean. Ocean acidification also causes dissolution of metals ions and destroys organic metal complexes and potentially could release organic pollutants back into the biosphere. In addition, ocean acidification will likely delay the biodegradation of organic contaminants resulting in an increase in bioavailability and toxicity of metals (Erses et al., 2008). One such example was reported by (Olaniran et al., 2011). The authors claimed that the biodegradation of 1,2-dichloroethane was inhibited by the presence of metals in an acidic ocean and the decreasing inhibition effect was followed as $Hg^{2+} > As^{3+} > Cd^{2+} > Pb^{2+}$.

1.5 Sperm motility and fertilisation success

Sperm, as the male reproductive cell, performs an essential biological function to transfer the male genome to the female oocyte – i.e. the process of fertilisation. For species with motile (swimming) sperm the first step in this process is sperm motility activation. Sperm motility is activated in response to diffusible molecules contained within the jelly layer of the oocyte; this initiates a chemotactic response in the sperm that allows sperm to move towards the oocyte and contact the jelly. Subsequently, the sperm acrosome reaction is triggered by a rise in internal calcium levels inside the sperm head. The sperm is then fused to the oocyte plasma membrane.

Studies regarding sperm movement are extensive (Kaupp et al., 2008, Guerrero et al., 2010, Yoshida and Yoshida, 2011, Alvarez et al., 2012); however, studies of the effects of environmental stressors on sperm motility of broadcast spawners are limited. In marine organisms, especially broadcast spawners where fertilisation occurs externally in the water column, a motile sperm is crucial. As sperm are released to the water column they must overcome local turbulence and the problems caused by sperm dilution. Further, as sperm are directly exposed to seawater they are potentially vulnerable to the presence of toxins within the aqueous environment, particularly toxins that may affect ATP metabolism, calcium signalling and microtubule contractions. If sperm lose the ability to swim they lose the ability to find oocytes in the water column to fertilise. Lewis and Galloway (2010) stated that, "*sperm are the most diverse cell type known suggesting strong evolutionary pressures acting on their production, which does not reconcile well with the idea of wastage*". Therefore, further research regarding sperm of aquatic organisms is urgently needed. It will be beneficial to know how sperm are affected by environmental stressors within aquatic ecosystems and the severity of the impacts of those stressors.

To achieve high rates of fertilisation success broadcast spawning species employ synchronous spawning strategies generally with high levels of sperm production as a means to maximise sperm-oocyte contact (Powell et al., 2001). Sperm and oocytes that are released into the water column are unprotected and therefore exposed to environmental stressors surrounding them; some of which may be able to disrupt the fertilisation process. Forbes et al. (2010) found that fertilisation and early post-fertilisation development is the most sensitive part of the life history, where external factors can easily disrupt these processes. Ringwood (1992) stated that fertilisation

assays are fast and easy compared to other larval assays, however, these tests are hardly completed with any concern for natural spawning that occurred in the environment for the sampled species.

The optimum sperm concentration will result in high fertilisation success, while high concentrations of sperm may result in decreased fertilisation success due to polyspermy. Marshall (2006) concluded that exposure of sperm to environmental contaminants will reflect on fertilisation success. Decreased sperm swimming ability due to environmental contaminants will have a similar effect on fertilisation success. Reducing sperm swimming ability will eventually reduce the chances of sperm collisions with oocytes and result in reduced fertilisation kinetics and fertilisation success of broadcast spawners (Lewis and Watson, 2012).

1.6   Species used in the study

One polychaete specie, *Arenicola marina*, and two echinoderm species, *Asterias rubens* and *Psammechinus miliaris* were used in this study. Each species is seasonally reproductive and therefore has a particular seasonal pattern of maturation and spawning. Gametes of *A. rubens* are available in early spring, *P. miliaris* in early summer, and *A. marina* in late autumn/early winter. Each of these broadcast spawning benthic invertebrates are keystone species within their particular habitats. Each occupies a differing trophic niche; *A. marina* is a deposit feeder, *A. rubens* is predatory, and *P. miliaris* is herbivorous. Due to their differing trophic niches, each species may presents a particularly vulnerability to toxin exposure (Sugni et al., 2007). For instance, *A. marina* which has a close association with sediments (which generally have heavier toxin burdens that the overlying water column) may respond differently than the echinoderm species that do not have as close an association with sediments. This niche-based approach may ultimately allow for the development of a more tailored (and hopefully more effective) environmental risk assessment.

Echinoderms are often used as bioindicators of environmental quality (Zito et al., 2005). As they are benthic species they are mainly vulnerable in the presence of micropollutants stored in marine sediments. As the experiments concern sperm motility and fertilisation, it is important to have species that will provide enough gametes to run the trials. Echinoderms produce enormous quantities of gametes and are fertilised externally (in seawater). The gametes are simple to develop, optically clear, and have

virtually identical embryos. The embryo is readily visible under light making it easier to identify the development of the embryo (Hermalin et al., 1981, Zito et al., 2005).

The polychaete, *Arenicola marina* is also used as a bioindicator species in ecotoxicological studies (Dean, 2008). Moreover, *A. marina* is easy to find and sample, occurs in high density, and has synchronised spermatogenesis periods.

1.7   Aims and objective of the study

The main aim of this research is to investigate the effects of environmental stressors on marine invertebrates by looking at sperm motility and fertilisation success as endpoints. The percentage of motile sperm and sperm swimming speed were measured for sperm motility. For fertilisation success, three types of experiments were performed; exposure of sperm to environmental stressors before fertilisation, exposure of oocytes to environmental stressors before fertilisation, and fertilisation following sperm and oocytes that were both exposed to environmental stress. These different experiments allow us to know which reproductive cell is more affected by the stressor. The emphasis of this study is ecotoxicological awareness of the effects of natural stressors and contaminants in the aquatic environment on marine invertebrates. The objectives are;

1) to explore the effects on sperm motility and fertilisation success on exposure of pharmaceuticals or organic contaminants; diclofenac, ibuprofen, and sildenafil citrate;
2) to assess the effects of copper and cadmium on sperm motility and fertilisation success;
3) to study the effects of sperm motility and fertilisation success when exposed to reduced pH seawater;
4) to determine the effects of nonylphenol and reduced pH seawater on sperm motility and fertilisation success.

The expected outcomes from this study will offer important insights into the mechanisms that permit marine invertebrates to survive and reproduce in polluted surroundings and habitats. We will better understand and predict the consequences of environmental stressors on their reproductive ecology of these key groups of marine invertebrates.

Chapter 2: The Effects of Diclofenac, Ibuprofen and Sildenafil Citrate (Viagra) on Sperm Motility and Fertilisation Success of Selected Marine Invertebrates

Abstract

Exposure to synthetic chemicals is a key environmental challenge faced by aquatic organisms. This chapter studies the time and concentration effects of the pharmaceuticals diclofenac, ibuprofen, and sildenafil citrate on sperm motility and successful fertilisation using the echinoderms, *Asterias rubens* and *Psammechinus miliaris*, and the polychaete worm *Arenicola marina*. Motility was reduced for all species when exposed to concentrations $\geq 0.1\mu g/L$ of diclofenac. Exposure to $\geq 1.0\mu g/L$ of ibuprofen only affected *P. miliaris* gametes and fertilisation success of *A. marina*. *A. rubens* and *P. miliaris* sperm increased in both percentage motility and swimming velocity when exposed to sildenafil citrate at concentrations $\geq 18ng/L$ and $\geq 50ng/L$, respectively. Pre-incubation of sperm with sildenafil citrate significantly increased fertilisation success in *A. rubens* and *P. miliaris* but not in *A. marina*. Pre-incubated *A. rubens* oocytes fertilised successfully in ibuprofen. According to EU Directive 93/67/EEC, diclofenac is classified as a vey toxic substance to gametes of *A. rubens, P. miliaris,* and *A. marina* (EC_{50}=100-1000$\mu g/L$) while ibuprofen is classified as very toxic to gametes of *P. miliaris* but non-toxic to gametes of *A. marina* (EC_{50}>10,000$\mu g/L$). The present study indicates that diclofenac may have negative impacts on the reproductive success of *P. miliaris, A. rubens,* and *A. marina*, whereas ibuprofen potentially may compromise *P. miliaris* reproduction. This study provides valuable insight into the mechanisms that allow marine invertebrates to survive and reproduce in contaminated and changing habitats.

2.1 Introduction

Pharmaceutical compounds are a growing class of contaminants within the broad category of pharmaceutical and personal care products (PPCPs). These compounds have become an environmental concern due to the large quantities which end up in the environment (Dietrich et al., 2002). Many pharmaceuticals and their metabolites are detected in wastewater and sewage treatment plants, which are implicated as the primary sources of environmental discharge (Daughton and Ternes, 1999). Despite most pharmaceuticals being detected in the nanogram-per-litre (ng/L) to low microgram-per-litre (µg/L) ranges in aquatic environments (Fent et al., 2006, Kasprzyk-Hordern et al., 2008, Triebskorn and Hetzenauer, 2012), the risks to aquatic biota cannot be excluded because of high biological and pharmacokinetic activity; for example, Jobling et al. (2003) demonstrated that exposure to 1ng/L and 10ng/L of ethinylestradiol affects embryo production of the molluscs *Potamopyrgus antipodarum* and *Pimephales promelas*.

Another concern regarding pharmaceutical contaminants is their physiological activity which may potentially affect non-target species (Fent et al., 2006, Ankley et al., 2007b). This is exemplified by declining vulture populations in India and Pakistan due to consuming animal diclofenac-treated carcasses, leading to renal failure and visceral gout (Oaks et al., 2004). Even though studies have found that pharmaceutical contamination may affect reproduction of aquatic animals (Hayashi et al., 2008, Memmert et al., 2013), research regarding pharmaceutical toxicity on the reproduction success of aquatic life, especially for marine invertebrates, remains scarce. This translates to inadequate risk assessment (Ringwood et al., 2003).

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely consumed drugs category (Fent et al., 2006). NSAIDs are primarily used for analgesic and anti-inflammatory purposes to relieve symptoms of rheumatic disorders and fever (Hayashi et al., 2008). NSAIDs, known as prostaglandin blockers, act as non-selective inhibitors for one or both isoforms of the cyclooxygenase enzyme, (cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2)) by interfering with the inflammatory mediator. They have been detected in significant quantities in municipal effluent (Tixier et al., 2003).

In the present study, two commonly used NSAIDs, diclofenac and ibuprofen were selected for laboratory experiments with selected marine invertebrates. Concentrations of diclofenac and ibuprofen in seawater range between 0.6-843ng/L and 0.01-2370ng/L, respectively (Fent et al., 2006, Gaw et al., 2014). Several studies recognise the relationship between diclofenac and ibuprofen exposure and impacts on aquatic organisms and ecology. For instance, exposure of *Salmo trutta* f. *fario*, (brown trout) to diclofenac at 0.5, 5.0 and 50.0 µg/L for up to 14 days resulted in significantly reduced haematocrit level and can adversely affect various organs and compromise the health of affected fish populations (Hoeger et al., 2005). Cytogenotoxic effects were seen in haemocytes of the freshwater zebra mussel (*Dreissena polymorpha*); further, Ericson et al. (2010a) found that exposure to ≥100µg/L diclofenac significantly affected growth of *Mytilus edulis* and *M. trossulus*.

Sildenafil citrate, also known as Viagra, is a phosphodiesterase type 5-inhibitor (PDE5 blocker) and is widely used to treat human male erectile dysfunction. Sildenafil citrate concentrations in wastewater are detected up to 10ng/L (Nieto et al., 2010, Fr. Schröder et al., 2010). The presence of sildenafil citrate and its metabolites in aquatic environments raises the question as to whether they may or may not affect aquatic organisms. There are almost no studies on the effects of sildenafil citrate on aquatic organisms. Rocco et al. (2012) published that sildenafil citrate affects DNA integrity of the teleost *Danio rerio* (zebrafish) and concluded that sildenafil citrate exerts genotoxic damage.

There is increasing awareness of the presence of pharmaceuticals and their metabolites in aquatic environments and their effects on aquatic flora and fauna. The European Council Directive 2001/83/EC (EC, 2001) concluded an environmental risk assessment (ERA) should be conducted before authorising the marketing of a medicinal product for human use. A two-phased (Phase I and Phase II) tiered assessment concept has been proposed for ERA of pharmaceuticals by The European Agency for the Evaluation of Medical Products (EMEA) (EMEA, 2005). Phase I is to estimate predicted environmental concentrations in surface water (PEC_{sw}). If the PEC_{sw} value is below 0.01µg/L, and exhibits no environmental concern, no further assessments are required. However, if the PEC_{sw} value is more than 0.01µg/L a Phase II environmental effect analysis is required.

This study assesses the effects of exposure to environmentally realistic concentrations of diclofenac, ibuprofen, and sildenafil citrate for a range of exposure periods on reproduction success of selected echinoderms, *A. rubens* and *P. miliaris,* and a polychaete worm *A. marina* where sperm motility and fertilisation success were assessed as endpoints. Recent advances in technology and computer analysis have enabled cell motility to be measured by computer assisted sperm analysis (CASA). This method has been applied extensively to sperm motility studies (WHO, 1992, Young et al., 1995, Kime et al., 1996). Moreover, several parameters, including motile percentage (% MOT), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), straightness (STR), and wobble (WOB), have been developed to characterise motility. Technological advances have therefore made it possible to investigate the toxicity effects of organic and metals contaminants on various organisms using cell motility bioassays. Thus, the percentage of motile sperm and curvilinear velocity were used to assess sperm quality in this study. Fertilisation success was evaluated both with and without pre-incubation of sperm and oocytes in the test pharmaceuticals. The results should allow for a better understanding of the effects of pharmaceutical contaminants on the reproductive success of ecologically important marine invertebrates and potentially permit extrapolations to predict population effects.

2.2 Material and methods

2.2.1 Collection and maintenance of animals

The seasonal nature of the reproductive strategies of the studied species dictates that gravid animals are available for no more than two or three months per year (Table 2.1). This enabled a research programme covering approximately nine months of the year.

Table 2.1. The availability of gravid animals and spawning time of the studied species.

Species	Fully Gravid
Asterias rubens	Early April – Late May
Psammechinus miliaris	Early July – Early September
Arenicola marina	Late October – Late December

2.2.1.1 *Asterias rubens*

Mature *A. rubens*, collected using fishing creels from the Amble coast from the end of March to early May, were transported in seawater to the laboratory at Newcastle University and held in flow-through tanks at 5°C and constant darkness until required. Animals were used within one week.

2.2.1.2 *Psammechinus miliaris*

P. miliaris were collected from two different locations around the west coast of Scotland. In 2010, only animals from the Isle of Cumbrae were used. Animals were collected from the Oban coast during July 2011 and 2012. Urchins were transported to the laboratory in portable tanks filled with seawater with ambient conditions and aerated by a portable electric pump. In the laboratory they were placed in flow-through tanks at 10°C and photoperiod 12L:12D until required. Animals were used within one week.

2.2.1.3 *Arenicola marina*

Mature *A. marina* were collected by digging during low tide, using a flat pronged fork from beaches at Alnwick during late October to late December of the three years of this investigation. Animals were selected for digging by identifying the head and tail burrow shafts. The size of the cast is related to the size of the worms allowing larger specimens (i.e. more likely to be mature) to be selected. Once removed from the sand, they were placed into buckets containing small amounts of seawater and sand, and returned to the laboratory where they were sexed by observation of the gametes present in the coelomic cavity under bright illumination. A high density of sperm morulae forms a milky white suspension, whereas oocytes give the coelomic fluid a granular orange appearance (Pacey and Bentley, 1992). Where this was inconclusive, a small drop of coelomic fluid was removed using a disposable syringe fitted with a 21-g hypodermic needle and examined under a light microscope. Following sexing, the animals were kept individually in plastic containers filled with 0.22μm filtered fresh seawater (FFSW) and kept at 5-6ºC, which is close to the ambient temperature for that time of year (November and December). The animals were checked daily, the water was changed, and plastic containers were regularly cleaned from mucus until use. The animals were left for at least 24 hours before being used in the experiments to allow gut contents to be voided.

2.2.2 Spawning induction, collection of gametes and preparation of test solutions

2.2.2.1 *Asterias rubens*

Stock solutions of 10^{-4} M 1-methyl adenine (1-MeAde) were prepared in 100ml FFSW. Individual seastars were placed in approximately 150ml FFSW in large bowls. Depending on the size of the animal, between 1-2ml of 10^{-4} M 1-MeAde solution was injected through one of the upper arms, just before it joins the oral disc, giving an approximate concentration within the animal of 1×10^{-6} M (Williams and Bentley, 2002a). Spawning commenced within 40 minutes. Once the seastars were removed the oocytes were collected, washed twice in FFSW and transferred to a beaker containing approximately 50ml FFSW. Oocytes were stored on ice until required. Oocyte density was determined by counting micro volumes (three replicates of 2µl of oocytes for each female used) under a compound microscope and calculating the average.

Males were spawned by injecting 1- MeAde using the same injection procedure, and sperm was collected as it was extruded from the gonopores using a pipette and deposited into Eppendorf tubes. It was kept 'dry' and stored on ice until used (Caldwell et al., 2002a).

2.2.2.2 *Arenicola marina*

Spawning was induced by injecting homogenised prostomia from donor gravid females (Pacey and Bentley, 1992). Donor females were left undisturbed for several minutes to evert their prostomia. The prostomia were then gripped in forceps and removed using scissors. The prostomia were cleaned of excess tissues under a binocular microscope and placed in a 1.5ml Eppendorf tube (three prostomia per tube) with 1ml of filtered (0.22µm) fresh seawater (FFSW). The prostomia were homogenised over ice using a sonicator and the homogenate drawn into a 1ml syringe with a 21g hypodermic needle (Williams, 1999). Approximately 300µl of homogenate was injected into each female (equivalent to one prostomium per female (Pacey and Bentley, 1992)). Females were injected the night before they were needed for experiments and left at 5-6ºC overnight to spawn. Oocytes were collected using a Pasteur pipette, left to settle in Eppendorf tubes and stored on ice until used (Williams, 1999).

Sperm maturation was induced by injection of 8,11,14 eicosatrienoic acid (1mg/ml) directly into the coelomic cavity. A concentration of 13.3µg/g of body mass was used for each animal (Pacey and Bentley, 1992). Spawning followed after approximately one hour. To prevent sperm activation before it was required, sperm was collected 'dry' (undiluted), deposited in an Eppendorf tube and kept on ice. The sperm samples were left to coalesce and the excess water was pipetted off.

2.2.2.3 *Psammechinus miliaris*

P. miliaris were induced to spawn by injection of 0.5-1ml of 1.5M isotonic potassium chloride. Gametes were collected in Eppendorf tubes and stored on ice until required (Caldwell et al., 2004).

2.2.3 Preparation of test solutions

Ibuprofen (CAS no 15687-27-1), diclofenac sodium salt (CAS 15307-79-6), and sildenafil citrate (CAS no171599-83-0) were obtained from Sigma-Aldrich UK, with chemical purity of 98%. These chemicals stock solutions were prepared in high-performance liquid chromatography grade methanol (Sigma-Aldrich UK). Methanol concentrations did not exceed 0.001% v/v in any experiment.

2.2.4 Sperm motility and sperm swimming velocity assay

Sperm motility was measured using a computer assisted sperm analysis system (CASA). Sperm movement was recorded using a camera attached to a Nikon microscope with a phase contrast objective lens at 20x10 magnification. The system captured images of sperm heads at a rate of 100 frames per second. Six to nine replicates of sperm suspension (1000µl) were prepared for each treatment according to the times and concentrations required (Table 2.2) and mounted on clean, concave glass slides. Sperm motility for each sample was recorded for five seconds in ten microscopic fields (50-100 sperm per field) providing a total of 60-90 fields for each treatment and time interval. Curvilinear velocity (µm/s) was assessed, which represents the time-averaged velocity of the sperm head along the actual trajectories of individual spermatozoa.

Table 2.2. The time and concentrations prepared for sperm motility and swimming velocity assay.

Species	Test Solution	Concentration	unit	Time (minutes)
Asterias rubens	Diclofenac	0.0, 0.01, 0.1, 1.0,10.0, 100.0, 1000.0	µg/L	
	Ibuprofen	0.0, 0.05, 0.1, 1.0, 2.0, 3.0, 30.0, 50	µg/L	0, 20, 40, 60
	Sildenafil citrate	0.0, 2.0, 10.0, 18.0, 50.0, 100.0	ng/L	
Psammechinus miliaris	Diclofenac	0, 0.01, 0.1, 1.0,10.0, 100.0	µg/L	
	Ibuprofen	0.0, 0.01, 0.1, 1.0,10.0, 100.0	µg/L	0, 20, 40, 60
	Sildenafil citrate	0.0, 2.0, 10.0, 18.0, 50.0, 100	ng/L	
Arenicola marina	Diclofenac	0.0, 0.01, 0.1, 1.0,10.0, 100.0, 1000.0	µg/L	
	Ibuprofen	0.0, 0.01, 0.1, 1.0,10.0, 100.0, 1000.0	µg/L	0, 30, 60, 90, 120
	Sildenafil citrate	0.0, 2.0, 10.0, 18.0, 50.0, 100.0, 1000.0	ng/L	

2.2.5 Effects of oocytes pre-incubation with pharmaceuticals on fertilisation success

Unfertilised oocytes were washed with FFSW to remove coelomic fluid before being counted and pooled. Two hundred and fifty oocytes were incubated in 1ml of test medium; either solvent control (FFSW), diclofenac, ibuprofen or sildenafil citrate at set concentrations and times in plastic Eppendorf tubes at 15ºC. After incubation, the oocytes were washed three times with FFSW and transferred into 24-well microplates to which pooled sperm from three males was added to give final concentrations of 2.5×10^6 sperm ml^{-1}. Formalin (10% v/v) was used to stop development and preserve the oocytes. Fertilisation success was scored by the presence of embryonic cleavage at 60 minutes post fertilisation using a Zeiss inverted microscope (Caldwell et al., 2004) (Caldwell et al., 2002b).

2.2.6 Effects of sperm pre-incubation with pharmaceuticals on fertilisation

Unfertilised oocytes not exposed to chemicals were obtained. Sperm at a concentration of 5×10^6 sperm ml^{-1} were incubated in a set of concentrations and times of solvent control (FFSW), diclofenac, and ibuprofen, or sildenafil citrate at 15ºC. After each set time, 250µl of sperm was added to the unfertilised oocytes to give a final concentration of 2.5×10^6 ml^{-1}. Fertilisation success was determined as in 2.2.4.

2.2.7 Effects of pharmaceuticals on in vitro fertilisation success

Samples with a concentration of 2.5×10^6 ml^{-1} sperm and 250 unfertilised oocytes were incubated in 1ml each of set concentrations of the test solutions, in 24-well microplates for 60 minutes. Fertilisation success was determined as in 2.2.4.

2.2.8 Statistical analysis

All statistical analyses were performed using SPSS (V17). Percentage data were arcsine transformed, while log transformed data was used for curvilinear velocity (VCL). All data were back transformed for presentation. No-observed-effect (NOEC) and lowest-observed-effect concentrations (LOEC) were determined by 2-way analysis of variance (ANOVA) when assumptions for normality and homoscedasticity were met (Shapiro-Wilk and Levene test, respectively). The significance level was set at $\alpha=0.05$. Significant ANOVAs were followed by a Dunnett's post hoc test to compare treatment means with control means. Steel's many-one rank test was applied to determine the NOEC or LOEC endpoints if tests for normality and homoscedasticity failed. Later, the Tukey post hoc test was used to identify differences among groups and identify any interaction effect between times and chemical concentrations. Data that did not fulfil normality and homoscedasticity assumptions were subjected to non-parametric Kruskal-Wallis tests followed by Wilcoxon-Mann-Whitney post hoc test. All figures and tables present the mean ± standard error (SE).

Probit analysis was used to calculate the EC_{50} (half minimal effective concentration) value with 95% confidence limits and fitting the regression equation arithmetically by taking the log of the concentrations used versus the probit value of percentage of immotile sperm and unfertilised oocytes. If the immotile or unfertilised

percentage in control was more than 10% the results with treatment samples were corrected using Abbot's formula (APHA, 1981):

Corrected % = $(Pe - Pc)/(100 - Pc) \times 100$ (Abbott, 1925)

Pe = Experimental percentage

Pc = Control percentage

2.3 Results

2.3.1 Sperm motility and sperm swimming velocity experiments

2.3.1.1 Diclofenac

Figure 2.1 presents data on sperm motility and curvilinear velocity of *A. rubens* when exposed to different diclofenac concentrations with increasing time. There was a significant decrease in percentage sperm motility and VCL when exposed to $\geq 1\mu g/L$ at ≥ 20 minutes. Sperm motility and VCL declined in a clear concentration- and time-dependent manner (Table 2.3).
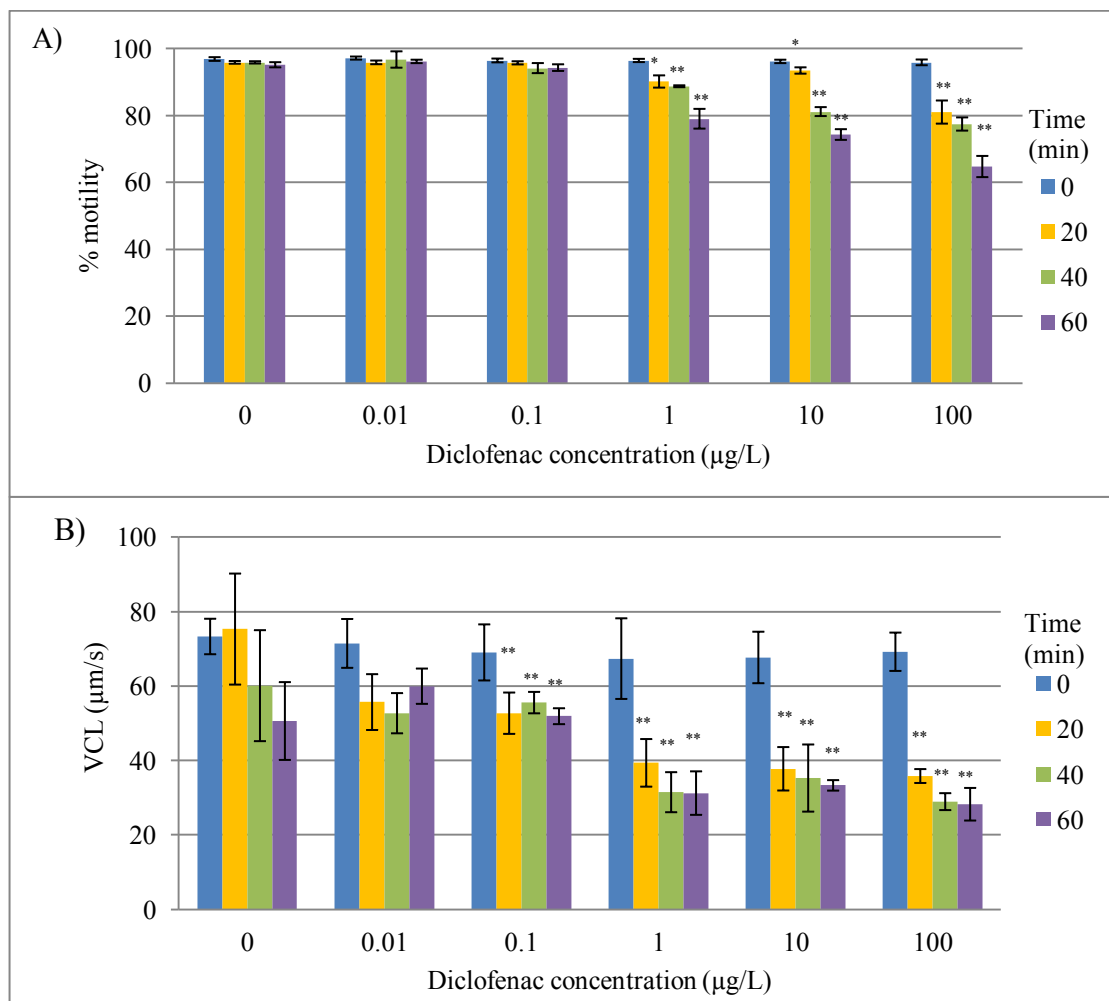
Figure 2.1. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Asterias rubens* spermatozoa after exposure to diclofenac for up to 60 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *$p<0.05$; **$p<0.01$.

Figure 2.2 presents data on the effects of exposure to diclofenac with increasing time on percentage sperm motility and VCL of *P. miliaris* sperm. Motility was negatively affected at concentrations $\geq 0.1 \mu g/L$ at 60 minutes. However, a significant reduction was seen at ≥ 20 minutes exposure to $1.0 \mu g/L$. A significant decline in VCL was observed when sperm were exposed to $\geq 0.1 \mu g/L$ at all time intervals. Sperm motility and VCL declined with a significant interaction between time and concentration ($F=2.573$, $p=0.001$; $F=2.301$, $p=0.003$ respectively; Table 2.3).
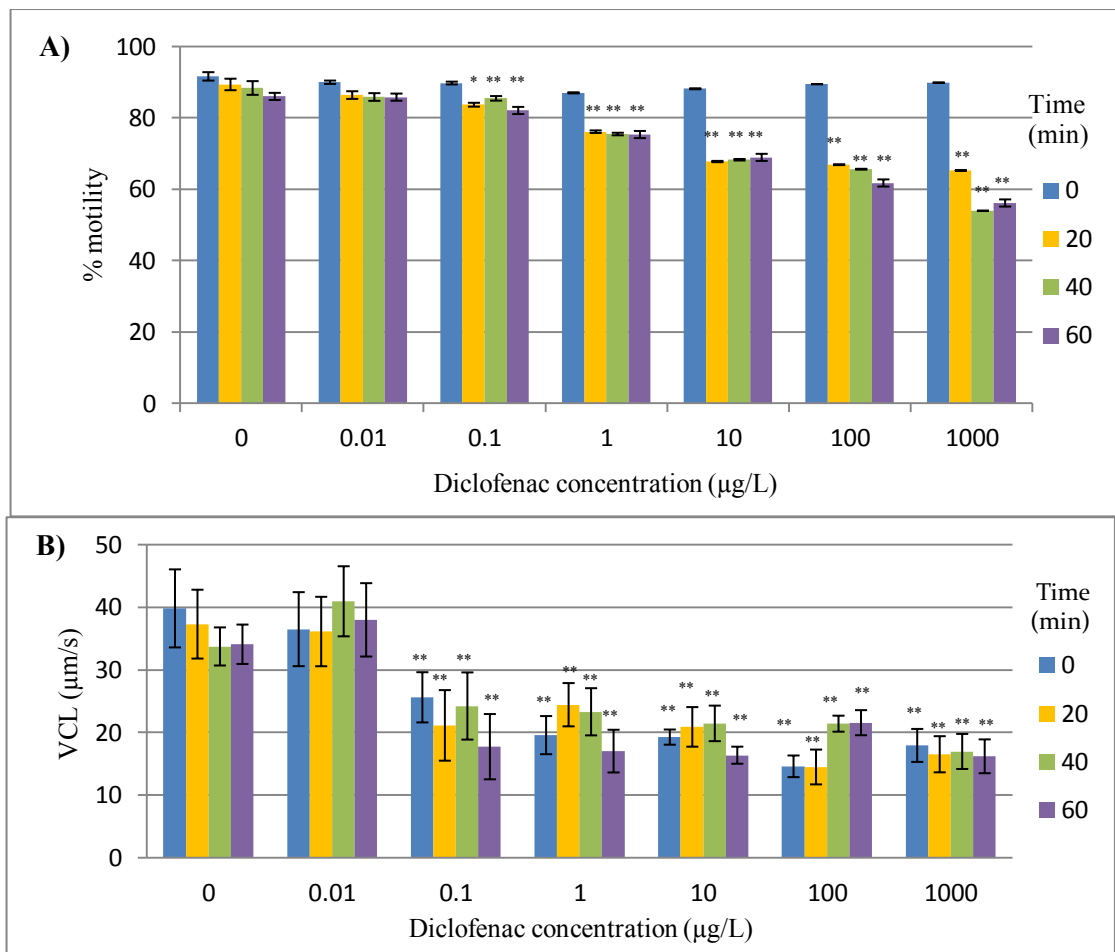
Figure 2.2. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Psammechinus miliaris* spermatozoa after exposure to diclofenac for up to 60 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Figure 2.3 shows the effect on percentage sperm motility and VCL of *A. marina* sperm when exposed to a concentration range of diclofenac with increasing time up to 120 minutes. Sperm motility reduced when exposed to $\geq 1\mu g/L$ at ≥ 90 minutes. Percentage motility was affected by concentration and time (F=1.776, p=0.018); however, for VCL diclofenac concentration had no significant effect. VCL was only reduced in time and interaction between time and concentration (Table 2.3).
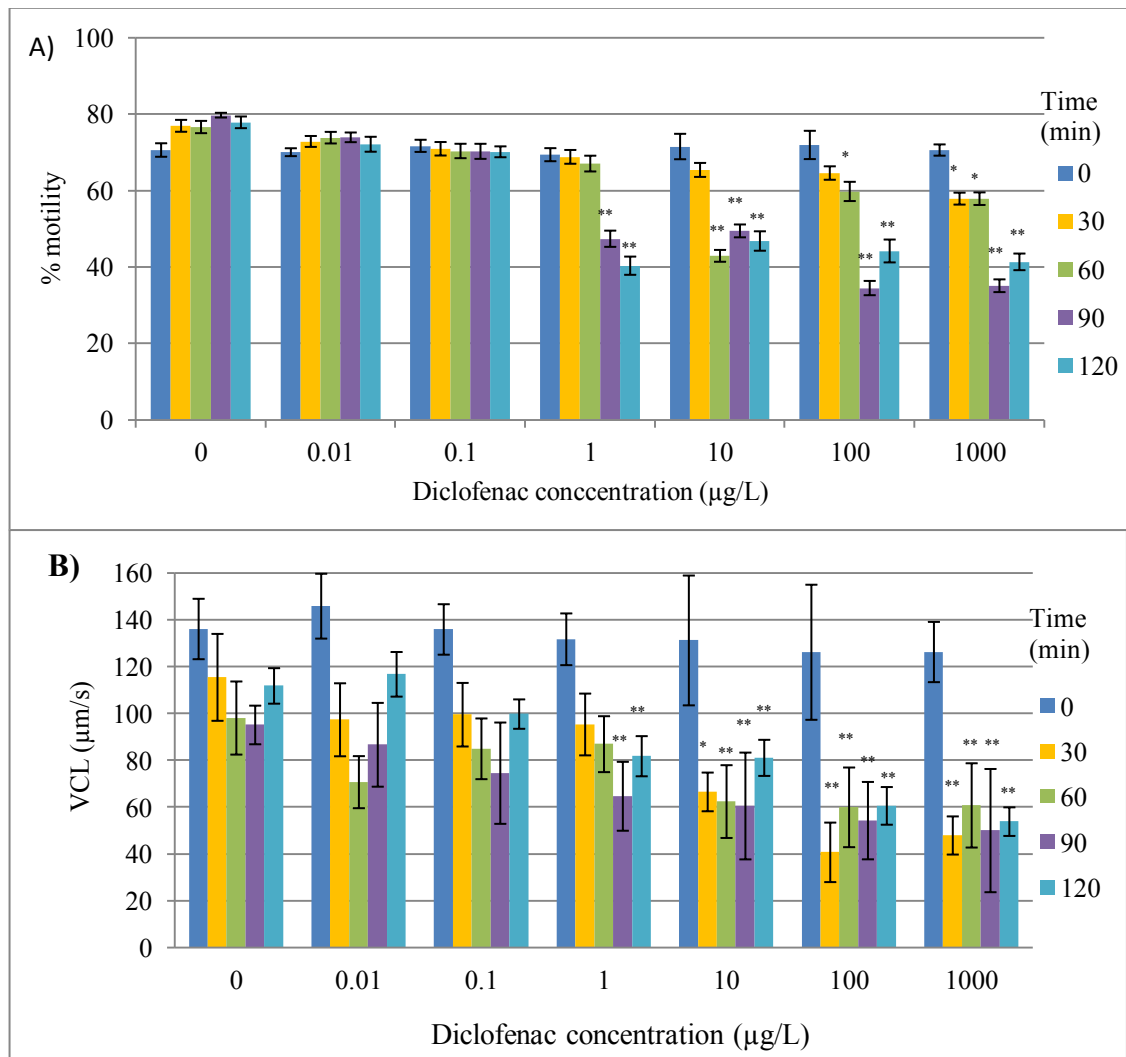
Figure 2.3. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Arenicola marina* spermatozoa after exposure to diclofenac for up to 120 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Table 2.3. Analysis of variance on arcsine transformed data for sperm motility and log transformed data for VCL for *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* spermatozoa, with time and diclofenac concentration as the main effects.

Species	Parameter	Source	SS	df	MS	F	Sig.
Asterias rubens	Sperm motility	Time	10015.7	3	3338.567	81.172	<0.001
		Concentration	423.257	6	70.543	1.715	0.018
		Time * concentration	1904.987	18	105.833	2.573	0.001
		Error	9213.066	224	41.13		
	Curvilinear velocity	Concentration	1.85	6	0.308	4.777	<0.001
		Time	1.786	3	0.595	9.224	0.001
		Concentration * time	2.673	18	0.149	2.301	0.003
		Error	14.458	224	0.065		
Psammechinus miliaris	Sperm motility	concentration	3357.673	5	671.535	40.945	<0.001
		Time	9239.685	3	3079.895	187.79	<0.001
		concentration * Time	1933.208	15	128.881	7.858	<0.001
		Error	2361.714	144	16.401		
	Curvilinear velocity	concentration	74.266	5	14.853	20.975	<0.001
		Time	49.054	3	16.351	23.091	<0.001
		concentration * Time	19.588	15	1.306	1.844	0.034
		Error	101.971	144	0.708		
Arenicola marina	Sperm motility	Time	13479.053	6	2246.509	607.053	<0.001
		Concentration	128.392	4	32.098	8.674	<0.001
		Time * concentration	157.722	24	6.572	1.776	0.018
		Error	777.143	210	3.701		
	Curvilinear velocity	Concentration	353.936	4	88.484	17.528	<0.001
		Time	76.537	6	12.756	2.527	0.022
		Concentration * time	481.393	24	20.058	3.973	<0.001
		Error	1060.119	210	5.048		

2.3.1.2 Ibuprofen

Figure 2.4 presents the results of exposure to ibuprofen with time for *A. rubens* sperm. No significant differences were observed in either sperm motility or VCL.
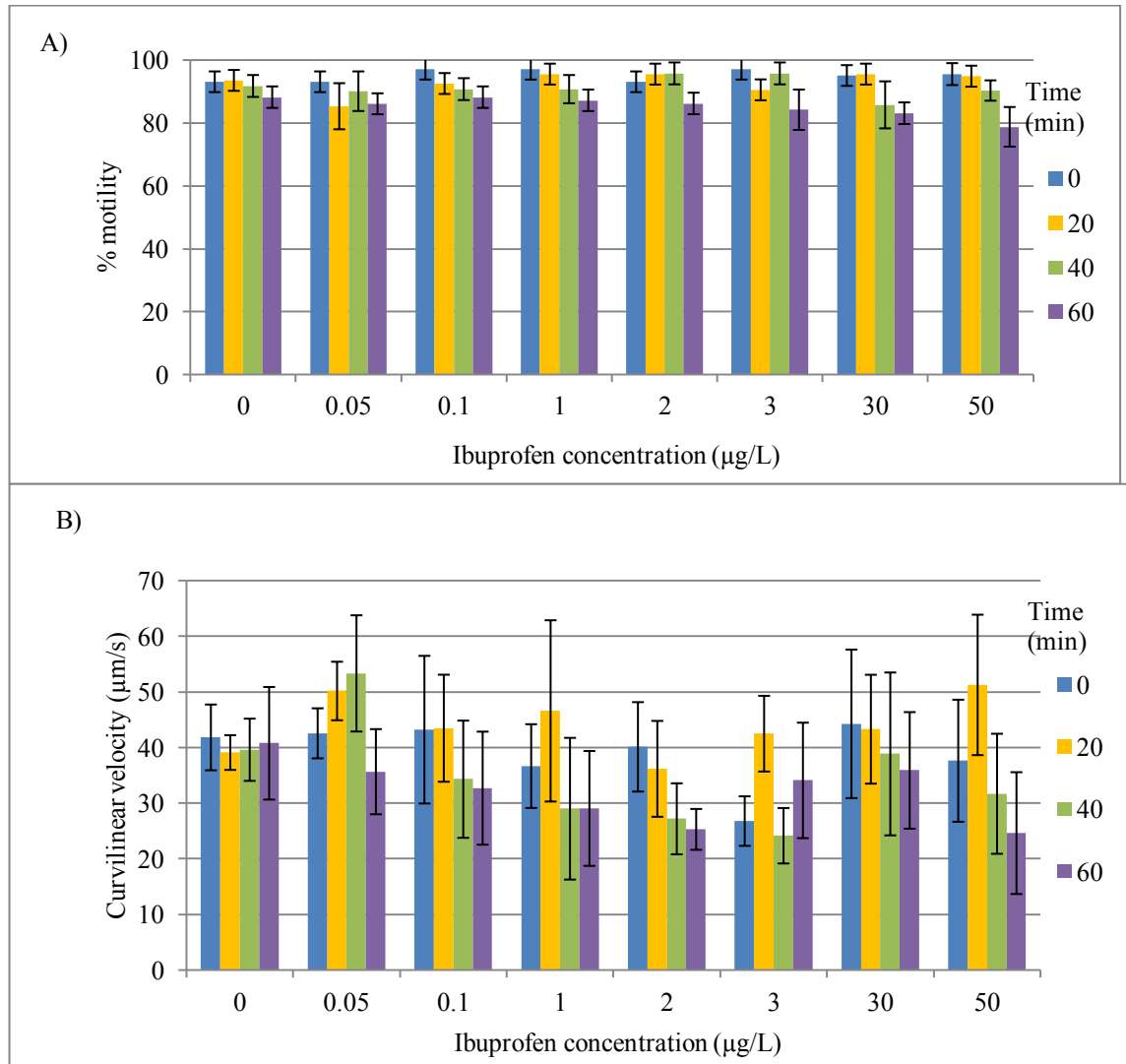
Figure 2.4. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Asterias rubens* spermatozoa after exposure to ibuprofen for up to 60 minutes.

The effects of exposure to ibuprofen with increasing time on *P. miliaris* sperm are shown in Figure 2.5. A significant decline in percentage sperm motility and VCL was found at ≥20 minutes exposure to ≥1µg/L. There was a significant interaction between time and concentration (F= 28.222, p<0.001; F=2.878, p=0.001; Table 2.4).
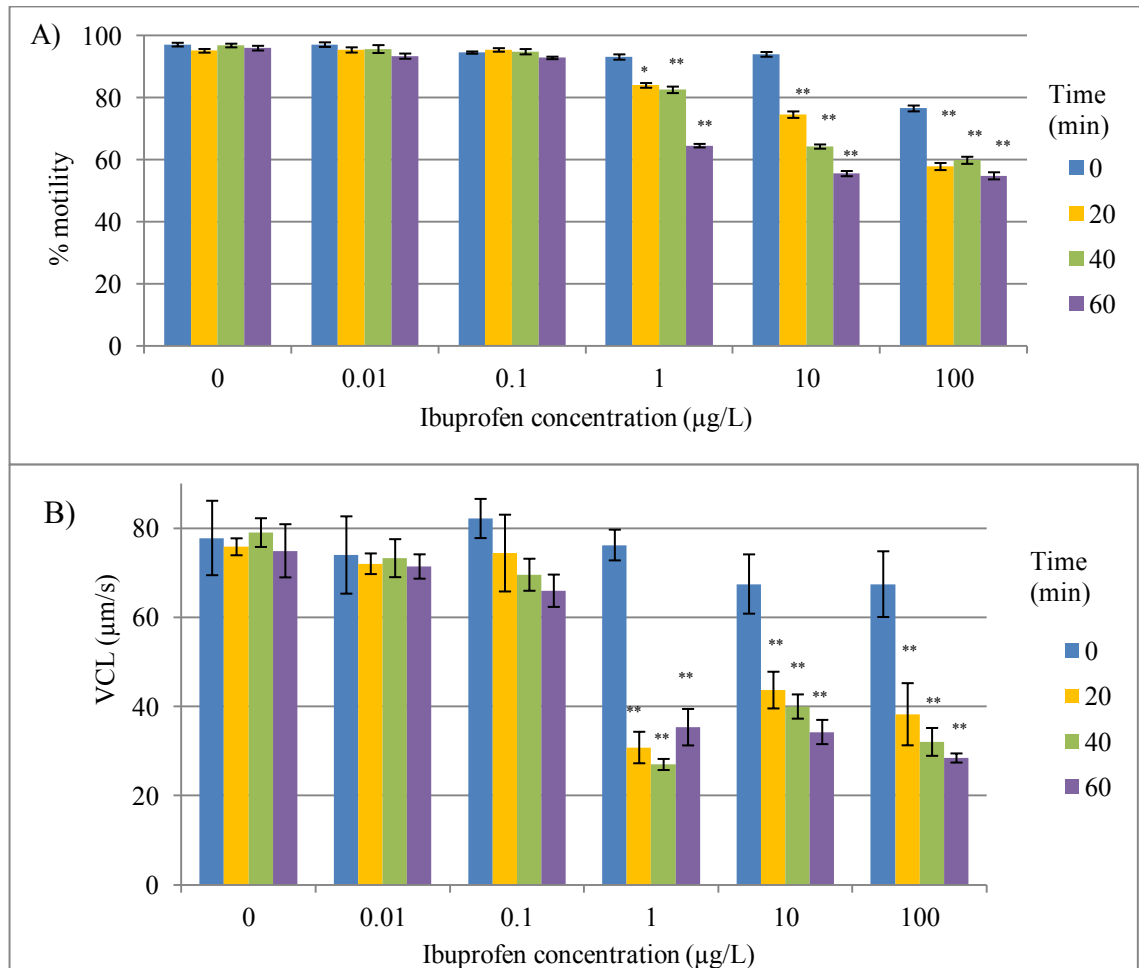
Figure 2.5. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Psammechinus miliaris* spermatozoa after exposure to ibuprofen for up to 60 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Figure 2.6 shows the result for *A. marina* sperm exposed to different concentrations of ibuprofen with increasing time. Sperm motility was unaffected by concentration or time; however, VCL was positively affected by $\geq 10\mu g/L$ at ≥ 20 minutes exposure (F=3.190, p<0.001; Table 2.4).
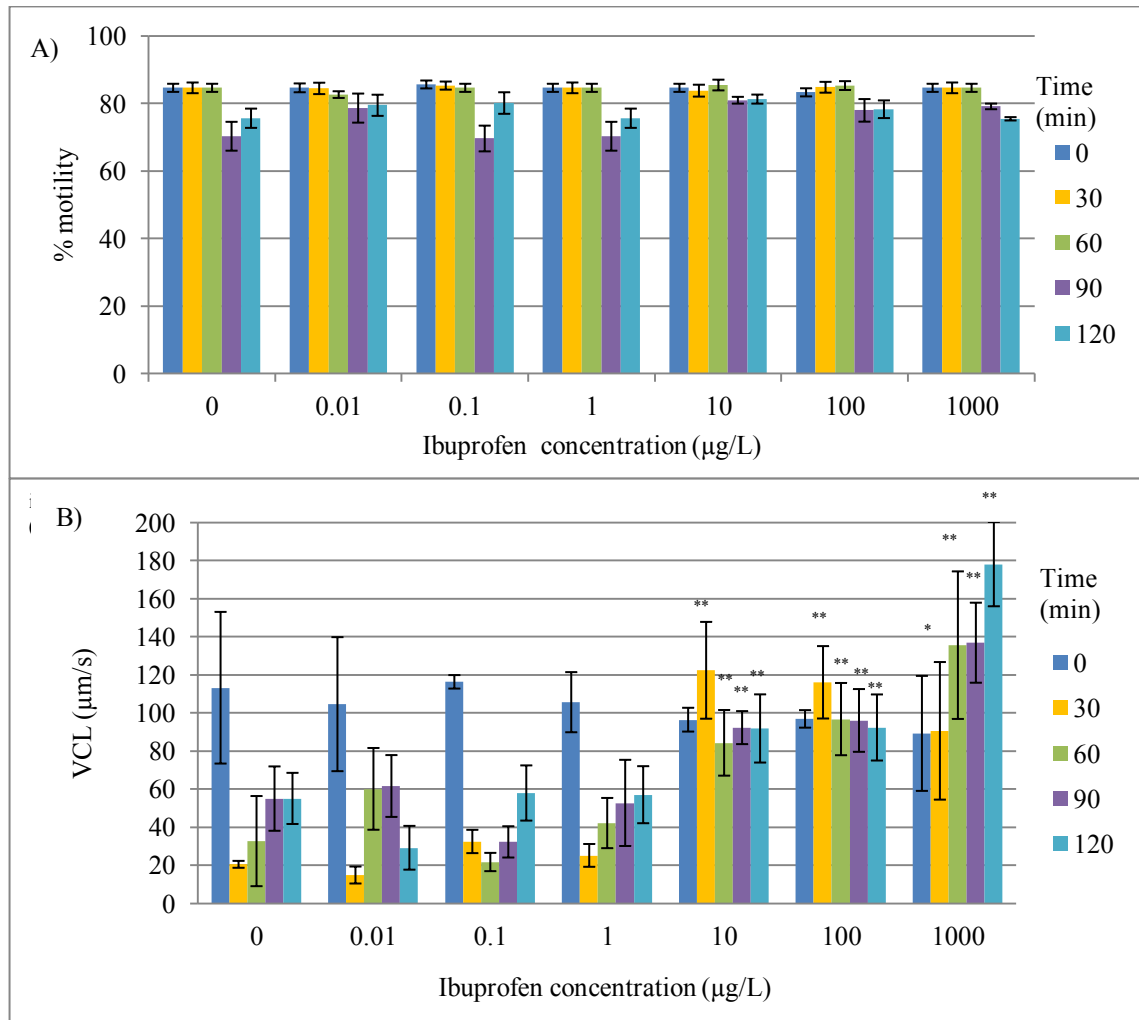
Figure 2.6. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Arenicola marina* spermatozoa after exposure to ibuprofen for up to 120 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Table 2.4. Analysis of variance on arcsine transformed data for sperm motility and log transformed data for VCL for *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* spermatozoa, with time and ibuprofen concentration as the main effects.

Species	Parameter	Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens	Sperm motility	concentration	16.385	7	2.341	1.827	0.082
		Time	85.538	3	28.513	22.254	<0.001
		concentration * Time	9.49	21	0.452	0.353	0.997
		Error	328	256	1.281		
	Curvilinear velocity	concentration	5098.545	7	728.364	0.918	0.493
		Time	5255.245	3	1751.748	2.207	0.088
		concentration * Time	6583.731	21	313.511	0.395	0.993
		Error	203177.23	256	793.661		
Psammechinus miliaris	Sperm motility	concentration	28228.976	5	5645.795	1143.68	<0.001
		Time	465.786	3	155.262	31.452	<0.001
		concentration * Time	2089.786	15	139.319	28.222	<0.001
		Error	710.857	144	4.937		
	Curvilinear velocity	concentration	27771.886	5	5554.377	32.993	<0.001
		Time	22422.844	3	7474.281	44.397	<0.001
		concentration * Time	7268.599	15	484.573	2.878	0.001
		Error	24242.614	144	168.351		
Arenicola marina	Sperm motility	Concentration	248.295	6	41.383	1.452	0.197
		Time	3342.076	4	835.519	29.321	<0.001
		concentration * Time	900.657	24	37.527	1.317	0.159
		Error	4986.667	175	28.495		
	Curvilinear velocity	Time	1.396	4	0.349	3.047	0.019
		Concentration	7.678	6	1.28	11.175	<0.001
		time * concentration	8.767	24	0.365	3.19	<0.001
		Error	20.04	175	0.115		

2.3.3.3 Sildenafil citrate

Figure 2.7 presents the results from *A. rubens* sperm exposed to different concentrations of sildenafil citrate with increasing time. Percentage sperm motility increased significantly at concentration $\geq$50ng/L (F=9.712, p<0.001; Table 2.5). However, VCL has significantly increased at $\geq$18ng/L (F=3.913, p=0.001;Table 2.5).
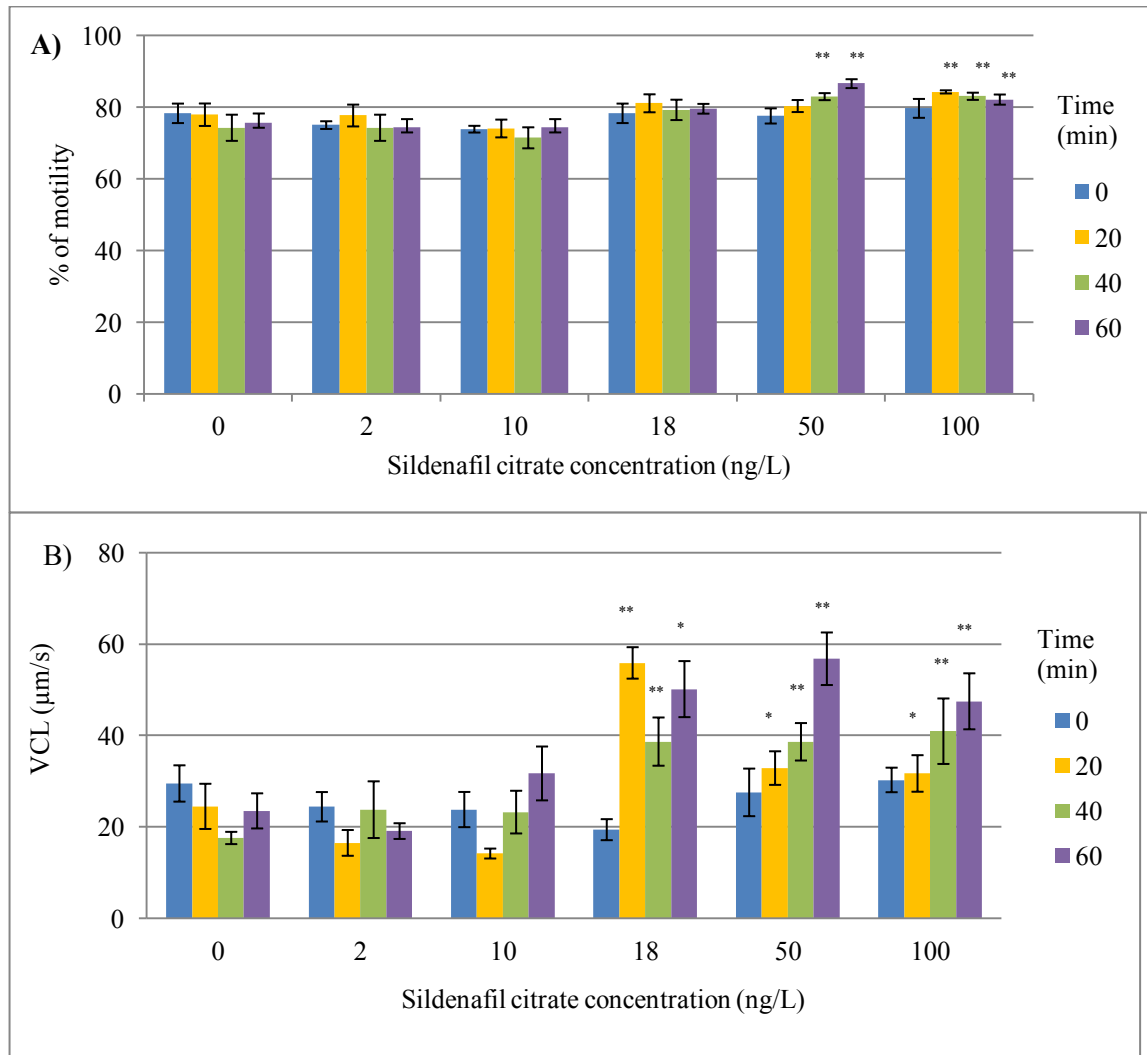
Figure 2.7. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Asterias rubens* spermatozoa after exposure to sildenafil citrate for up to 60 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

The effects on *P. miliaris* sperm exposed to sildenafil citrate are presented in Figure 2.8. Exposure to ≥50ng/L significantly increased percentage sperm motility. A reduction in VCL was seen when exposed to 2ng/L and 10ng/L at 60 minutes; however, VCL increased when exposed to concentrations ≥18ng/L.
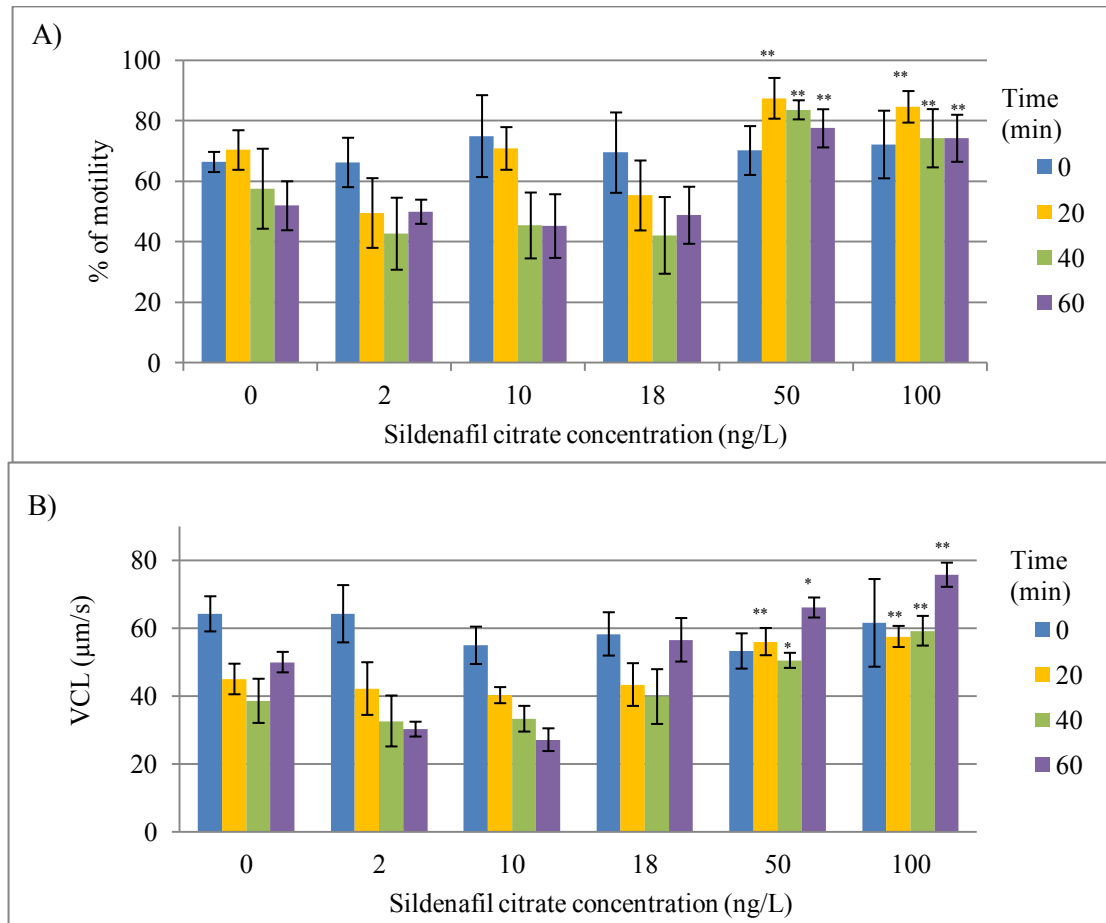
Figure 2.8. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Psammechinus miliaris* spermatozoa after exposure to sildenafil citrate for up to 60 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks $*p<0.05$; $**p<0.01$.

Figure 2.9 shows the results for *A. marina* sperm exposed to sildenafil citrate. No significant differences were found for sperm motility or curvilinear velocity at any concentration or time.
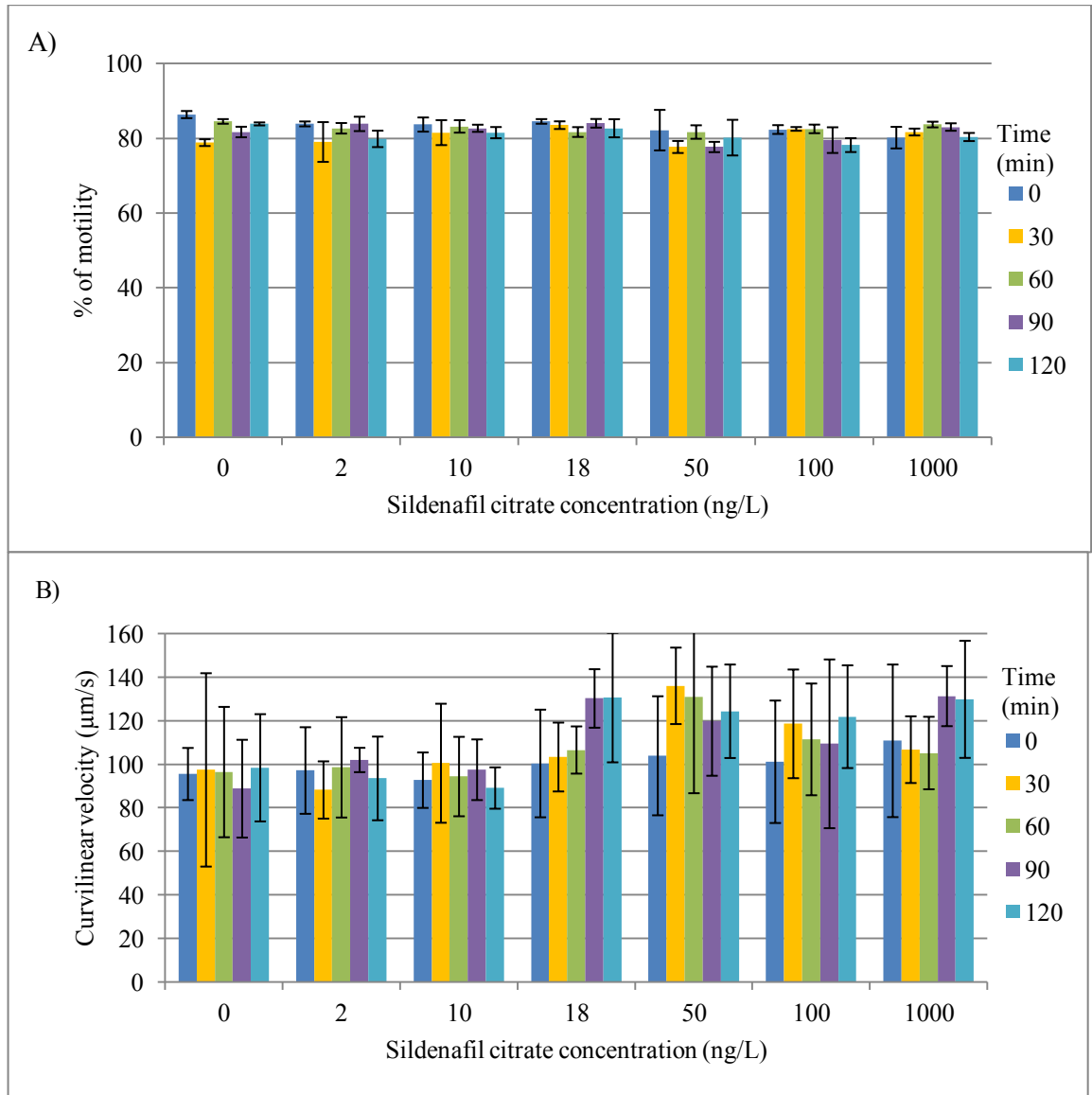
Figure 2.9. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Arenicola marina* spermatozoa after exposure to sildenafil citrate for up to 120 minutes.

Table 2.5. Analysis of variance on arcsine transformed data for sperm motility and log transformed data for VCL for *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* spermatozoa, with time and sildenafil citrate concentration as the main effects.

Species	Parameter	Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens	Sperm motility	Concentration	2369.771	5	473.954	9.712	<0.001
		Time	164.381	3	54.794	1.123	0.341
		Concentration * Time	603.197	15	40.213	0.824	0.65
		Error	9369.809	192	48.801		
	Curvilinear velocity	concentration	2.395	3	0.798	3.913	0.01
		Time	11.706	5	2.341	11.474	<0.001
		concentration * Time	13.005	15	0.867	4.249	<0.001
		Error	39.176	192	0.204		
Psammechinus miliaris	Sperm motility	Concentration	18550.7	5	3710.15	5.944	<0.001
		Time	6425.83	3	2141.94	3.431	0.019
		Concentration * Time	8009.3	15	533.953	0.855	0.615
		Error	89887.6	144	624.219		
	Curvilinear velocity	Time	2.117	3	0.706	24.024	<0.001
		Concentration	0.616	5	0.123	4.193	0.001
		Time * Concentration	0.349	15	0.023	0.792	0.685
		Error	4.23	144	0.029		
Arenicola marina	Sperm motility	Concentration	254.362	6	42.394	1.505	0.179
		Time	221.552	4	55.388	1.967	0.102
		Concentration * Time	422.781	24	17.616	0.625	0.912
		Error	4929	175	28.166		
	Curvilinear velocity	Concentration	34402.547	6	5733.758	1.669	0.131
		Time	6489.771	4	1622.443	0.472	0.756
		Concentration * Time	24874.78	24	1036.449	0.302	0.999
		Error	601087.8	175	3434.787		

2.3.2 The effect of pre-incubation of sperm with pharmaceutical contaminants on fertilisation

2.3.2.1 Diclofenac

Incubation of sperm in diclofenac before fertilisation had a negative effect on fertilisation success for *A. rubens* (Figure 2.10(A)). A pronounced declined in fertilisation success was observed at 0.1, 1, 10, 100 and 1000μg/L. Sperm incubated for 60 minutes with 1, 10, 100 and 1000μg/L had mean fertilisation success of 72.82±1.15%, 68.22±1.35%, 61.33.7±2.51% and 30.88±1.78% respectively, whereas controls had mean fertilisation success of 96.72±0.96%. There was a significant interaction between concentration and exposure period (F=2.035, p=0.017; Table 2.6).

A similar pattern was noted for *P. miliaris* sperm (Figure 2.10 (B)). Control sperm maintained fertilisation success of 98.22±0.26% to 95.22±1.41% throughout the experiment. Interaction between diclofenac concentration and time was also significant (F=1.704, p=0.04; Table 2.6). Fertilisation success for sperm incubated in 1μg/L declined to 82.88±0.84% after 20 minutes and continued to decline with time. After 60 minutes only 30.88±1.79% of oocytes were fertilised.

Incubation of sperm before fertilisation in diclofenac had a negative effect on the fertilisation success of *A. marina* (Figure 2.10 (C)). A significant reduction was seen at 30 minutes and 10μg/L exposure. There was a significant interaction between concentration and time (F=2.478, p<0.001; Table 2.6).
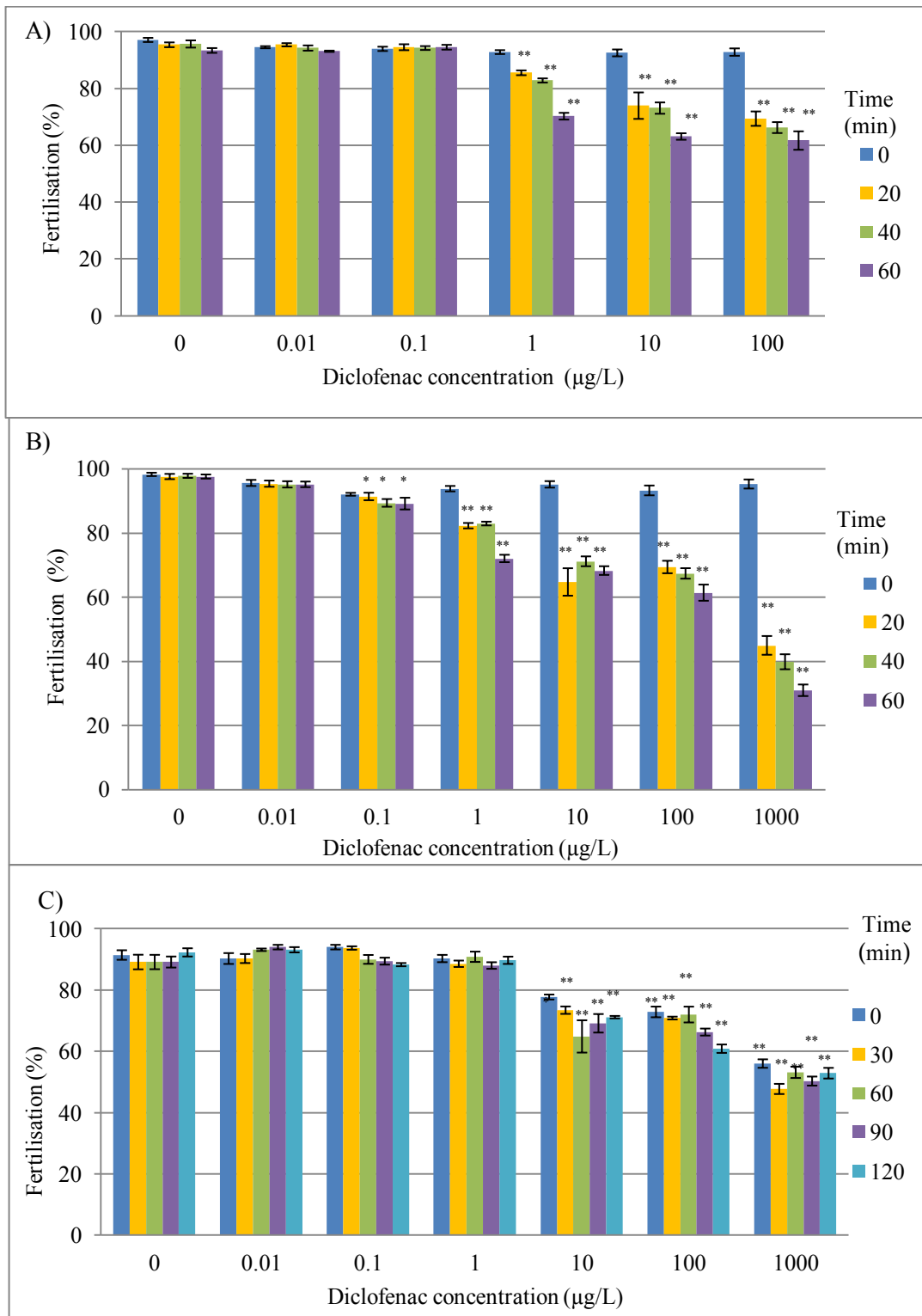
Figure 2.10. Fertilisation percentage of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris*, and C) *Arenicola marina* after incubation of sperm with diclofenac at different concentrations and times prior to fertilisation. Treatments significantly different from the control within the same time intervals are indicated by asterisks *$p < 0.05$; **$p < 0.01$.

Table 2.6. Analysis of variance on arcsine transformed of percentage of fertilisation success for *Asterias rubens*, *Psammechinus miliaris*, and *Arenicola marina* with time and diclofenac concentration as main effects.

Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens					
Time	286.135	3	95.378	7.527	<0.001
Concentration	33786.163	6	5631.027	444.356	<0.001
Concentration * time	388.575	18	21.588	1.704	0.040
Error	2838.599	224	12.672		
Psammechinus miliaris					
Concentration	14935.024	5	2987.005	251.875	<0.001
Time	155.517	3	51.839	4.371	<0.001
Concentration * time	361.910	15	24.127	2.035	0.017
Error	1707.79	144	11.859		
Arenicola marina					
Concentration	25405.539	6	4234.257	321.830	<0.001
Time	210.458	4	52.614	3.999	0.004
Concentration * time	782.443	24	32.602	2.478	<0.001
Error	2762.933	210	13.157		

2.3.2.2 Ibuprofen

Incubation of sperm in ibuprofen before fertilisation resulted in no significant differences compared to controls in *A. rubens* (Figure 2.11 (A)). Reduction in fertilisation success was only affected by time (F=20.058, p<0.001; Table 2.7). The control sperm maintained fertilisation success of 97.11±0.29% to 96.22±0.42% throughout the experiment. However, for *P. miliaris* sperm a pronounced decline in fertilisation success was observed at 1, 10, and 100μg/L. Sperm incubated for 60 minutes with 1, 10, and 100μg/L had mean fertilisation success of 70.29±1.18%, 63.14±1.14% and 61.71±3.25% respectively, whereas controls had mean fertilisation success of 94±0.84%. There was a significant interaction between concentration and exposure period (F=2.73, p<0.001; Table 2.7). Incubation of sperm before fertilisation in ibuprofen had a slightly negative effect on the fertilisation success of *A. marina* (Figure 2.11 (C)). The control sperm maintained a fertilisation rate of 91.22±1.42% to 90.8±1.23% throughout the experiment. At 60 minutes, pre-incubation fertilisation success had reduced significantly at concentrations of 1μg/L and above.

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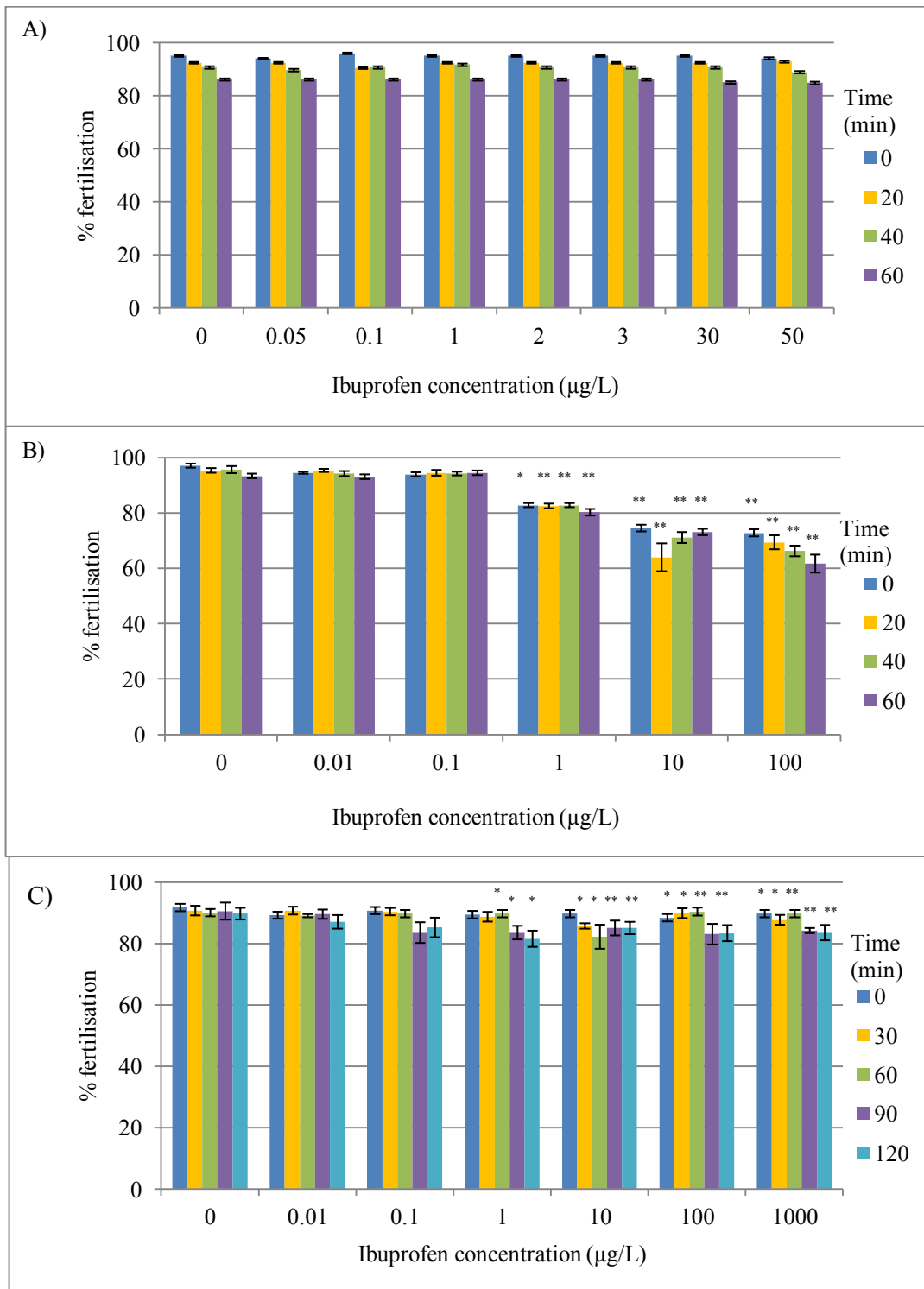
Figure 2.11. Percent fertilisation of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris* and C) *Arenicola marina* after incubation of sperm with ibuprofen at different concentrations and times prior to fertilisation. Treatments significantly different from the control within the same time intervals are indicated by asterisks *$p<0.05$; **$p<0.01$.

Table 2.7. Analysis of variance on arcsine transformed data of percentage of fertilisation success for *Asterias rubens*, *Psammechinus miliaris* and *Arenicola marina* with time and ibuprofen concentration as main effects.

Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens					
concentration	6.274	7	0.896	0.628	0.733
Time	85.872	3	28.624	20.058	<0.001
concentration * Time	6.990	21	0.333	0.233	1.000
Error	365.333	256	1.427		
Psammechinus miliaris					
concentration	22258.762	5	4451.752	230.927	<0.001
Time	244.000	3	81.333	4.219	0.007
concentration * Time	791.714	15	52.781	2.738	0.001
Error	2776.000	144	19.278		
Arenicola marina					
concentration	736.857	6	122.810	6.074	<0.001
Time	921.143	4	230.286	11.389	<0.001
concentration * Time	700.857	24	29.202	1.444	0.093
Error	3538.500	175	20.220		

2.3.2.3 Sildenafil citrate

The effect of sperm pre-incubation in sildenafil citrate before fertilisation is shown in Figures 2.12(A), 2.12(B) and 2.12(C) for *A. rubens*, *P. miliaris*, and *A. marina* respectively. Sildenafil citrate gave a positive effect on fertilisation success of *A. rubens* at 20 minutes and 50ng/L and above. The control sperm was maintained within a range of 93.88±0.56% to 92.88±0.26%. There was a significant interaction between time and concentration (F=6.86, p<0.001; Table 2.8). A similar pattern was observed for *P. miliaris*. A significant increase of fertilisation success was found at 20, 40 and 60 minutes and 50 and 100ng/L. Fertilisation success of the sperm control at 60 minutes was 81.71±3.16 while at 60 minutes and 100ng/L sildenafil citrate exposure resulted in a fertilisation success of 85.29%. There was significant interaction between concentration and time (F=9.868, p <0.001; Table 2.8). There were no significant effects observed for *A. marina* treatments.
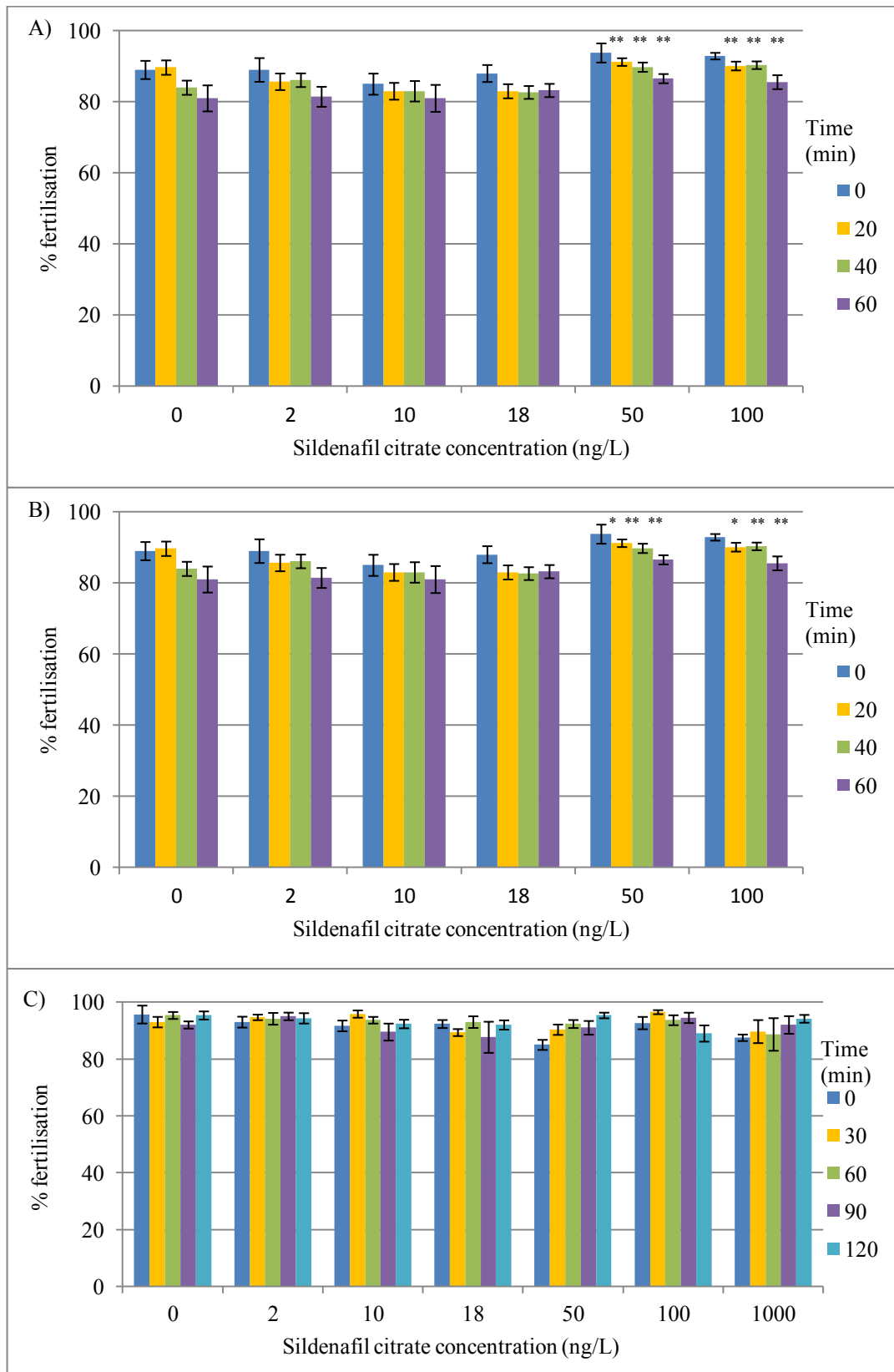
Figure 2.12. Fertilisation percentage of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris* and C) *Arenicola marina* after incubation of sperm with sildenafil citrate at different concentrations and times prior to fertilisation. Treatments significantly different from the control within the same time intervals are indicated by asterisks *$p<0.05$; **$p<0.01$.

Table 2.8. Analysis of variance on arcsine transformed data for fertilisation success of *Asterias rubens*, *Psammechinus miliaris* and *Arenicola marina* with time and sildenafil citrate concentration as main effects.

Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens					
Concentration	1373.579	5	274.716	11.044	<0.001
Time	2471.125	3	823.708	33.114	<0.001
Concentration * Time	2423.514	15	161.568	6.495	<0.001
Error	4776.000	192	24.875		
Psammechinus miliaris					
Concentration	3843.030	5	768.606	55.722	<0.001
Time	7791.923	3	2597.308	188.297	<0.001
Concentration * Time	2041.756	15	136.117	9.868	<0.001
Error	1986.286	144	13.794		
Arenicola marina					
Concentration	495.857	6	82.643	2.571	0.051
Time	138.219	4	34.555	1.075	0.370
Concentration * Time	895.048	24	37.294	1.160	0.285
Error	5624.500	175	32.140		

2.3.3 Pre-incubation of oocytes with pharmaceutical contaminants

2.3.3.1 Diclofenac

Figure 2.13 (A-C) shows the results of pre-incubation of oocytes in diclofenac before fertilisation for *A. rubens, P. miliaris*, and *A. marina* respectively. A significant decrease was found for fertilisation success of *A. rubens* when exposed to 10, 100 and 1000 µg/L at 20 minutes and above. Oocyte controls were in the range of 97.78±1.04% and 94.24±2.82% throughout the experiments. Fertilisation occurred at 60 minutes exposure of 10, 100 and 1000µg/L which was 73.77±2.70%, 62.00±5.03 and 38.44±3.59% respectively. A similar pattern was observed with *P. miliaris*. A reduction in fertilisation success was seen at 20 minutes and above for 10 and 100µg/L. There was a significant interaction between concentration and time (F=2.427, p=0.004; Table 2.9). For *A. marina* a significant decline was observed at 10, 100 and 1000µg/L. Oocyte controls at 60 minutes had a fertilisation success of 93.12±0.21%. There was a reduction to 49.32±2.33% at 60 minutes for the 1000µg/L exposure. The interaction between concentration and time was also significant (F=3.74, p<0.001; Table 2.9).
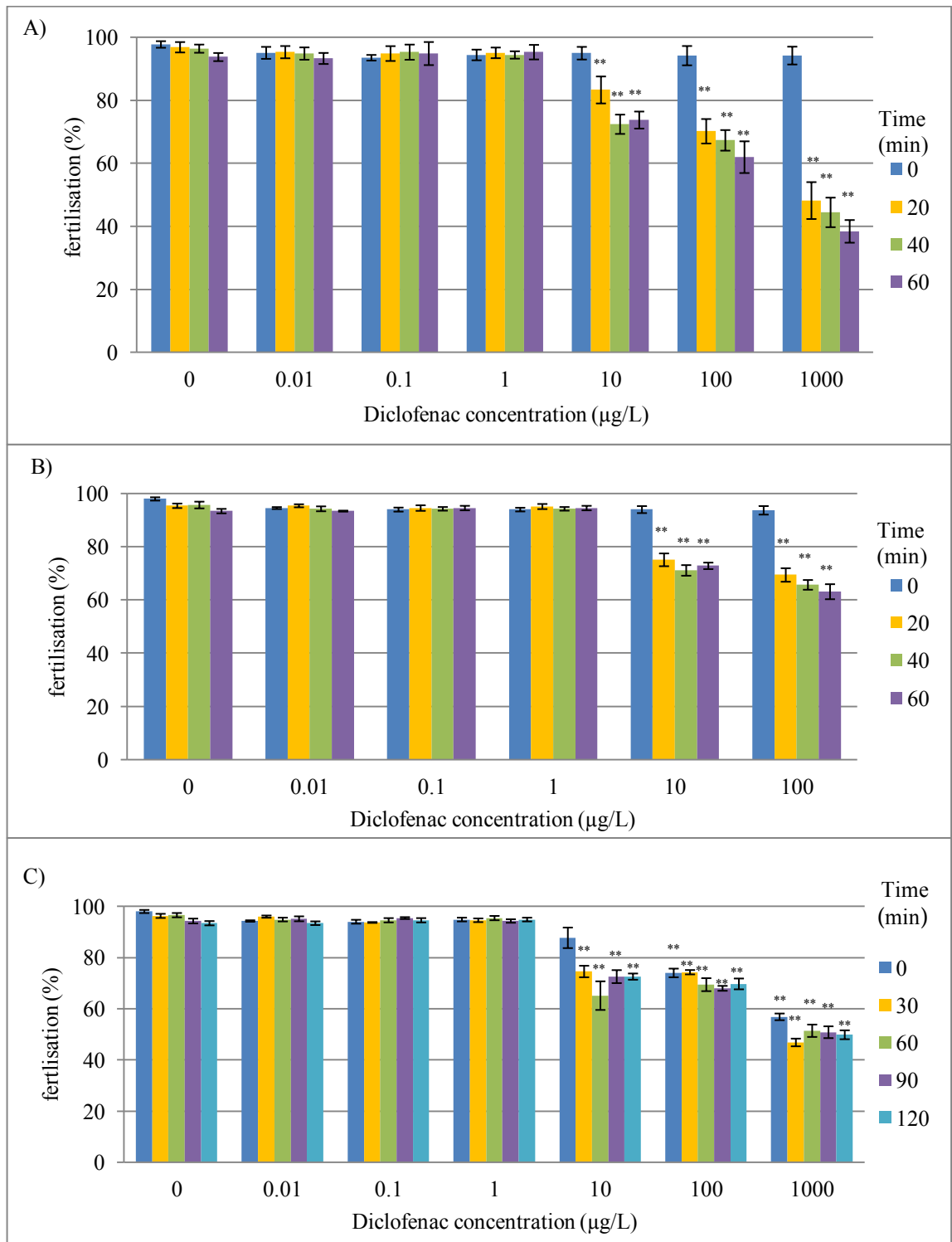
Figure 2.13. Fertilisation percentage of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris*, and C) *Arenicola marina* after incubation of oocytes with diclofenac at different concentrations and times prior to fertilisation. Treatments significantly different from control within the same time intervals are indicated by asterisks *$p<0.05$; **$p<0.01$.

Table 2.9. Analysis of variance on arcsine transformed data for fertilisation success of *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* with time and diclofenac concentration as main effects.

Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens					
Time	104.569	3	34.856	2.826	0.040
Concentration	40150.577	6	6691.763	542.465	<0.001
Time * concentration	527.973	18	29.332	2.378	0.002
Error	2763.230	224	12.336		
Psammechinus miliaris					
Concentration	16550.402	5	3310.080	268.974	<0.001
Time	113.108	3	37.703	3.064	0.030
Time * concentration	448.065	15	29.871	2.427	0.004
Error	1772.107	144	12.306		
Arenicola marina					
Concentration	35467.307	6	5911.218	420.878	<0.001
Time	467.803	4	116.951	8.327	<0.001
Time * concentration	1261.133	24	52.547	3.741	<0.001
Error	2949.441	210	14.045		

2.3.3.2 Ibuprofen

Figure 2.14 (A) does not indicate any effect of pre-incubation of oocytes of *A. rubens* in ibuprofen. Control oocytes maintained a fertilisation success of 97.78±0.31% to 95.33±0.41% throughout the experiment. There were no significant effects of either ibuprofen concentration or incubation period, and no significant interaction between concentration and time. For *P. miliaris* oocytes (Figure 2.14 (B)), a significant reduction was only observed when incubated in 100μg/L at 60 minutes exposure. However, oocyte incubation in 100μg/L had an effect where a reduction of fertilisation success was observed at 20, 40, and 60 minutes exposure. There was a significant interaction between concentration and time (F=9.85, p<0.001; Table 2.10). For *A. marina* the only significant reduction occurred at 1000μg/L.
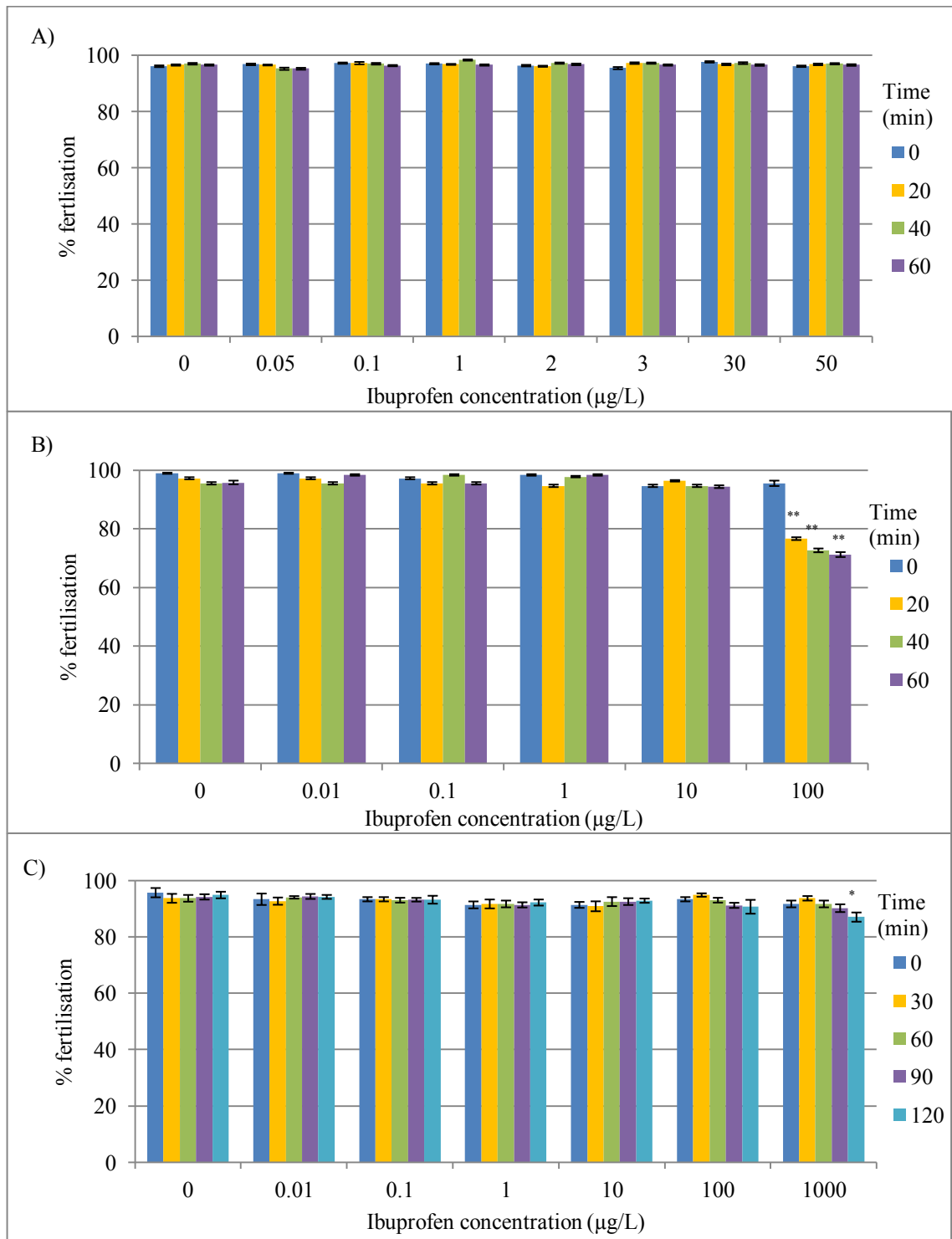
Figure 2.14. Fertilisation percentage of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris*, and C) *Arenicola marina* after incubation of oocytes with ibuprofen at different concentrations and times prior to fertilisation. Treatments significantly different from the control within the same time intervals are indicated by asterisks *$p < 0.05$; **$p < 0.01$.

Table 2.10. Analysis of variance on arcsine transformed data for fertilisation success, with time and ibuprofen concentration as main effects.

Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens					
concentration	9.163	7	1.309	1.844	0.079
Time	3.955	3	1.318	1.857	0.137
concentration * Time	16.184	21	0.771	1.085	0.364
Error	181.778	256	0.710		
Psammechinus miliaris					
concentration	2410.762	5	482.152	334.717	<0.001
Time	74.786	3	24.929	17.306	<0.001
concentration * Time	213.000	15	14.200	9.858	<0.001
Error	207.429	144	1.440		
Arenicola marina					
concentration	368.629	6	61.438	6.136	<0.001
Time	7.648	4	1.912	0.191	0.943
concentration * Time	289.086	24	12.045	1.203	0.245
Error	1752.167	175	10.012		

2.3.3.3 Sildenafil citrate

Figure 2.15 presents the results of oocytes pre-incubation in different concentrations and times. There was no significant difference in *A. rubens, Psammechinus miliaris and Arenicola marina* fertilisation success in any treatment (Figure 2.15(A), (B) and (C)).
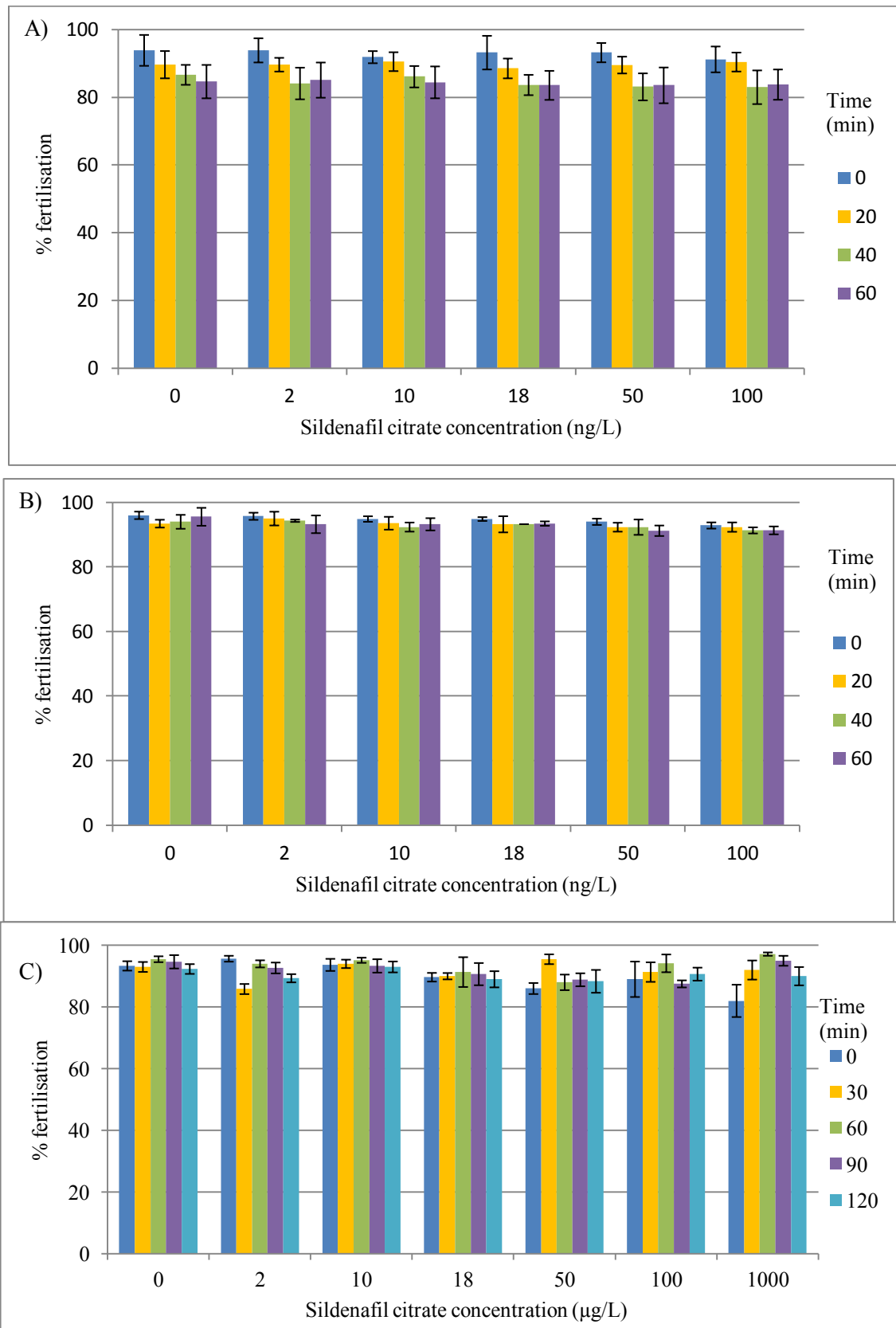
Figure 2.15. Fertilisation percentage of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris*, and C) *Arenicola marina* after incubation of oocytes with sildenafil citrate at different concentrations and times prior to fertilisation.

Table 2.11. Analysis of variance on arcsine transformed data for fertilisation success, with time and sildenafil citrate concentration as main effects.

Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens					
Concentration	10.412	5	2.082	0.151	0.979
Time	7764.866	3	2588.289	188.097	<0.001
Concentration * Time	71.606	15	4.774	0.347	0.989
Error	2642.000	192	13.760		
Psammechinus miliaris					
Concentration	562.500	5	112.500	6.359	0.0568
Time	5539.119	3	1846.373	104.371	0.7111
Concentration * Time	810.738	15	54.049	3.055	0.0823
Error	2547.429	144	17.690		
Arenicola marina					
Concentration	544.381	6	90.730	2.411	0.059
Time	353.143	4	88.286	2.346	0.056
Concentration * Time	1379.524	24	57.480	1.527	0.064
Error	6585.333	175	37.630		

2.3.4 Exposure of sperm and oocytes to pharmaceutical contaminants

2.3.4.1 Diclofenac

Figure 2.16 shows the results of incubation of both oocytes and sperm with diclofenac on fertilisation success. A significant decrease in fertilisation success was observed at 1, 10, and 100μg/L compared to the control. For *A. rubens*, fertilisation success reduced to 46.22±1.98% of the highest diclofenac concentration compared to controls of 98.6±1.24% (F=140.58, p=<0.001; Table 2.12). For *P. miliaris*, fertilisation success of the control was 97.9±1.36%, which declined to 61.2±1.56% at 100μg/L (F=77.68, p<0.001; Table 2.12). Percentage fertilisation success also declined in *A. marina* dropping from a mean of 98.4±0.57% for controls to 22.2±1.26% at 1000μg/L (F=295.70, p<0.001; Table 2.12).
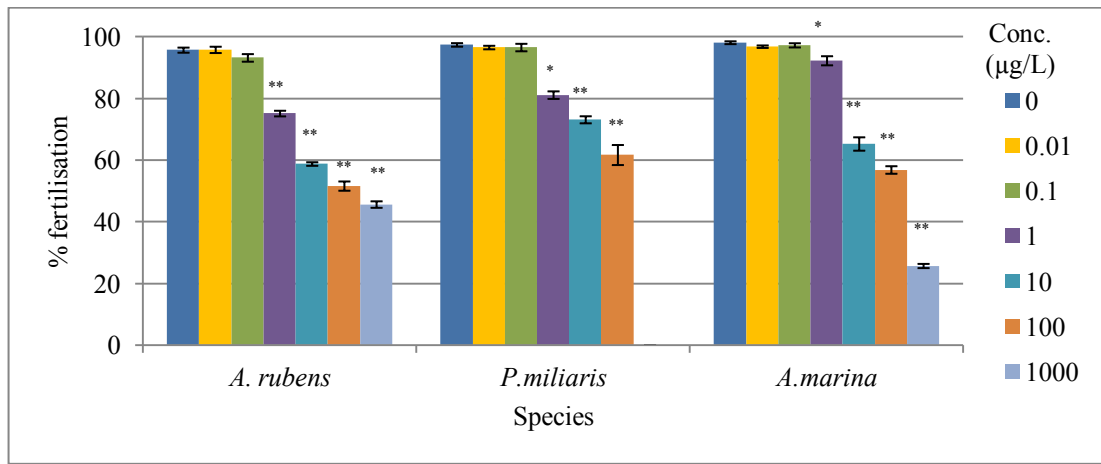
Figure 2.16. Fertilisation percentage of oocytes from *A. rubens, P. miliaris,* and *A. marina* after incubation of both sperm and oocytes with diclofenac for 60 minutes at different concentrations. Treatments significantly different from the control are indicated by asterisks $*p<0.05$; $**p<0.01$.

Table 2.12. Analysis of variance on arcsine transformed data for fertilisation success, with diclofenac concentration as main effect.

Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens					
Concentration	10784.541	6	1797.424	140.582	<0.001
Error	715.991	56	12.786		
Psammechinus miliaris					
Concentration	7016.000	5	1403.200	77.682	<0.001
Error	650.286	36	18.063		
Arenicola marina					
Concentration	17163.102	6	2860.517	295.707	<0.001
Error	406.286	42	9.673		

Ibuprofen

Figure 2.17 shows the fertilisation success of oocytes of *A. rubens*, *P. miliaris,* and *A. marina* following exposure of 60 minutes to ibuprofen. A significant reduction in fertilisation success was observed when gametes were exposed to 1µg/L and above for *P. miliaris*. Percentage fertilisation for the control was 98.23±1.12% and reduced to 51.22±2.33% at the highest incubation concentration. Exposure to 1000 µg/L resulted in

significant decline for *A. marina*. However, fertilisation success can be considered high with 88.23±0.93%. The results showed no effect for *A. rubens*.
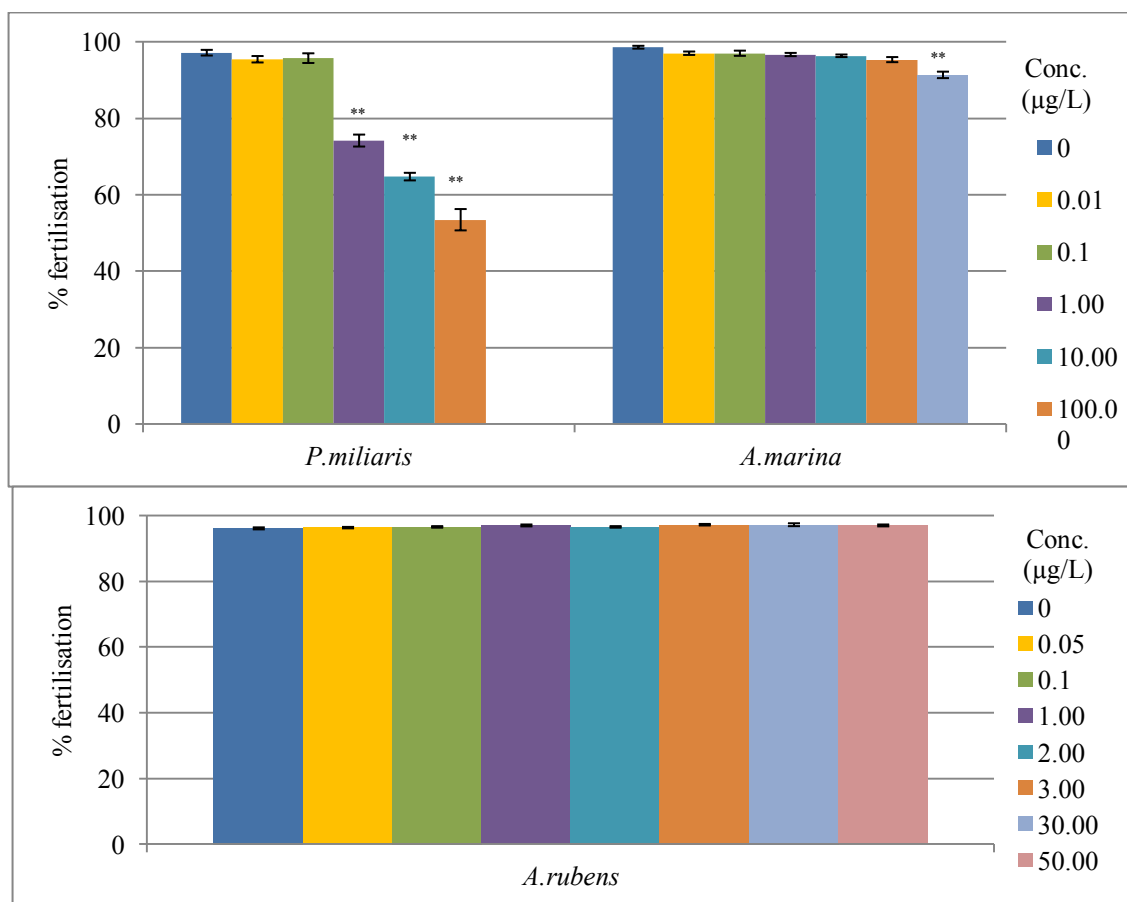
Figure 2.17. Percent fertilisation of gametes from *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* after incubation of sperm and oocytes with ibuprofen for 60 minutes at different concentrations. Treatments significantly different from the control are indicated by asterisks $*p<0.05$; $**p<0.01$.

Table 2.13. Analysis of variance on arcsine transformed data for fertilisation success with ibuprofen concentration as the main effect.

Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens					
Concentration	2.764	7	0.395	0.517	0.819
Error	48.889	64	0.764		
Psammechinus miliaris					
Concentration	12269.619	5	2453.924	149.225	<0.001
Error	592.000	36	16.444		
Arenicola marina					
Concentration	194.580	6	32.430	16.908	<0.001
Error	69.048	36	1.918		

2.4.4.3 Sildenafil citrate

Figure 2.18 presents the results of fertilisation success of gametes from *A. rubens*, *P. miliaris* and *A. marina* incubated with sildenafil citrate for 60 minutes. Fertilisation success of *A. rubens* increased at and above 18ng/L. Control gamete fertilisation was 90.22± 1.12% and 93.28±0.94% at the highest concentration. A similar pattern was observed in *P. miliaris*. Percentage fertilisation increased with exposure to ≥18ng/L. No effect was found for *A. marina*.
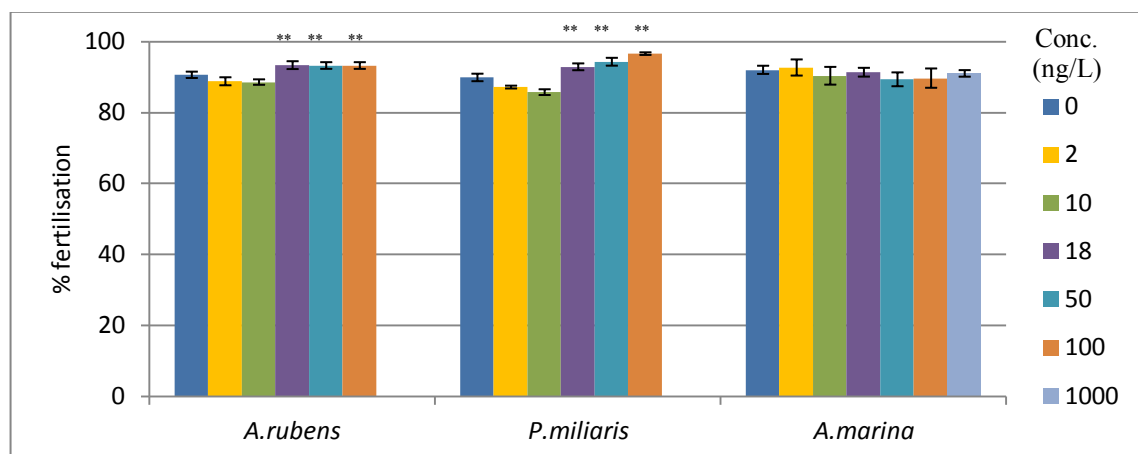
Figure 2.18. Fertilisation percentage of gametes from *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* after incubation of sperm and oocytes with sildenafil citrate for 60 minutes at different concentrations. Treatments significantly different from the control are indicated by asterisks *$p<0.05$; **$p<0.01$.

Table 2.14. Analysis of variance on arcsine transformed data for fertilisation success of sildenafil citrate concentration as the main effect.

Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens					
concentration	320.012	5	64.002	7.436	<0.001
Error	421.733	49	8.607		
Psammechinus miliaris					
Concentration	1030.476	5	206.095	41.884	<0.001
Error	177.143	36	4.921		
Arenicola marina					
Concentration	54.124	6	9.021	0.348	0.906
Error	932.667	36	25.907		

Table 2.15. No-observed-effects (NOEC) or lowest-observed-effects concentration (LOEC), half-minimal effective concentration (EC$_{50}$) values and toxicity substance classification for *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*.

Species	Test	Pharmaceutical	NOEC or LOEC (µg/L)	EC$_{50}$ (µg/L)	classification +
A. rubens	Sperm motility	Diclofenac	NOEC= 0.10	60min = 2335.8	Toxic
		Ibuprofen	NOEC= 50.00	N/A	N/A
		Sildenafil citrate	NOEC= 0.18	60min= 2.25 x 10^{12}	Non Toxic
	Fertilisation success, pre-incubation of sperm	Diclofenac	NOEC= 0.10	60min = 2610	Toxic
		Ibuprofen	NOEC= 50.00	N/A	N/A
		Sildenafil citrate	NOEC= 0.010	60min= 7.15 x 10^{13}	Non toxic
	Fertilisation success, pre-incubation of oocyte	Diclofenac	NOEC= 1.00	60min =1679.59	Toxic
		Ibuprofen	NOEC= 50.00	N/A	N/A
		Sildenafil citrate	NOEC= 0.10	N/A	N/A
	Fertilisation success, exposure of sperm and oocyte	Diclofenac	LOEC = 0.01	60min = 616.48	Very toxic
		Ibuprofen	NOEC= 50.00	N/A	N/A
		Sildenafil citrate	NOEC = 0.01	2.37 x 10^{12}	Non toxic
P. miliaris	Sperm motility	Diclofenac	NOEC= 0.01	60min = 378.22	Very toxic
		Ibuprofen	NOEC= 0.1	60min = 845.98	Very toxic
		Sildenafil citrate	NOEC = 0.018	7.23 x 10^{10}	Non toxic
	Fertilisation success, pre-incubation of sperm	Diclofenac	NOEC= 0.01	60min =298.42	Very toxic
		Ibuprofen	NOEC= 0.10	60min = 437.03	Very toxic
		Sildenafil citrate	NOEC= 0.10	60min = 6.241 x 10^{10}	Non toxic
	Fertilisation success, pre-incubation of oocyte	Diclofenac	NOEC=1.00	60min =429.37	Very toxic
		Ibuprofen	NOEC= 1.00	60min = 4.56 x10^{6}	Non toxic
		Sildenafil citrate	NOEC= 0.01	N/A	N/A
	Fertilisation success, exposure of sperm and oocyte	Diclofenac	LOEC = 0.01	60min =247.31	Very toxic
		Ibuprofen	NOEC= 0.10	60min =792.96	Very toxic
		Sildenafil citrate	NOEC= 0.010	60min = 4.335 x 10^{10}	Non toxic

A. marina	Sperm motility	Diclofenac Ibuprofen Sildenafil citrate	NOEC= 0.10 NOEC= 1000.0 NOEC= 1.0	120min =106.77 N/A N/A	Very toxic N/A N/A
	fertilisation success, pre-incubation of sperm	Diclofenac Ibuprofen Sildenafil citrate	NOEC= 1.00 NOEC= 0.10 NOEC = 1.0	120min = 565.53 120 min = 3.24×10^{9} N/A	Very toxic Non toxic N/A
	fertilisation success, pre-incubation of oocyte	Diclofenac Ibuprofen Sildenafil citrate	NOEC= 1.00 NOEC= 100 NOEC= 1.00	120min = 552.34 120min = 5.16×10^{19} N/A	Very toxic Non toxic N/A
	fertilisation success, exposure of sperm and oocyte	Diclofenac Ibuprofen Sildenafil citrate	LOEC = 0.01 NOEC= 1.00 NOEC= 1.0	60min =112.61 60min =1.14×10^{19} N/A	Very toxic Non toxic N/A

+ References from EU Directive 93/67/EEC, N/A = Not applicable

2.4   Discussion

To evaluate the effects of diclofenac, ibuprofen, and sildenafil citrate (Viagra) exposure on sperm motility and fertilisation success, the sperm and/or oocytes of *A. rubens, P. miliaris,* and *A. marina* were exposed to higher concentrations than those reported from seawater in previous studies, i.e. diclofenac = 0.6-843ng/L (Fent et al., 2006, Ankley et al., 2007b); ibuprofen = 0.01-2370ng/L (Fent et al., 2006, Ankley et al., 2007b); and sildenafil citrate = up to 10ng/L (Nieto et al., 2010, Fr. Schröder et al., 2010).

2.4.1 NOEC, LOEC and EC$_{50}$ of sperm motility and fertilisation success.

According Table 2.15, *A. rubens* sperm proved more tolerant to ibuprofen and diclofenac than *P. miliaris* sperm, as is shown by the 60 minutes sperm motility NOEC wherein *A. rubens* (0.10μg/L) was an order of magnitude higher than *P. miliaris* (0.01μg/L) and for *A. marina* the NOEC (0.10μg/L) could not be determined until after 120 minutes. *P. miliaris* sperm was severely affected by ibuprofen wherein the 60 minutes NOEC for sperm motility was 0.10μg/L. Regression log-concentration probits were obtained for both pharmaceuticals to estimate the effective concentration (EC$_{50}$) at 95% significance (Table 2.15). The 60 minutes EC$_{50}$'s for diclofenac effects on sperm motility were 2335.80μg/L for *A. rubens*, 378.22μg/L for *P. miliaris,* while the 120min EC$_{50}$ for *A. marina* was 106.77μg/L. The 60 minutes ibuprofen sperm motility EC$_{50}$ for *P. miliaris* was 845.98μg/L. According to the EU Directive 93/67/EEC classification there was no toxic effect of ibuprofen against sperm motility of *A. rubens* and *A. marina* as the NOEC was higher than 50μg/L and 1000μg/L respectively.

For fertilisation success of sperm pre-incubated in diclofenac, the 60 minutes NOEC for *A. rubens* was 0.1μg/L and 0.01μg/L for *P. miliaris*, whereas the 120 minutes NOEC for *A. marina* was 1.0μg/L. While sperm pre-incubation NOECs for *P. miliaris* and *A. marina* were both 0.10μg/L.

The 60 minutes EC$_{50}$'s for fertilisation success of sperm pre-incubated in diclofenac were 2610 and 298.42μg/L for *A. rubens* and *P. miliaris* respectively, and the 120 minutes EC$_{50}$ for *A. marina* was 565.53μg/L. The 60 minutes EC$_{50}$'s for fertilisation

success of sperm pre-incubated in ibuprofen was 437.03μg/L for *P. miliaris* and the 120 minutes EC_{50} value for *A. marina* was 3.24 x 10^9μg/L. The percentage of fertilisation success from pre-incubated sperm of *A. rubens* in ibuprofen did not differ significantly from controls indicating that *A. rubens* sperm were insensitive to ibuprofen exposure.

The 60 minutes NOEC for *A. rubens* and *P. miliaris* and the 120 minutes NOEC for *A. marina* for fertilisation success following pre-incubation of oocytes with diclofenac was 1.0μg/L. The 60 minutes EC_{50} values for diclofenac for *A. rubens* and *P. miliaris* were 1679.59μg/L and 429.37μg/L respectively, and was 4.56 x 10^6μg/L for ibuprofen for *P. miliaris*. The 120 minutes EC_{50} values for *A. marina* for diclofenac and ibuprofen were 552.34μg/L and 5.155 x 10^{18}μg/L respectively. There were no toxic effects on *A. rubens* oocytes until exposed to 50μg/L of ibuprofen therefore an EC_{50} could not be calculated.

Since a NOEC cannot be obtained using Dunnett's post hoc (no significant difference from control) for all three test species, the 60 minutes LOEC was determined for fertilisation success following exposure of both sperm and oocytes to diclofenac; this calculated as 0.01μg/L for all three species. The 60 minutes EC_{50} values for fertilisation success of *A. rubens*, *P. miliaris,* and *A. marina* were 616.48μg/L, 247.31μg/L and 1112.6μg/L, respectively. The 60 minutes NOECs for fertilisation success of sperm and oocytes exposed to ibuprofen were 0.10μg/L for *P. miliaris* and 1.00μg/L for *A. marina* while the 60 minutes EC_{50}'s for *P. miliaris* and *A. marina* were 792.96μg/L and 1.14 x 10^{19}μg/L. No toxic effects were observed for *A. rubens*.

For sperm motility in sildenafil citrate, 60 minutes NOEC for *A.rubens* and *P.miliaris* was 0.18μg/L while EC_{50}'s for sperm motility of *A.rubens* was 60min= 2.25 x 10^{12} and 7.23 x 10^{10} for *P.miliaris*. The 60 minutes NOEC for *A. rubens* and *P. miliaris* for fertilisation success following pre-incubation of sperm and incubation of sperm and oocytes with sildenafil citrate was 0.01μg/L.

To the authors best knowledge, this study provides the first data on toxicity of diclofenac, ibuprofen and sildenafil citrate to marine invertebrate gametes, and as such it

may potentially make a valuable contribution to risk assessment of these pharmaceuticals.

2.4.2 Toxicity level of pharmaceuticals to gametes of *A. rubens, P. miliaris* and *A. marina.*

According to EU Directive 93/67/EEC (Table 2.16), diclofenac is classified as toxic (EC_{50}=1679.58-2335.8μg/L) to *A. rubens* gametes in terms of exposure of sperm and oocytes separately based on endpoints of percentage sperm motility and fertilisation success of pre-incubated gametes. However, diclofenac toxicity increased and is classified as very toxic (EC_{50}=616.48μg/L) to *A. rubens* gametes when both sperm and oocytes were exposed simultaneously. Diclofenac is classified as very toxic to *P. miliaris* (EC_{50}=247.31-429.37μg/L) and *A. marina* (EC_{50}=106.7-552.34μg/L) gametes; whereas ibuprofen is classified as non-toxic to *A. rubens* and *A. marina* gametes but is classified as very toxic to *P. miliaris* gametes (EC_{50} = 792.96μg/L). Sildenafil citrate is classified as non-toxic to *A.rubens* (2.25×10^{12}- 7.15×10^{13}μg/L) and *P.miliaris* (4.335-7.23×10^{10}μg/L) gametes.

Table 2.16. Toxicity level of pharmaceutical classification from EU Directive 93/67/EEC.

Classification	EC_{50} Concentration (μg/L)
Non-toxic	>100,000
Harmful	10,000-100,000
Toxic	1000-10,000
Very toxic	<100-1000
Extremely toxic	<100

These gamete exposure tests indicated acute toxicity effects (immotile sperm and unfertilised oocytes) for *A. rubens*, *P. miliaris,* and *A. marina* sperm motility and fertilisation success and show that ibuprofen possesses lower acute toxicity compared to diclofenac. Even though diclofenac is classified as very toxic to gametes of *A. rubens, P. miliaris,* and *A. marina* and ibuprofen as very toxic to gametes of *P. miliaris*, it should be clearly pointed out that the values obtained in this study are much higher than environmental concentrations detected in seawater (diclofenac=0.6-843ng/L, ibuprofen=0.01-2370ng/L; (Kasprzyk-Hordern et al., 2008, Kim et al., 2007, Flippin et

al., 2007). Diclofenac is more toxic then ibuprofen and sildenafil citrate is less toxic to gametes of the three test species involved.

2.4.3 General discussion

In this chapter, diclofenac was found to significantly reduce sperm curvilinear velocity (swimming speed), the percentage of motile sperm, and fertilisation success with sperm pre-incubated in diclofenac with concentration- and time-dependent relationships for *A. rubens, P. miliaris,* and *A. marina*. In broadcast spawning marine invertebrates, sperm limitation is related to population dynamics (Levitan and Petersen, 1995b). The three test species are broadcast spawners where vast numbers of spawned motile sperm are crucial if sperm are to reach oocytes for fertilisation, especially when sperm density is low (Levitan, 2000b). Moreover, in the fertilisation process, a decrease in sperm swimming speed may lead to fewer sperm-oocyte interactions, which may significantly reduce population fertilisation success (Caldwell et al., 2004).

Fertilisation success reduced significantly when oocytes were pre-incubated in diclofenac. The reduction might relate to diclofenac being strongly associated with the oocyte surface membrane thereby affecting the binding of sperm/oocytes in the fertilisation process. The present results suggest that diclofenac exposure may have negative consequences for both sperm and oocytes of *A. rubens, P. miliaris,* and *A. marina*. By extrapolation, a reduction of fertilisation success caused by diclofenac may have harmful implications for the population dynamics for these species and, as all three are ecologically important within their various habitats; their local ecosystem may be disrupted. The fact that sperm treated with diclofenac reduced sperm motility and fertilisation success suggests that the sperm of all three species are sensitive and can be affected by diclofenac.

Exposure of sperm to ibuprofen only effected *P. miliaris* with a recorded decline in sperm swimming speed and percentage sperm motility at ≥ 20 minutes of exposure to 1.0μg/L. There was no reduction in sperm motility of *A. rubens* and *P. miliaris*. However, pre-incubation of sperm in ibuprofen reduced the fertilisation success in *A. marina* and *P. miliaris*. Oocytes from *A. marina* were more robust compared to sperm. A significant reduction in fertilisation success was only observed at pre-incubation of

oocytes in 1000μg/L at 120 minutes. Exposure of sperm and oocytes at 60 minutes caused a reduction in fertilisation of *P. miliaris* and *A. marina* at 1.0μg/L and 1000μg/L respectively. Gametes of *A. rubens* were highly tolerant to ibuprofen.

Sperm of *A. rubens* and *P. miliaris* showed positive effects in terms of motility and subsequent fertilisation success when exposed to sildenafil citrate. *A. rubens* sperm were influenced more than *P. miliaris*, wherein 18ng/L and above, sperm motility and fertilisation success increased. Compared to *P. miliaris*, only at 50ng/L was a positive response found. Even though sperm of *A. rubens* and *P. miliaris* do react to sildenafil citrate, *A. marina* sperm did not show any response.

The present study suggests that sildenafil citrate was not toxic to *A. rubens* and *P. miliaris* gametes. Even though sildenafil citrate positively affect sperm and oocytes of *A. rubens* and *P. miliaris* to increase motility and fertilisation success, the concentrations detect in surface waters, 10ng/L (Nieto et al., 2010, Fr. Schröder et al., 2010), is lower than the concentrations that improved reproductive success in this thesis.

The effects of sperm and oocyte responses for each species were different. This in itself is a very interesting observation. Each of the species occupies a different trophic level giving each different abilities in the biomagnification process (Sugni et al., 2007). Therefore, the effects of exposure diclofenac ibuprofen and sildenafil citrate to the gametes of test species could be diverse.

Numerous studies have examined the acute toxicity effects of anti-inflammatory drugs to aquatic organisms. Diclofenac is more toxic than other anti-inflammatories such as ibuprofen, naproxen, propranolol, and metoprol (based on algae, cladoceran, and macrophyte studies (Láng and Kőhidai, 2012, Cleuvers, 2003, Cleuvers, 2004a)). Similar results were obtained in the present study where the toxicity of diclofenac was greater that ibuprofen. The authors suggest the reason for toxicity is related to the logarithmised octanol-water partitioning coefficient (log K_{ow}). The toxicity of a substance increases with the log K_{ow}, diclofenac (log $K_{ow} = 4.4$) > ibuprofen (log $K_{ow} = 3.5$).

NSAIDs are known as prostaglandin blockers that inhibit COX-1 and/or COX-2 enzymes. In humans, these enzymes play important roles to reduce or prevent inflammation. However, the role of COX-1 and COX-2 in aquatic fauna varies. The COX-2 enzyme in humans is similar in fish, making fish a potential target of NSAIDs (Zou et al., 1999). Research regarding ibuprofen effects on *Mytilus galloprovincialis* indicated that activities of cyclooxygenase enzymes reduced and induced oxidative stress (Gonzalez-Rey and Bebianno, 2012). In sea urchins, Schuel (1984) discovered that eicosanoids play an important role in preventing polyspermic fertilisation. The oocytes of *Arbacia punctulata* and *Strongylocentrotus lividus* were treated to several cyclooxygenase inhibitors by using several anti-inflammatory drugs resulting in increased polyspermic fertilisation. Polyspermic fertilisation normally leads to abnormal development and embryo death, making it crucial to prevent these events. Diclofenac and ibuprofen inhibit prostaglandin synthesis by targeting the COX enzymes resulting in a higher possibility of polyspermic fertilisation.

Sildenafil citrate, also known as Viagra, was first introduced as a phosphodiesterase-5 (PDE5) inhibitor. In the biochemical process of sperm movement and chemotaxis in sea urchins, cyclic guanosine monophosphate (cGMP) plays an important role regulating motility and chemoattraction (Kaupp et al., 2003, Darszon et al., 2001, Garbers, 1989, Darszon et al., 2005, Neill and Vacquier, 2004). Changes in cGMP will regulate speract to induce sea urchin sperm motility and chemotaxis. The level of cGMP activity is controlled by guanylyl cyclase which is rich in the sperm flagellum (Garbers, 1989, Ward et al., 1985). However, the enzyme PDE5 works to accept cGMP, then break cGMP down and reduce sperm motility. As sildenafil citrate is a PDE inhibitor, it works to block PDE5 by attaching on the catalytic site of PDE5 and preventing it from accepting cGMP. This can increase guanylyl cyclase activity of cGMP and increase sperm motility. Su and Vacquier (2006) showed that sildenafil citrate is able to block the activity of PDE5 of sea urchin sperm to regulate cGMP and finally increase sperm motility. Similarly, a study showed sildenafil citrate increased sperm motility in humans (Glenn et al., 2009).

In broadcast spawners, fertilisation occurs externally in the water column. Any external stress, such as pollutants, could affect sperm function by spontaneous generation of reactive oxygen species (ROS) (Kazama et al., 2014), inducing sperm

DNA damage (Lewis and Galloway, 2009), disrupting sperm swimming ability (Caldwell et al., 2004), and the ability of the sperm to undergo the acrosome reaction (Pillai et al., 1997). Disruption of sperm function may lead to fertilisation failure. For broadcast spawners, fertilisation is the first stage in their life history. For them to survive, synchronous spawning and high production of sperm are strategies to ensure the highest chances of sperm/egg collision for fertilisation and thus maintain the species' population dynamics.

The reduction in percentage sperm motility and fertilisation success after exposure to diclofenac confirmed that this pharmaceutical could be active within the aquatic environment. This indicates the diclofenac may be a threat to the reproductive success of marine invertebrates. Under the EU Water Framework Directive (2013) (WFD 2013/39/EU), diclofenac is listed on the 'watch list' of emerging aquatic pollutants. Therefore, the proposed level of diclofenac in fresh and transitional waters (the estuarine and coastal area up to one nautical mile or 1.85 km from shore) should be monitored to ensure it remains lower than 10ng/L. Monitoring pharmaceuticals and their metabolites in coastal environments should be considered complimentary to perspective risk assessment. The decision by the European Council to place diclofenac on the watch list of emerging pollutants was timely as diclofenac may now justifiably be considered a threat to marine invertebrates.

However, further studies are needed to understand the effect of these pharmaceuticals to each life stage. As there are mixed contaminants in the aquatic environment, the effects of this mixture on the environment and on each contaminant should be studied. Studies regarding natural environmental stressors like ocean acidification, climate change and global warming should be included to determine how they affect the response of aquatic flora and fauna to pharmaceuticals and to improve and validate risk assessment procedures.

2.5   Conclusions

This chapter supports prior evidence that diclofenac presents a likely risk to the reproductive success of marine invertebrates with significant reductions noted for sperm motility and fertilisation success; however, the acute toxicity concentrations were more

than 100 times higher than those measured in the environment. Diclofenac is very toxic to sperm and oocytes of *A. rubens* (EC_{50}=616.48µg/L), *P. miliaris* (EC_{50}=792.96µg/L), and *A. marina* (EC_{50}=112.61µg/L), as classified by EU Directive 93/67/EEC.

Ibuprofen also threatens the fertilisation success of *P. miliaris.* Ibuprofen was nontoxic to *A. marina* and *A. rubens* gametes but is classified as very toxic to *P. miliaris* gametes (EC_{50} =792.86).

Positive effects were obtained for sperm motility and fertilisation success of *A. rubens* and *P. miliaris* with increased percentage sperm motility and fertilisation success with sildenafil exposure. However, there were no significant effects for *A. marina*.

In summary, diclofenac was more toxic than ibuprofen and sildenafil citrate was least toxic. Therefore, keeping environmental concentrations of diclofenac and ibuprofen under control is important to maintain sustainable ecosystems. Any impairment of reproductive success is of concern as each species are keystone species within their habitats. These results provide valuable insight into the ecotoxicological science of the reproductive success of invertebrates.

Chapter 3: Cadmium and Copper Effects on Sperm Motility and Fertilisation Success of Selected Marine Invertebrates

Abstract

Aquatic ecosystems are persistently exposed to numerous xenobiotics from anthropogenic sources. Elevated metal concentrations are one form of chemical pollution prevalent in most coastal seas. This chapter examines the effect of copper and cadmium on sperm motility and fertilisation success of three species of marine invertebrate; the polychaete worm, *Arenicola marina*, and the echinoderms *Psammechinus miliaris* and *Asterias rubens*. Exposure of sperm to copper concentrations greater than 10μg/l significantly reduced the average swimming speed (measured as curvilinear velocity) and percentage of sperm motility for all test species. For cadmium, a significant reduction was only observed when exposed to 1000μg/l. Incubation of gametes with ≥10μg/l of copper significantly reduced the fertilisation success of all test species. Cadmium proved less toxic with fertilisation inhibited at 1000μg/l for *A. rubens* and *A. marina* and 100μg/l for *P. miliaris*. Pre-incubation of sperm in copper inhibited fertilisation at ≥10μg/l, while pre-incubation in cadmium inhibited fertilisation at concentrations of 100μg/l for *P. miliaris* and 1000μg/l for *A. rubens* and *A. marina* respectively. The effects of pre-incubation of oocytes in copper and cadmium were concentration and time dependent. A significant reduction of fertilisation success was only observed when oocytes were pre-incubated in 1000μg/l of copper for *A. rubens* and ≥100 μg/l for *P. miliaris* and *A. marina.* Pre-incubation with cadmium inhibited fertilisation at 1000μg/l for all species. In conclusion, the potential exists for impairment of echinoderm and polychaete reproductive success in metal contaminated sites, possibly threatening the species population in those environments.

3.1   Introduction

Metals have long been recognised as major marine pollutants. They are slowly released into water bodies and are generally found in very low concentrations (Ansari et al., 2004). Some metals are essential for life and some are merely beneficial, whereas many are highly toxic (Lane and Morel, 2000, Uthus, 2003, Zeng et al., 2005). Metals have a tendency to accumulate in the tissues of organisms and can be amplified along food chains. Metal toxicity varies depending on the response of different organisms to metal exposure. At low levels some metals such as copper or cobalt are essential for enzyme function but will readily become toxic at higher concentrations.

Bryan (1976a) reported that several aquatic species are sensitive to copper in the concentration range 1-10µg/l, while Nelson et al. (1988) observed that 2µg/l of copper dichloride ($CuCl_2$) had significant toxic effects on young bay scallops and surf clams. Even though cadmium is not an essential element for any organism, cadmium reportedly increases phytoplankton photosynthesis and growth at a concentration of 100µg/l (Miao and Wang, 2006). However, cadmium has been listed by the US EPA and the EC's priority list as one of the most toxic metals in industrial discharges and has been reported to affect reproductive success of marine invertebrates (Filosto et al., 2008, Giudici and Guarino, 1989). Other studies report that increased exposure to cadmium and copper may result in reduced gamete viability in marine bivalves (Fitzpatrick et al., 2008c) and may cause significant physiological changes (Akberali and Trueman, 1985).

The pollution of marine ecosystems by metals is a global problem and is aggravated by the ability of these ecosystems to concentrate and accumulate metals within food chains (Watling, 1983). Marine organisms can take up these metals through their digestive system and increase the potential for their entry to higher trophic levels including into the human food chain. It is therefore important to continually monitor the levels of metals within the environment.

Metal contaminants may affect the reproductive success of organisms by reducing the quality and/or quantity of gametes and consequently affect fertilisation success, embryo development, larval viability, and species fitness and survival (Fitzpatrick et al., 2008b, Filosto et al., 2008, Arizza et al., 2009). Sperm motility is a crucial factor in determining sperm quality (at least for species reliant on swimming sperm, including marine broadcast spawners).

This chapter examines the effects of cadmium and copper on sperm motility and fertilisation success of a polychaete worm, *Arenicola marina* and two echinoderms, *Asterias rubens* and *Psammechinus miliaris*. The objectives are: 1) to determine the swimming speed (curvilinear velocity, VCL); and 2) to study fertilisation success when exposed to certain concentrations of copper and cadmium. The results will increase knowledge of the effects of metal exposure on reproductive processes of ecologically important marine invertebrates.

3.2   Materials and Methods

Materials and methods are as described in chapter 2 except for chemical preparation.

3.2.1 Chemical preparation:

All experiments were performed with analytical grade metal salts, $CdCl_2$ and $CuSO_4$ (purchased from Sigma Aldrich UK), dissolved in $0.22\mu m$ filtered fresh seawater and then serially diluted to obtain a concentration range (0.1, 1, 10, 100, 1000$\mu g/l$). All experiments included a control of filtered seawater only. Glassware and equipment were acid washed before use.

3.3 Results

3.3.1 Sperm motility and curvilinear velocity

3.3.1.1 Copper

Figure 3.1 shows the data for *A. rubens* sperm when exposed to copper over time. Sperm motility and curvilinear velocity were affected by increasing time of exposure and copper concentration. Significant reductions in sperm motility and curvilinear velocity were seen when exposed to ≥10μg/l copper after 20 minutes; however, a significant interaction between time and concentration was only observed for percent of sperm motility (F=2.149, p=<0.001; Table 3.1).
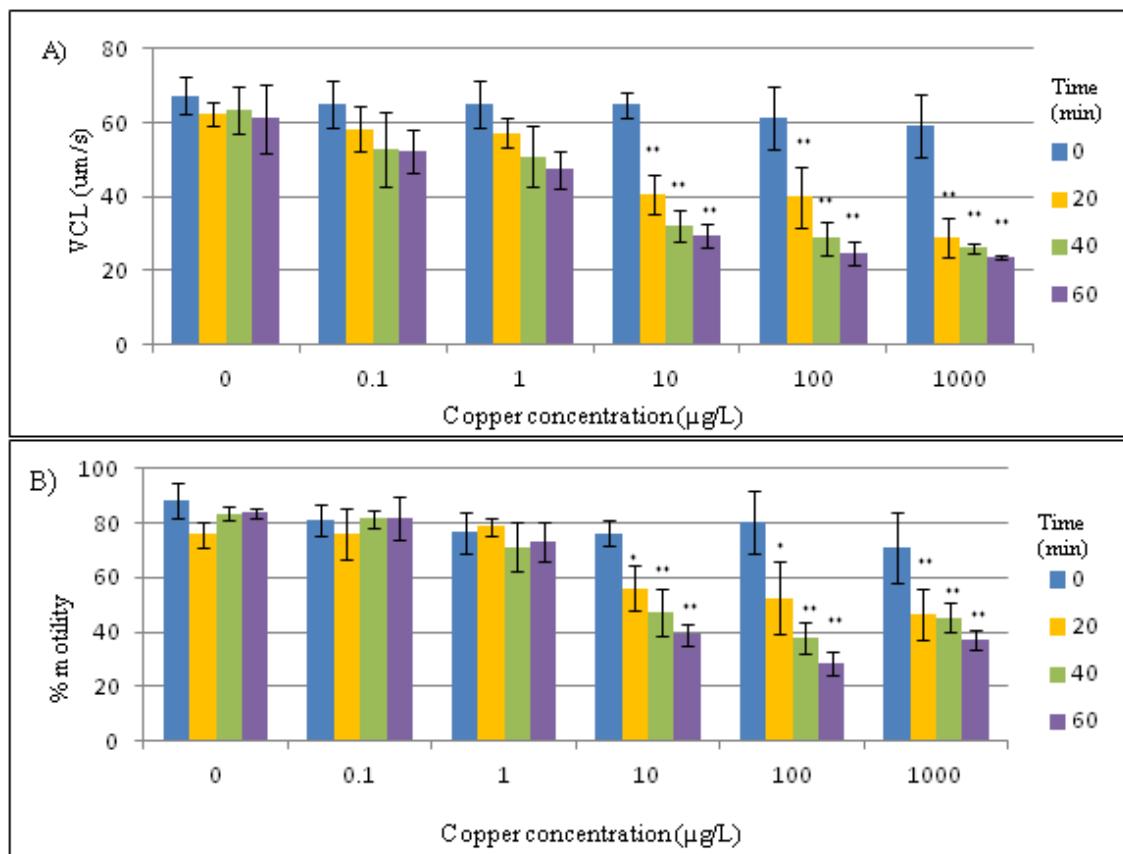
Figure 3.1. A) Percentage of curvilinear velocity (VCL) and B) sperm motility of *Asterias rubens* sperm after exposure to copper for up to 60 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

There was a significant decrease in percentage of sperm motility and curvilinear velocity when exposed to ≥10µg/l of copper at almost all time intervals for *P.miliaris* gametes. Sperm motility and curvilinear velocity were negatively affected by copper concentration and time of exposure and there was a significant interaction between time and concentration (F=17.05, p=<0.001; F=1.72, p=0.049 respectively; Table 3.1).
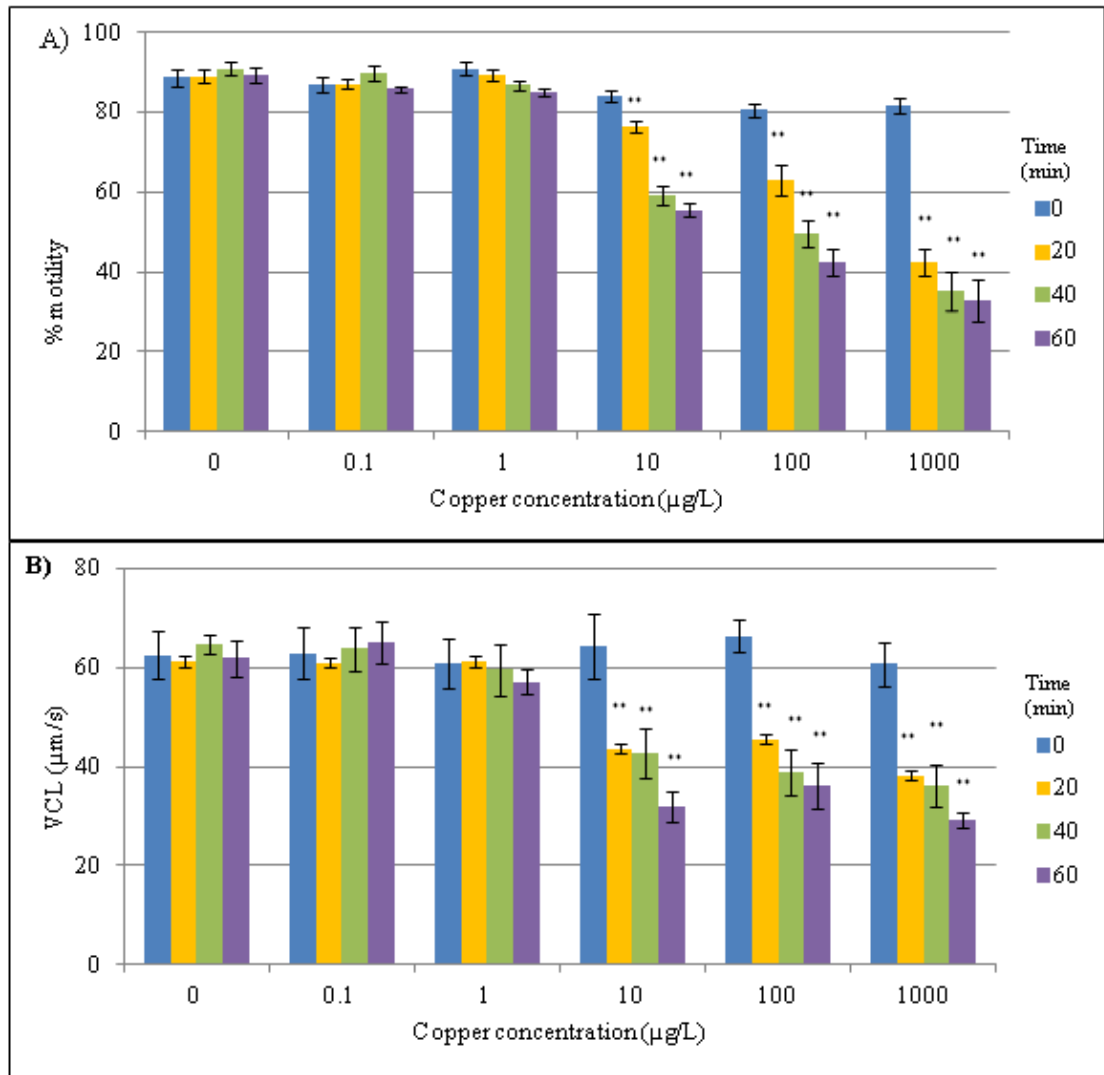
Figure 3.2. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Psammechinus miliaris* sperm after exposure to copper for up to 60 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

The effects on sperm motility and curvilinear velocity of increasing copper concentrations over time on *A. marina* sperm are shown in Figure 3.3. A significant reduction in curvilinear velocity and percentage sperm motility were observed when exposed to ≥ 10ug/l of copper for more than 20 minutes. These measures declined in a clear concentration and dependent-time manner (F=74.393, p=<0.001; F=4.3849, p=<0.001 respectively, Table 3.1).
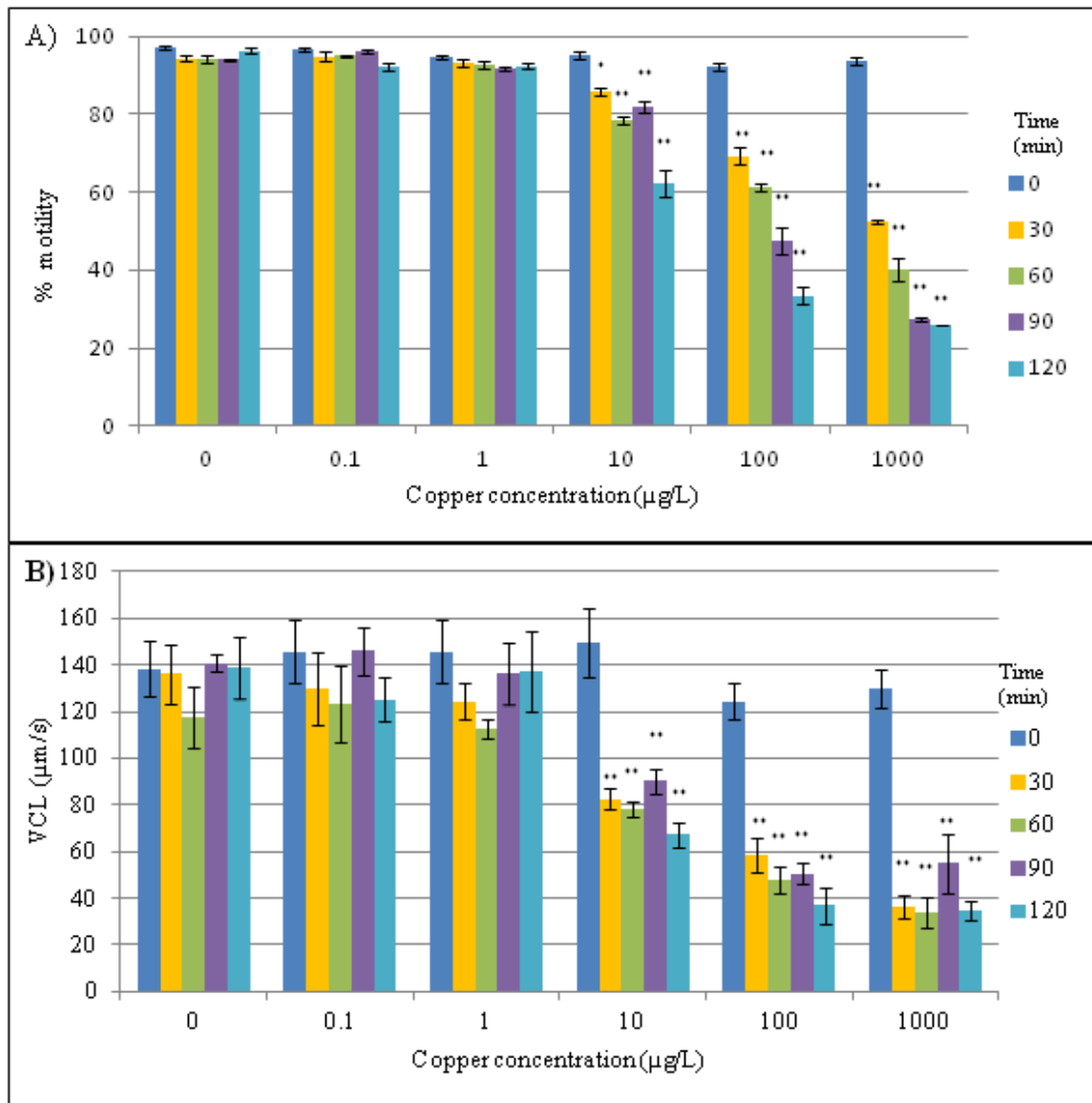
Figure 3.3. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Arenicola marina* spermatozoa after exposure to copper for up to 120 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Table 3.1. Analysis of variance on arcsine transformed data for sperm motility and log transformed data for VCL for *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* sperm, with time and copper concentration as the main effects.

Species	Parameter	Source	SS	df	MS	F	Sig.
Asterias rubens	Sperm motility	Concentration	49712.81	5	9942.562	20.353	<0.001
		Time	9218.354	3	3072.785	6.29	<0.001
		Time*Concentration	15745.53	15	1049.702	2.149	<0.001
		Error	93792.09	192	488.5		
	Curvilinear velocity	Concentration	13248.76	5	2649.753	7.396	<0.001
		Time	10846.8	3	3615.599	10.092	<0.001
		Time*Concentration	7695.347	15	513.023	1.432	0.136
		Error					
Psammechinus miliaris	Sperm motility	Concentration	55604.7	5	11120.94	200.436	<0.001
		Time	12936.56	3	4312.185	77.72	<0.001
		Time*Concentration	14190	15	946	17.05	<0.001
		Error	10652.89	192	55.484		
	Curvilinear velocity	Concentration	8077.038	5	1615.408	8.972	<0.001
		Time	11569.91	3	3856.637	21.419	<0.001
		Time*Concentration	4656.697	15	310.446	1.724	0.049
		Error	34570.37	192	180.054		
Arenicola marina	Sperm motility	Concentration	69901.68	5	13980.34	982.006	<0.001
		Time	18753.55	4	4688.388	329.321	<0.001
		Time*Concentration	21181.82	20	1059.091	74.393	<0.001
		Error	2562.571	180	14.237		
	Curvilinear velocity	Concentration	239021.7	5	47804.35	69.526	<0.001
		Time	88611.18	4	22152.8	32.219	<0.001
		Time*Concentration	60290.42	20	3014.521	4.384	<0.001
		Error	123763	180	687.572		

3.3.1.2 Cadmium

Figure 3.4 shows the data for *P. miliaris* sperm when exposed to increasing concentration of cadmium over time. There was a significant decline in curvilinear velocity and percentage of sperm motility when exposed to ≥10µg/l of cadmium at for longer than 20 minutes. Curvilinear velocity and percentage motility were affected by concentration, time and interaction between the terms (F= 31.72 p=<0.001; F=13.23, p=<0.001 respectively, Table 3.2).
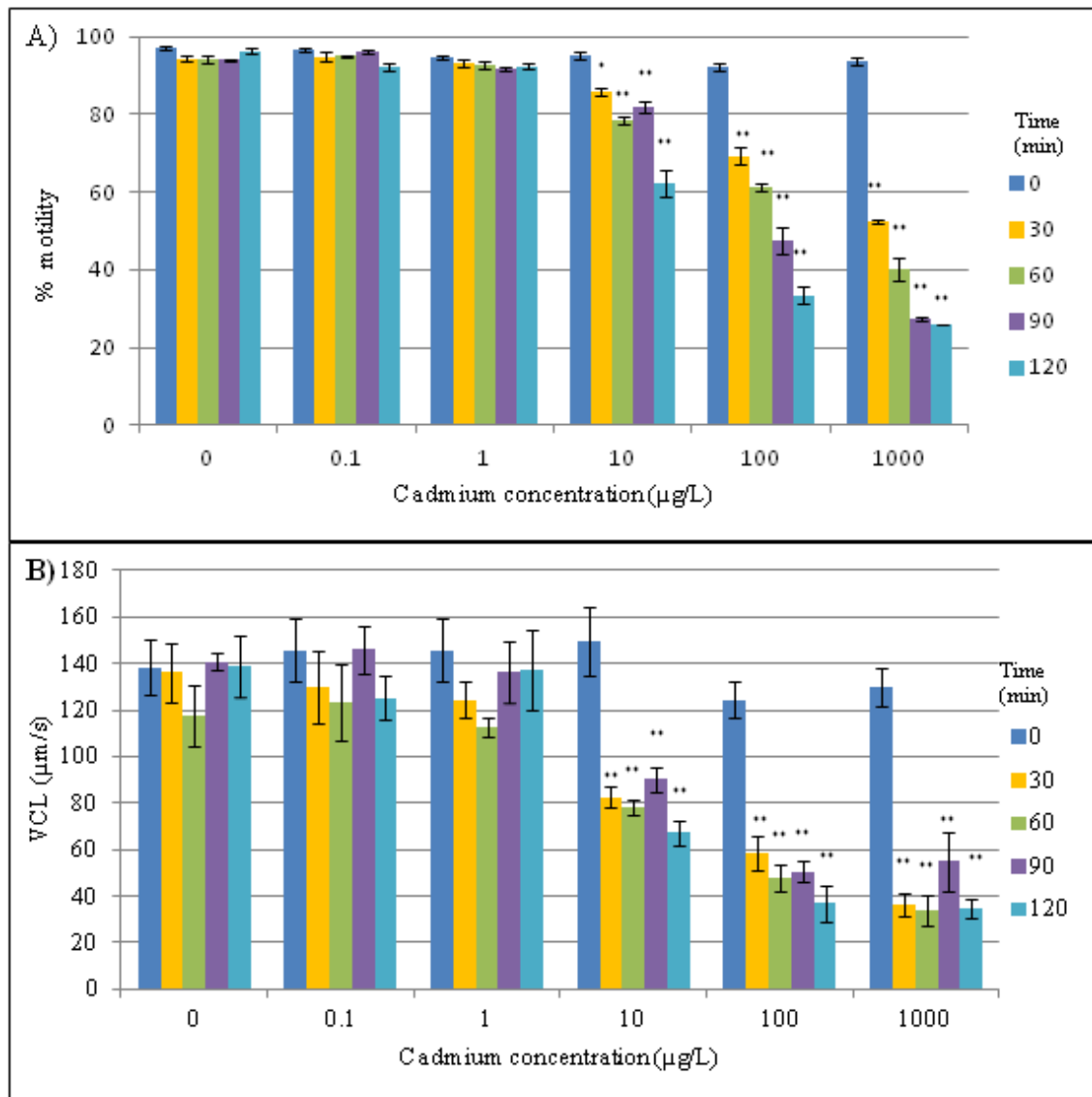
Figure 3.4. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Psammechinus miliaris* sperm after exposure to cadmium for up to 60 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Figure 3.5 presents the data for *A. rubens* sperm exposed to increasing concentration of cadmium over time. Exposure to 1000µg/l for more than 20 minutes significantly reduced sperm motility and curvilinear velocity. There was also significant interaction between concentration and time (F=31.48, p=<0.001; F=5.49, p=<0.001 respectively, Table 3.2).
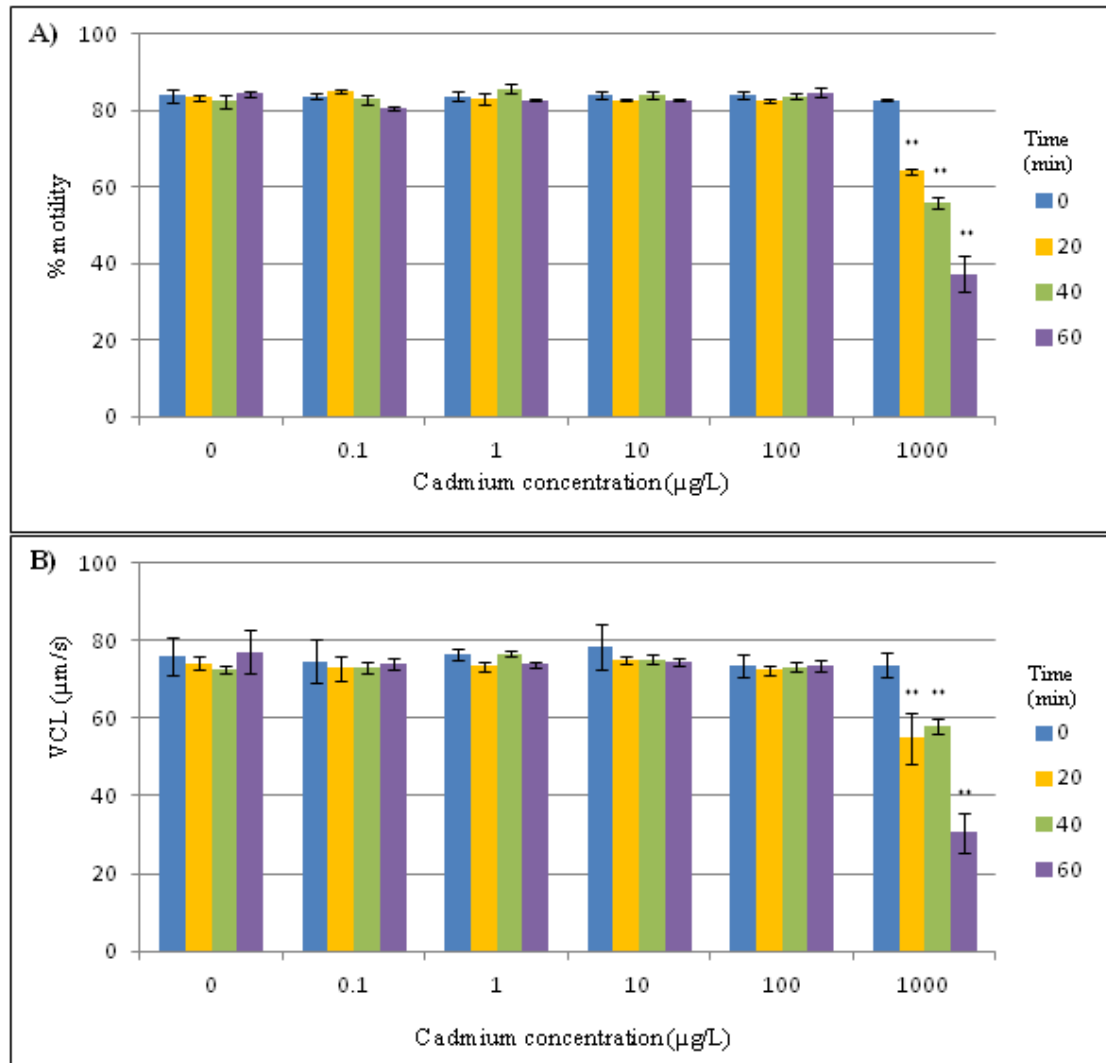
Figure 3.5. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Asterias rubens* sperm after exposure to cadmium for up to 60 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Figure 3.6 presents the data on the effects of cadmium exposure over time on *Arenicola marina* sperm. Only the highest cadmium exposure (1000ug/l at 60 minutes for percentage motility and 1000μg/l at 90 minutes for VCL) caused a significant reduction in curvilinear velocity and percentage of sperm motility. These measures declined with a clear interaction of concentration and time (F=15.693.23, p=<0.001; F=3.23, p=<0.001 respectively, Table 3.2).
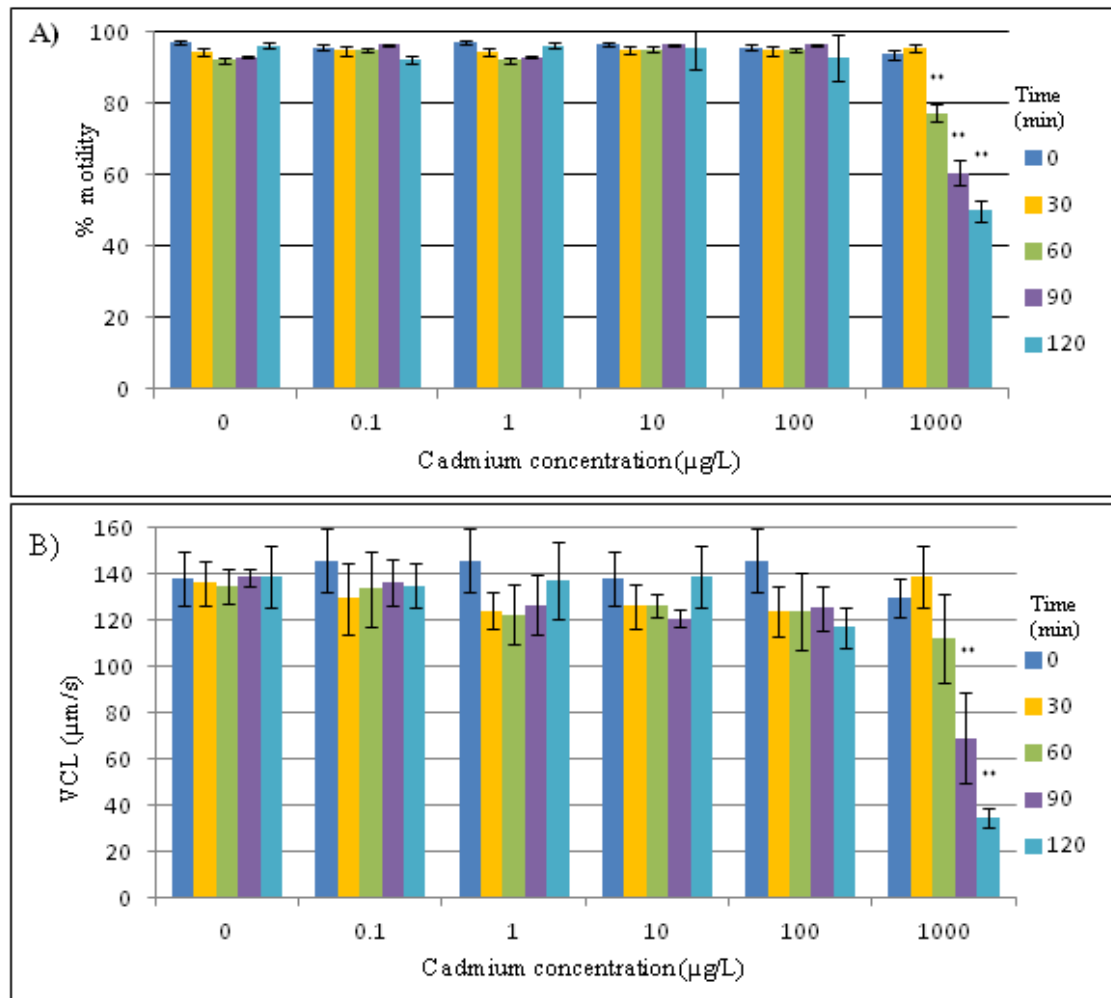
Figure 3.6. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Arenicola marina* sperm after exposure to cadmium for up to 120 minutes. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Table 3.2. Analysis of variance on arcsine transformed data for sperm motility and log transformed data for VCL for *Psammechinus miliaris, Asterias rubens* and *Arenicola marina* sperm, with time and cadmium concentration as the main effects.

Species	Parameter	Source	SS	df	MS	F	Sig.
Psammechinus miliaris	Sperm motility	Concentration	12181.26	5	2436.253	132.677	<0.001
		Time	4730.903	3	1576.968	85.881	<0.001
		Time*Concentration	8736.903	15	582.46	31.72	<0.001
		Error	3525.556	192	18.362		
	Curvilinear velocity	Concentration	35601.86	5	7120.372	43.788	<0.001
		Time	13150.37	3	4383.458	26.957	<0.001
		Time*Concentration	32259.15	15	2150.61	13.226	<0.001
		Error	31220.82	192	162.608		
Asterias rubens	Sperm motility	Concentration	16502.87	5	3300.574	196.263	<0.001
		Time	1882.981	3	627.66	37.323	<0.001
		Time*Concentration	7940.13	15	529.342	31.476	<0.001
		Error	3228.889	192	16.817		
	Curvilinear velocity	Concentration	11102.8	5	2220.56	24.596	<0.001
		Time	2009.293	3	669.764	7.419	<0.001
		Time*Concentration	7443.942	15	496.263	5.497	<0.001
		Error	17334.21	192	90.282		
Arenicola marina	Sperm motility	Concentration	10390.95	5	2078.19	71.666	<0.001
		Time	3641.6	4	910.4	31.395	<0.001
		Time*Concentration	9104.857	20	455.243	15.699	<0.001
		Error	5219.714	180	28.998		
	Curvilinear velocity	Concentration	67274.39	5	13454.88	12.968	<0.001
		Time	30481.17	4	7620.292	7.345	<0.001
		Time*Concentration	67211.03	20	3360.552	3.239	<0.001
		Error	186751.4	180	1037.508		

3.3.2 Pre-incubation of sperm with metals

3.3.2.1 Copper

Incubation of sperm in copper before fertilisation had a negative effect on the fertilisation success for *A. rubens* (Figure 3.7(A)). Control sperm maintained a fertilisation success of 95.7±0.76% to 91.44±0.76% throughout the experiment. The interaction between copper concentration and time was significant (F=1.826, p= 0.034, Table 3.3). Fertilisation success for sperm incubated in $10\mu gl^{-1}$ copper declined to 73.3±1.63% after 20 minutes but the inhibition rate subsequently declined with time. After 60 minutes only 31.54±4.66% of oocytes were fertilised. A similar pattern was recorded for *P. miliaris* sperm (Figure 3.7(B)). A pronounced decline in fertilisation success was observed at 10, 100 and 1000μg/l. Sperm incubated for 60 minutes with 10, 100 and 1000ug/l copper had a mean fertilisation success of 60.4±2.82%, 53.7±1.17% and 32.29±2.50% respectively, whereas controls had a mean fertilisation success of 96±0.5%. There was a significant interaction between concentration and exposure period (F=25.85, p=<001, Table 3.3). Incubation of sperm before fertilisation in copper had a negative effect on the fertilisation success of *A. marina* (Figure 3.7(C)). Control sperm maintained a fertilisation success of 94.44±0.89% to 98.8±0.45%. With 20 minutes pre-incubation in copper, fertilisation success reduced significantly at concentrations of 10ug/l and above. There was a significant interaction between concentration and time (F=76.75, p= <0.001, Table 3.3).
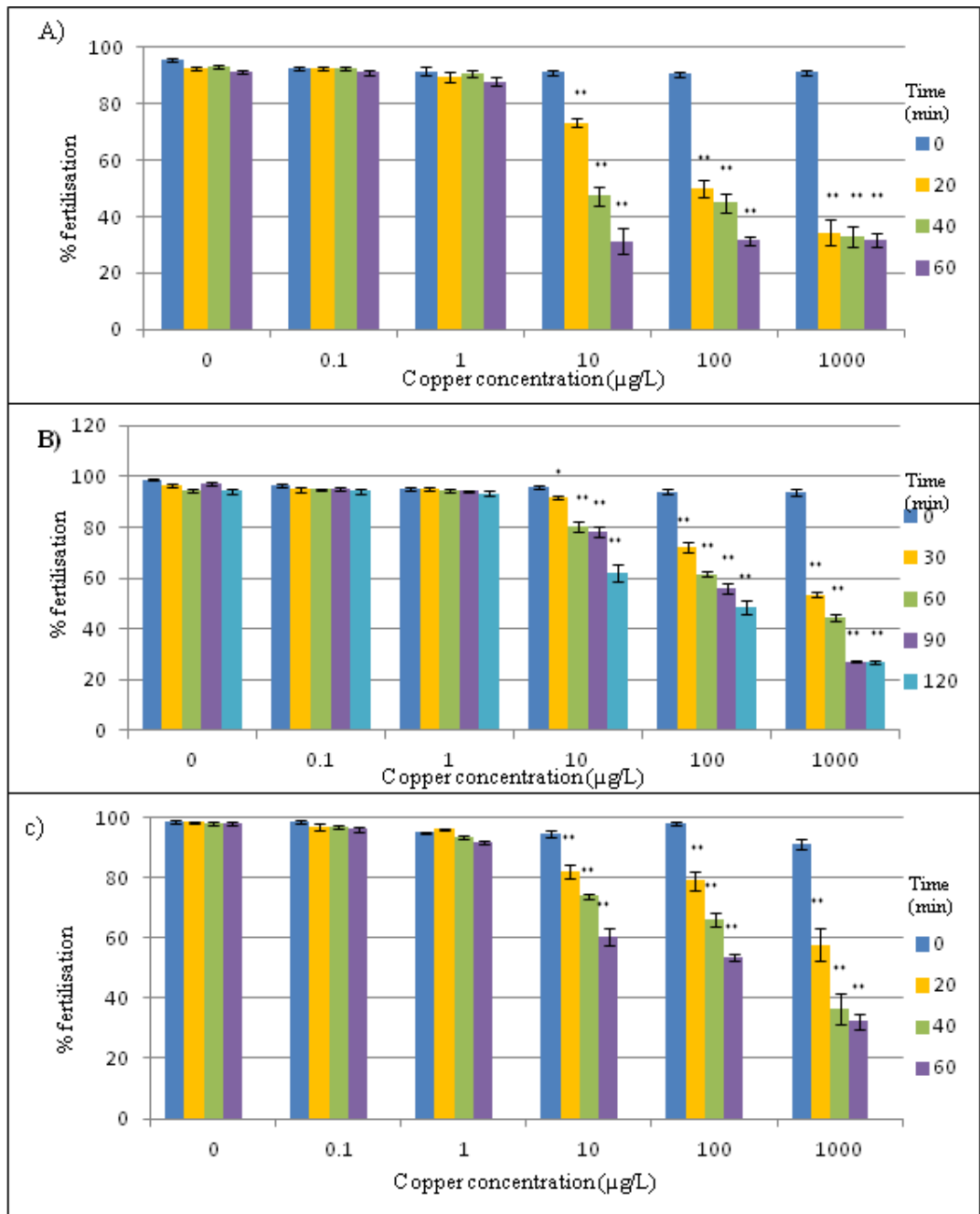
Figure 3.7. Percent fertilisation of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris* and C) *Arenicola marina* after incubation of sperm with copper sulphate at different concentrations and times prior to fertilisation. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Table 3.3. *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Analysis of variance on arcsine transformed fertilisation success, with time and copper concentration as main effects.

Source	SS	df	MS	F	Sig.
Asterias rubens					
Concentration	19289.425	5	3857.885	7.637	<0.001
Time	16572.496	3	5524.165	10.936	<0.001
Time*Concentration	13834.456	15	922.297	1.826	0.034
Error	96989.391	192	505.153		
Psammechinus miliaris					
Concentration	42533.500	5	8506.700	223.452	<0.001
Time	16103.907	3	5367.969	141.005	<0.001
Time*Concentration	14759.759	15	983.984	25.847	<0.001
Error	7309.333	192	38.069		
Arenicola marina					
Concentration	64249.067	5	12849.813	1084.300	<0.001
Time	16505.743	4	4126.436	348.199	<0.001
Time*Concentration	18191.171	20	909.559	76.751	<0.001
Error	2133.143	180	11.851		

3.3.2.2 Cadmium

The data for mean percentage fertilisation success after pre-incubation of sperm for 0, 20, 40 and 60 minutes in increasing concentrations of cadmium chloride are presented in Figure 3.8. Mean fertilisation success of control sperm for *A. rubens* (Figure 3.8(A)) was maintained within the range of 99.1±0.35% to 98.0±0.18%. There was a significant decline in fertilisation success when sperm were pre-exposed to 1000μg/l cadmium for longer than 20 minutes. The interaction between concentration and time was significant (F=165, p= <0.001, Table 3.4). The incubation of sperm in cadmium had a negative effect on the fertilisation success of *P. miliaris* at 100 and 1000 μg/l (Figure 3.8(B)). Control sperm maintained a fertilisation success of 98.4±0.44 to 98.2±0.52%. The interaction between concentration and time was significant (F=96.58, p= <0.001, Table 3.4). Fertilisation success for sperm incubated in 1000 μg/l had declined to 82.00±2.23% after 20 minutes but the inhibition rate subsequently declined with time. After 60 minutes only 33.11±2.24% of gametes were fertilised. For *A. marina*, control sperm maintained a fertilisation success of 98.8±0.45 to 94.26±0.89. A significant

reduction in fertilisation success was observed for sperm incubated with 1000μg/l of cadmium for longer than 90 minutes. The interaction between concentration and time was significant (F=32.96, p= <0.001, Table 3.4).
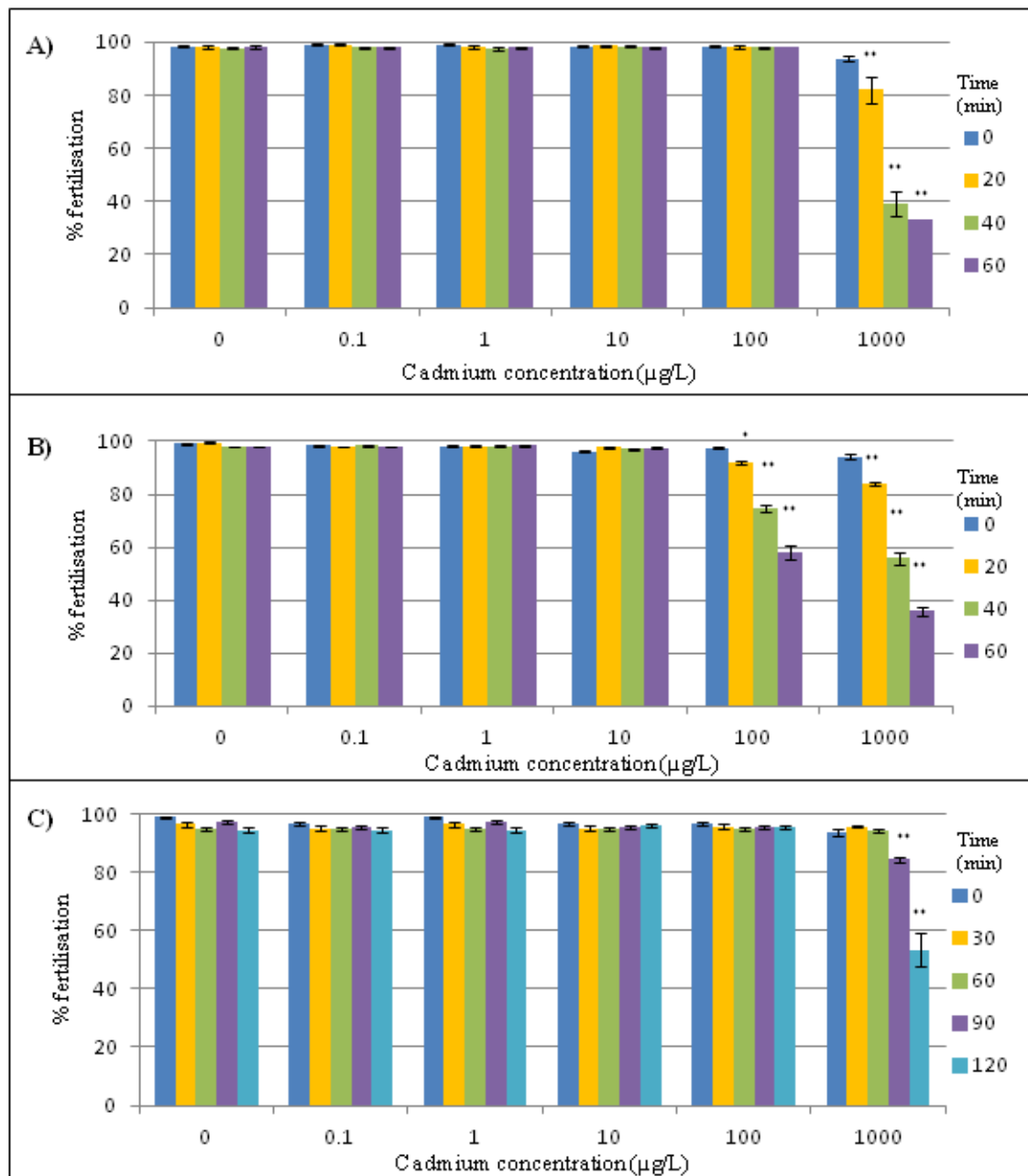
Figure 3.8. Percent fertilisation of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris* and C) *Arenicola marina* after incubation of sperm with cadmium chloride at different concentrations and times prior to fertilisation. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Table 3.4. *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Analysis of variance on arcsine transformed fertilisation success, with time and cadmium concentration as main effects.

Source	SS	df	MS	F	Sig.
Asterias rubens					
Concentration	30628.759	5	6125.752	810.265	<0.001
Time	8983.907	3	2994.636	396.106	<0.001
Time*Concentration	18803.315	15	1253.554	165.810	<0.001
Error	1451.556	192	7.560		
Psammechinus miliaris					
Concentration	39534.833	5	7906.967	563.479	<0.001
Time	4609.833	3	1536.611	109.504	<0.001
Time*Concentration	20327.611	15	1355.174	96.575	<0.001
Error	2694.222	192	14.032		
Arenicola marina					
Concentration	3903.886	5	780.777	71.767	<0.001
Time	2031.857	4	507.964	46.691	<0.001
Time*Concentration	7171.686	20	358.584	32.960	<0.001
Error	1958.286	180	10.879		

3.3.3   Pre-incubation of oocytes with metal

3.3.3.1 Copper

Data for *A. rubens* is presented in Figure 3.9(A). Two-way ANOVA for *A. rubens* copper-exposed oocytes revealed a significant reduction in fertilisation success when exposed to 1000µg/l for 20 minutes and above. After 60 minutes 19.78±2.79% of oocytes were fertilised. Control oocytes maintained a fertilisation success of 97.55±0.715% to 97.11±0.45%. Interaction between concentration and time was significant (F=134, p= <0.001, Table 3.5). Incubation of oocytes in copper had a negative effect on the fertilisation success of *P. miliaris* (Figure 3.9(B)). A pronounced decline in fertilisation success was observed at 100µg/l with increasing concentration. Sperm incubated for 60 minutes with 1000µg/l had a mean fertilisation success of 32.22±1.27%, whereas controls had a mean fertilisation success of 98.44±0.55%. Inhibition was concentration- and time-dependent (F=132.56, p=<0.001, Table 3.5). Incubation of oocytes in copper before fertilisation also negatively affected the fertilisation success of *A. marina* (Figure 3.9 (C)). Control oocytes maintained a fertilisation success of 98.57±0.43 to 94.28±0.89%. At 30 minutes of oocyte incubation,

fertilisation significantly reduced at 10µg/l copper and above. The interaction between concentration and time was significant (F=96.69, p= <0.001, Table 3.5).
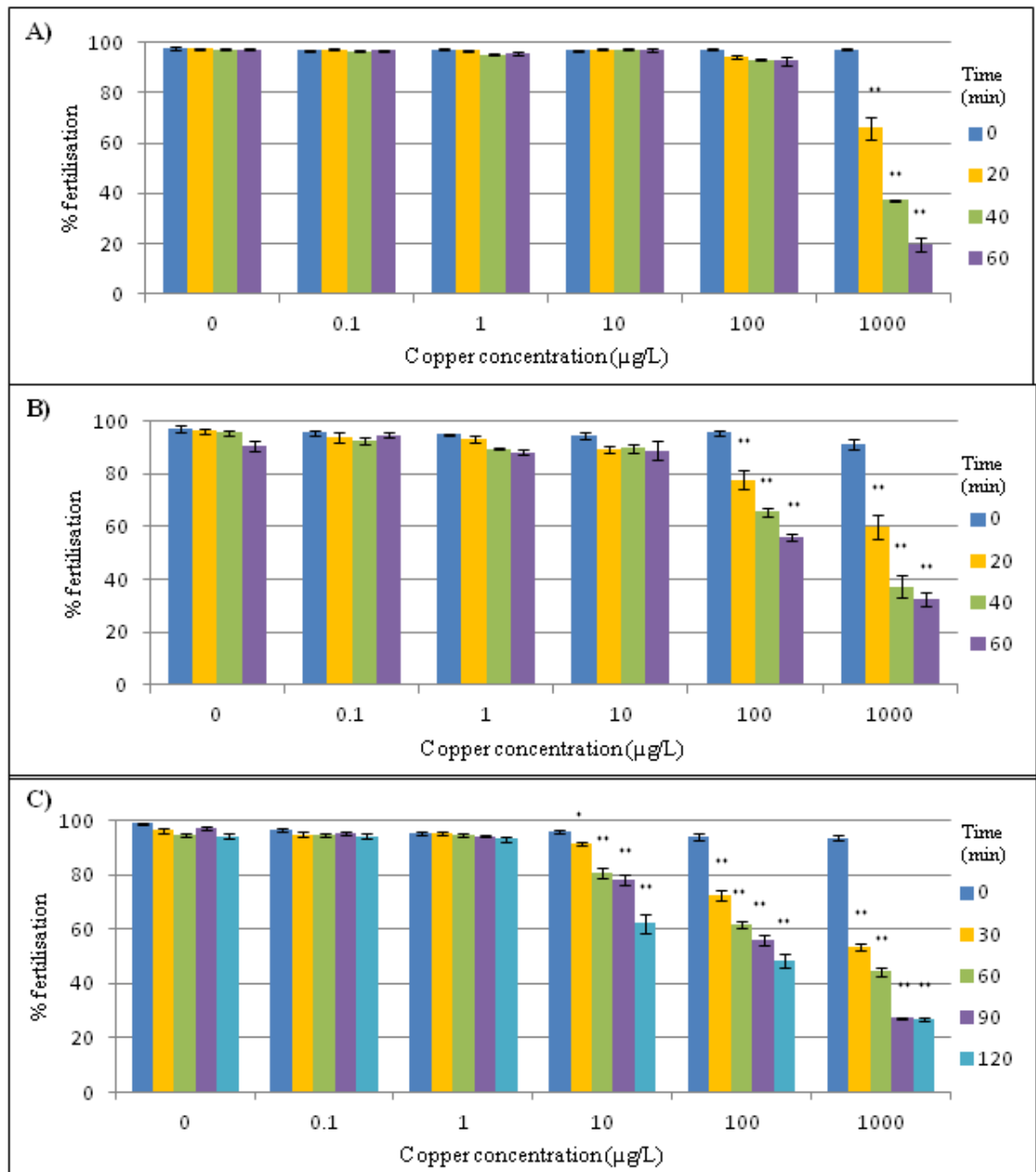
Figure 3.9. Percent fertilisation of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris* and C) *Arenicola marina* after incubation of oocytes with copper sulphate at different concentrations and times prior to fertilisation. Treatments significantly different from the control within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Table 3.5. *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Analysis of variance on arcsine transformed fertilisation success, with time and copper concentration as main effects.

Source	SS	df	MS	F ratio	P value
Asterias rubens					
Concentration	52712.093	5	10542.419	824.685	<0.001
Time	5435.741	3	1811.914	141.738	<0.001
Time*Concentration	25695.648	15	1713.043	134.004	<0.001
Error	2454.444	192	12.784		
Psammechinus miliaris					
Concentration	42533.500	5	8506.700	223.452	<0.001
Time	16103.907	3	5367.969	141.005	<0.001
Time*Concentration	14759.759	15	983.984	25.847	<0.001
Error	7309.333	192	38.069		
Arenicola marina					
Concentration	64249.067	5	12849.813	1084.300	<0.001
Time	16505.743	4	4126.436	348.199	<0.001
Time*Concentration	18191.171	20	909.559	76.751	<0.001
Error	2133.143	180	11.851		

3.3.3.2 Cadmium

Incubation of oocytes in cadmium chloride before fertilisation had a negative effect on the fertilisation success of *A. rubens* (Figure 3.10(A)). Fertilisation success for oocytes incubated in 1000µg/l declined to 91.11±0.58% after 20 minutes but the inhibition rate subsequently declined with time. After 60 minutes, only 52.22±1.71% of gametes were fertilised. Control oocytes maintained a fertilisation success of 99.11±0.35 to 98.00±0.01%. The interaction between concentration and time was also significant (F=142.17, p= <0.001, Table 3.6). A similar pattern was recorded for *P. miliaris* oocytes (Figure 3.10(B)) with a pronounced decline in fertilisation success observed at 1000µg/l. Oocytes incubated for 60 minutes with 1000µg/l had a mean fertilisation success of 32.22±1.27%, whereas controls had a mean fertilisation success of 98.44±0.55%. Concentration and time had significant interactions (F=132.56, p=<0.001, Table 3.6). Incubation of oocytes in cadmium before fertilisation also negatively affected fertilisation success of *A. marina* (Figure 3.10(C)). Fertilisation success reduced significantly after 60 minutes of exposure at 1000µg/l dropping to 74.57±2.49% and

declined further to 54.28±3.61 at 120 minutes. The interaction between concentration and time was significant ($F=29.44$, $p= <0.001$, Table 3.6). Control oocytes maintained a fertilisation success of 98.85±0.40 to 95.86±0.88%.
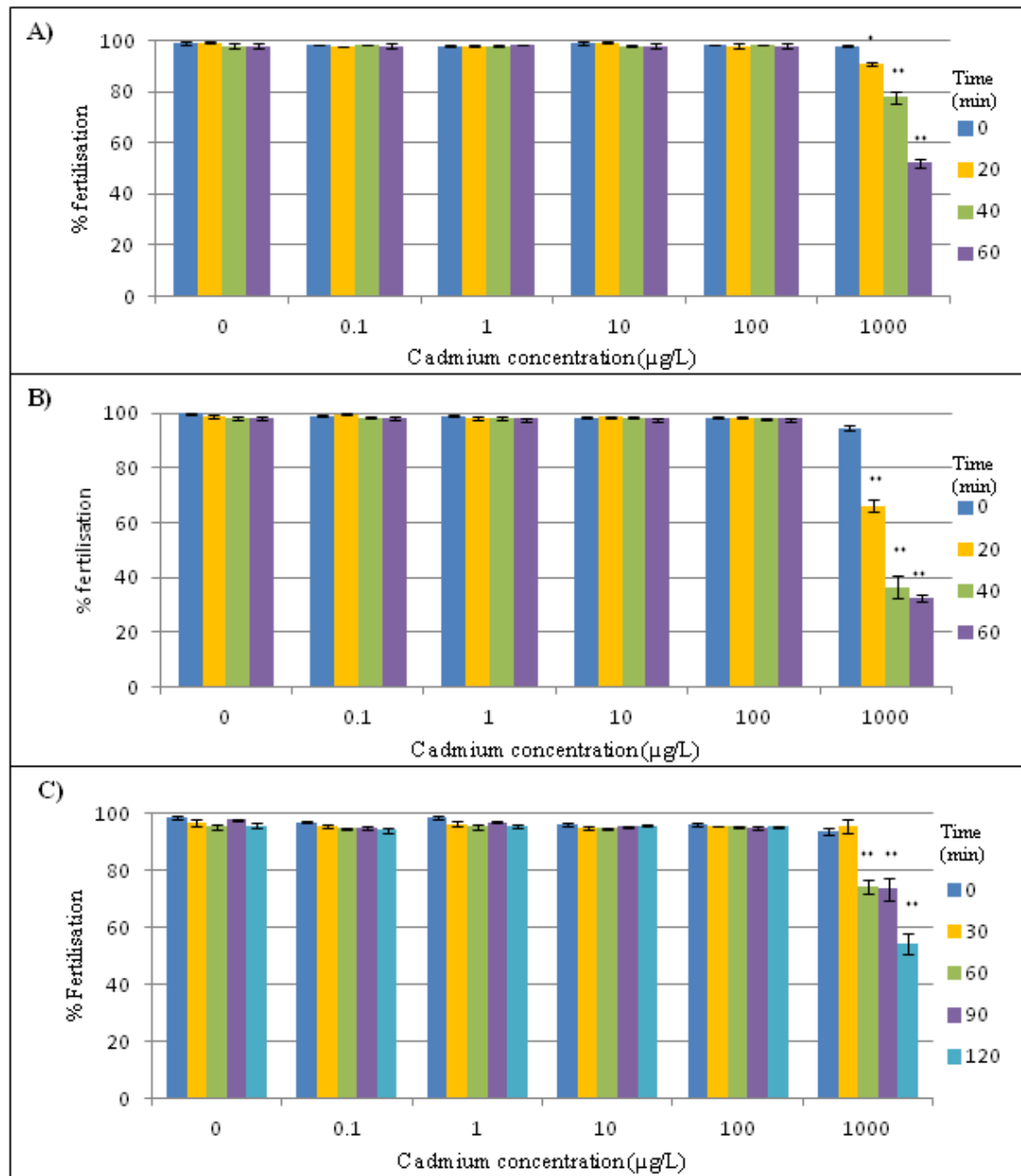
Figure 3.10. Percent fertilisation of oocytes from A) *Asterias rubens*, B) *Psammechinus miliaris* and C) *Arenicola marina* after incubation of oocytes with cadmium chloride at different concentrations and times prior to fertilisation. Treatments significantly different from the control within the same time intervals are indicated by asterisks *$p<0.05$; **$p<0.01$.

Table 3.6. *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Analysis of variance on arcsine transformed fertilisation success, with time and cadmium concentration as main effects.

Source	SS	df	MS	F	Sig.
Asterias rubens					
Concentration	10362.148	5	2072.430	491.917	<0.001
Time	2062.074	3	687.358	163.153	<0.001
Time*Concentration	8984.370	15	598.958	142.170	<0.001
Error	808.889	192	4.213		
Psammechinus miliaris					
Concentration	44790.389	5	8958.078	871.990	<0.001
Time	4723.315	3	1574.438	153.258	<0.001
Time*Concentration	20428.352	15	1361.890	132.568	<0.001
Error	1972.444	192	10.273		
Arenicola marina					
Concentration	48037.124	5	9607.425	1384.418	<0.001
Time	7842.838	4	1960.710	282.536	<0.001
Time*Concentration	13420.876	20	671.044	96.697	<0.001
Error	1249.143	180	6.940		

3.3.4 Incubation of sperm and oocytes in metals

3.3.4.1 Copper

Figure 3.11 shows the results of incubating both oocytes and sperm with copper sulphate on fertilisation success. A significant decrease in fertilisation success was observed at 10, 100 and 1000µg/l and after 60 minutes fertilisation compared to the control. For *A. rubens*, fertilisation success was reduced to 19.22±2.48% at the highest copper concentration compared to controls of 97.6±0.71% (F=91.87, p=<0.001). For *P. miliaris*, fertilisation of the control was 96.9±0.36% which declined to 41.2±0.33% at 1000µg/l (F=156, p=<0.001). Percentage fertilisation success also declined in *A. marina* dropping from a mean of 99.4±0.37% for controls to 46±4.26% at 1000µg/l (F=11.262, p=<0.001, Table 3.7).
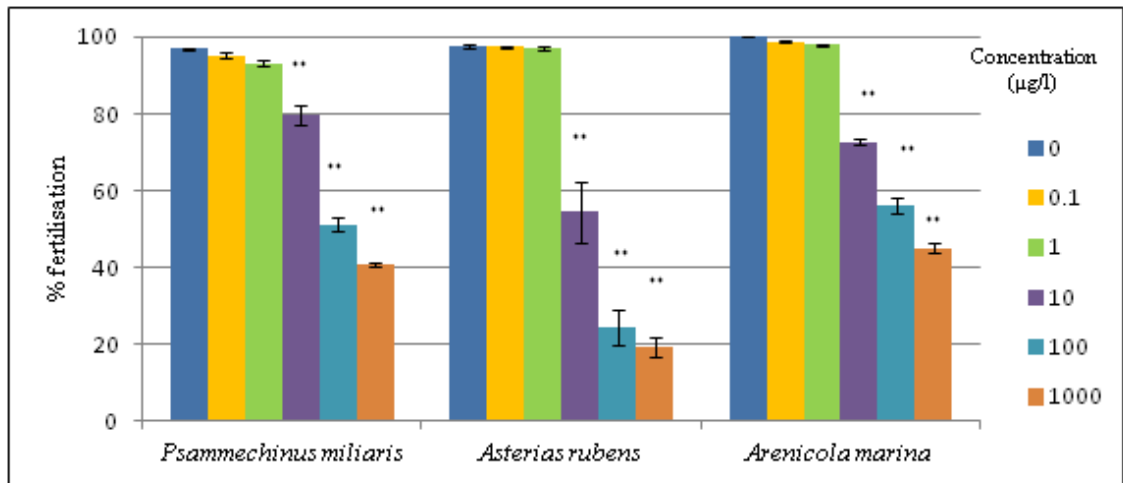
Figure 3.11. Percent fertilisation of oocytes from *A. rubens, P. miliaris* and *A. marina* after 60 minutes incubation of sperm and oocytes with different concentration of copper. Treatments significantly different from the control are indicated by asterisks *p<0.05; **p<0.01.

Table 3.7. *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Analysis of variance on arcsine transformed fertilisation levels, with time and copper concentration as main effects.

Source	SS	df	MS	F ratio	P value
Asterias rubens					
Concentration	63039.9	5	12608	91.874	<0.001
Error	6587.11	48	137.231		
Psammechinus miliaris					
Concentration	26812.1	5	5362.42	323.181	<0.001
Error	796.444	48	16.593		
Arenicola marina					
Concentration	20326.3	5	4065.26	504.653	<0.001
Error	290	36	8.056		

3.3.4.2 Cadmium

Figure 3.12 presents the fertilisation results of incubating sperm and oocytes in increasing cadmium chloride concentrations for 60 minutes. Fertilisation success reduced in *A. rubens* and *A. marina* at 1000µg/l (Figure 3.12); however, fertilisation was still evident even at this concentration. There were no significant differences between controls and 0.1, 1, 10 or 100µg/l cadmium. Percentage fertilisation success declined

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earlier in *P. miliaris*, dropping from a mean of 99.8 ± 0.57% for controls to 29.4 ± 7.79% at 1000µg/l (F=499.305, p=<0.001, Table 3.8); however, fertilisation decline was significant at 100µg/l. A significant fertilisation failure was recorded for *A. rubens* at 1000µg/l with a mean fertilisation success of 40.67±1.41 (F=1565, p=<0.001, Table 3.12). A similar pattern was found for *A. marina* which also decreased significantly at 1000µg/l to 46.57±11.29% compared with 99.4±0.98% for the control (F=499.04, P=<0.001, Table 3.8).
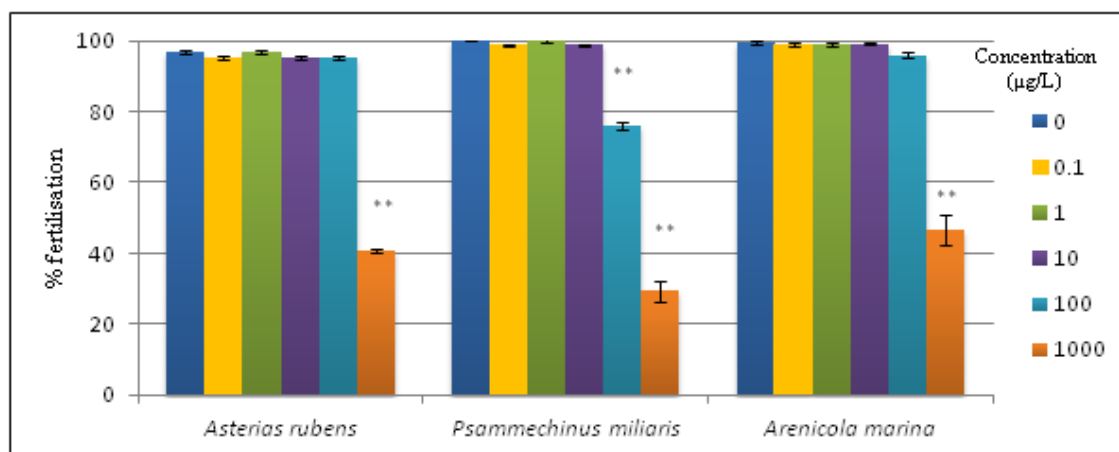
Figure 3.12. Percent fertilisation of oocytes from *A. rubens*, *P. miliaris* and *A. marina* after 60 minutes incubation of sperm and oocytes with different concentration of cadmium. Treatments significantly different from the control by asterisks *p<0.05; **p<0.01.

Table 3.8. *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Analysis of variance on arcsine transformed fertilisation levels, with time and cadmium concentration as main effects.

Source	SS	df	MS	F ratio	P value
Asterias rubens					
Concentration	28101.048	5	5620.210	499.046	<0.001
Error	405.429	36	11.262		
Psammechinus miliaris					
Concentration	22901.204	5	4580.241	1565.399	<0.001
Error	140.444	48	2.926		
Arenicola marina					
Concentration	28101.048	5	5620.210	499.046	<0.001
Error	405.429	36	11.262		

Table 3.9. No observable effect concentration (NOEC) and effective concentration 50 (EC_{50}) value of copper and cadmium toxicity.

Metal	Test	*P. miliaris*		*A. rubens*		*A. marina*	
		NOEC (µg/l)	60 minutes EC_{50} (µg/l)	NOEC (µg/l)	60 minutes EC_{50} (µg/l)	NOEC (µg/l)	120 minutes EC_{50} (µg/l)
Copper	Sperm motility	1.0	58.16	1.0	304.88	1.0	274.83
	Pre-incubation of sperm	1.0	118.33	1.0	248.13	1.0	345.3
	Pre-incubation of oocytes	1.0	277.61	100.0	1176.82	1.0	1281.97
	Exposure sperm and oocytes	1.0	275.73	1.0	378.35	1.0	541.76
Cadmium	Sperm motility	10.0	144.63	100.0	1660.54	100.0	4624.69
	Pre-incubation of sperm	10.0	787.35	100.0	3327.87	100.0	6003.82
	Pre-incubation of oocytes	100.0	7366.62	100.0	3123.17	100.0	6190.82
	Exposure sperm and oocytes	10.0	1932.81	100.0	3178.84	100.0	4020.33

The NOEC and EC_{50} values are presented in Table 3.9. The NOEC for the *P. miliaris* sperm motility test exposed to copper sulphate and cadmium chloride were 1.0 and 10.0µg/l, respectively; whereas the 60 minutes EC_{50}s were 58.16 and 144.63µg/l, respectively. In fertilisation tests, *P. miliaris* sperm pre-incubated in copper and cadmium had NOECs of 1.0 and 10.0µg/l, respectively, while the 60 minutes EC_{50} values were 118.33 and 787.35µg/l, respectively. The NOECs for pre-incubated oocytes were 1.0 and 100.0µg/l, respectively for copper and cadmium and the 60 minutes EC_{50} values were 277.61 and 7366.62µg/l, respectively. The comparative values for when both sperm and oocytes were pre-exposed to the metals were 1.0 and 10.0µg/l (NOEC) and 275.73 and 1932.81µg/l for the 60 minutes EC_{50}, for copper and cadmium respectively.

The sperm motility NOECs for *A. rubens* exposed to copper and cadmium were 1.0 and 100µg/l, while for sperm pre-incubation they were 1.0 and 100.0µg/l respectively. The 60 minutes sperm motility EC_{50} values were 304.88 and 1660.54µg/l and 248.13 and 3327.87µg/l for the sperm pre-incubation test. The NOECs for pre-incubated were both 100.0µg/l whereas the 60 minutes EC_{50} values were 1176.82 and 3123.17 µg/l. The figures for when both sperm and oocytes were pre-exposure were 1.0 µg/l and 100 µg/l (NOEC) and 378.35 and 3178.84 µg/l (EC_{50}).

The NOEC sperm motility values for *A. marina* were 1.0 and 100µg/l (copper and cadmium respectively); sperm pre-incubation generated the same values, whereas the EC_{50} values for the sperm motility and sperm pre-incubation tests were 274.83 and 345.3 µg/l for copper and 4624.69 and 6003.83 µg/l for cadmium. Similar values were determined for oocytes pre-incubation - NOECs were 1.0 and 100.0µg/l while EC_{50} values were 881.97 and 6190.82µg/l, respectively. Pre-incubation of both sperm and oocytes in copper and cadmium produced EC_{50} values of 541.76 and 4020.33µg/l respectively.

3.4   Discussion

3.4.1   Toxicity of metals to gametes of marine invertebrates

In this present study, a comparison of the toxicity of copper and cadmium against sperm motility and fertilisation success clearly showed that copper was the more toxic

metal. This conclusion supports prior work on the sea urchin, *Diadema setosum* by Thongra-Ar (1997) who reported a 20 minutes EC_{50} based on sperm toxicity for fertilisation success of 17µg/l for copper and 628µg/l for cadmium. In terms of EC_{50} values for cadmium toxicity to marine invertebrate sperm, adverse effects on fertilisation capacity were shown to arise due to sperm toxicity, for example in the sea urchin, *Anthocidaris crassispina* (30 minutes EC_{50} of 1700µg/l) (Vaschenko et al., 1999). Many other studies report sperm toxicity EC_{50} values for a variety of sea urchin species ranging between 380-1700µg/l (Kobayashi, 1994, Dinnel et al., 1989, Nacci, 1986).

A study on the serpulid polychaete *Hydroides elegans*, found the EC_{50} for embryogenic inhibition to be 47µg/l when oocytes were exposed to copper prior to fertilisation (Xie et al., 2005). An EC_{50} for gametes of the polychaete *Alitta* (*Nereis*) *virens* exposed to copper was reported at 139.8µg/l (Caldwell et al., 2011b); however, Watson et al. (2008) reported that exposure to copper, at up to 500µg/l, for the same species, resulted in no reduction in fertilisation success. As copper and cadmium concentrations in seawater range between 0.05-0.25µg/l and 0.08-0.25µg/l (OSPAR, 2002), sperm and oocytes of these three species are not likely under threat from these metals as the EC_{50} values obtained in this study were higher than those measured in the environment.

3.4.2   Gamete sensitivity to metals between species

In terms of gamete sensitivity to metals across the three test species, *P. miliaris* sperm were more sensitive to copper than sperm from *A. rubens*; *A. marina* sperm were less sensitive to copper. For oocytes, copper was more toxic to *P. milaris* than *A. rubens* but less toxic for *A. marina*. *A. marina* sperm were the most resistant to copper, followed by *A. rubens* with *P. milaris* sperm the most affected. Conversely, *P. miliaris* oocytes were less sensitive to cadmium followed by *A. marina* then *A. rubens*. The differences in the sensitivity of gametes from each species might be explained by the different trophic levels they occupy. Moreover, as the polychaete worm is generally found living in more contaminated sites, and particularly as it is a sediment dweller (sediments have a higher metal burden that overlying water)*,* it is perhaps unsurprising that *A. marina* gametes were less sensitive to copper and cadmium (except the oocytes with cadmium exposure) compared to the echinoderms. In addition, this can be explained by the metal detoxification system and tolerance of this worm to these metals. (Mouneyrac et al.,

2003) indicated that the polychaete *Hediste diversicolor,* can increase its tolerance to cadmium, copper, and zinc by secreting mucus that prevents or reduces the metal availability.

3.4.3 General discussion

The results of the present study show that exposure to 10, 100 and 1000μg/l of copper sulphate and 1000μg/l of cadmium chloride significantly decreased the swimming speed and percentage motility of each of the three tested invertebrate species.

Sperm of free-spawning organisms, such as echinoderms and polychaetes, are immotile at the time of release but become spontaneously motile upon dilution in seawater (Rothschild, 1948). This activation has been shown to be triggered by changes in extracellular osmotic pressure and the concentration of specific ions relative to that of the animal's seminal plasma. Mechanisms of sperm motility in marine invertebrates are explained by Fitzpatrick et al. (2008b). Dilution of sperm in seawater may activate the metabolic pathways to trigger the flagella to make a movement (Rothschild, 1948). Sperm is active and motile once cytochrome C is activated; however, cytochrome C activity is suppressed by sulfhydryl groups, which result in immotile sperm (Fitzpatrick et al., 2008a). Cytochrome C is a vital component of the electron transport chain (ETC) in mitochondria that drives energy production (Kim et al., 2010). Following the release of sperm to the water column and their consequent dilution, cytochrome C is released from inhibition by the sulfhydryl group which allows respiration and produces the energy for the sperm's motility (Fitzpatrick et al., 2008a).

Dissolution of copper produces cuprous ions which readily bind with sulfhydryl group and inhibit ATP production by interfering with electron transport along the ETC (Ay et al., 1999). ATP works by supplying energy and is one of the important factors to make a sperm swim. Inhibition of ATP will therefore result in reduced swimming speed (Casson, 2012). In the sea urchin *Arbacia crassipina*, cadmium and phenol have been shown to modify the structure of mitochondrial cristae, affecting the ATP supply for sperm movement (Au et al., 2000a) and consequently decreasing sperm velocity.

Further evidence has been reported that copper may be acting at regulatory sites within the sperm flagellum. Studies report that sperm motility reduction is caused by

inherited defects associated with the sperm tail (Lukac et al., 2013, Aly et al., 2012) and midpiece mitochondria (Feng et al., 2011). These results are similar to Young and Nelson (1974) who found that sperm motility of *Arbacia punctulata* decreased with increasing copper exposure (Young and Nelson, 1974). As the role of copper in sea urchin metabolism is well documented, excess copper ions exert their affect at a variety of enzymatic sites. Morisawa and Mohri (1972) argued that cytochrome C oxidase in the midpiece of the sea urchin sperm accounts for most of the copper found in these cells and another study claimed that sea urchin sperm are able to bind copper to the extent of 300 times the amount of water (Barnes and Rothschild, 1950). Moreover, evidence has shown that copper arrests the decline in oxygen consumption of dilute suspensions of sea urchin sperm (Rothschild and Tuft, 1950) which resulted in reduced swimming speed and motility. In a study of the marine mussel*, Mytilus edulis*, it was shown that copper accumulates in the sperm mitochondria (Earnshaw et al., 1986) and caused the formation of reactive oxygen species (ROS), reducing the mitochondrial membrane potential and resulting in oxidative damage (Krumschnabel et al., 2005). Hence, interfering with the mitochondrial activity in sperm by metals resulted in reduced sperm swimming speed and may also affect the percentage of motile sperm.

Reduction in sperm swimming speed can also be related to an alteration of sperm morphology. Studies have reported that exposure to > 5mg/L of cadmium to sea urchins resulted in mitochondrial damage, breakage of the flagella, and swelling of the midpiece (Au et al., 2000b, Au et al., 2000c). Au et al. (2000a) also demonstrated a time dependent adverse effect of cadmium on sperm motility. Exposure of cadmium for a period of four weeks to adult sea urchins resulted in cytological distortion to sperm cell development. Sperm were observed to have developed short incomplete 'broken tails' and also had deformation of mitochondrial cristae. Cytological alteration of the sperm tail and midpiece mitochondria will result in impaired sperm motility. All of the reasons that affect sperm motility can cause a reduction in fertilisation success. This is because reduced sperm swimming speed may result in a reduction of sperm-egg collisions and a decline in fertilisation success (Lewis and Galloway, 2010).

Conducting fertilisation success experiments whereby both oocytes and sperm are simultaneously exposed to pollutants reveals little about the sensitivity of either germ cell to the toxin. Therefore, oocytes and sperm were incubated independently in copper and cadmium and fertilised with germ cells that were not toxin exposed. The paternal environment can induce epigenetic damage in sperm that can be stably transmitted to

offspring (De Boer et al., 2010, Jablonka and Raz, 2009). Ritchie and Marshall (2013) suggested that the sperm environment may affect offspring developmental success. The exposure to a stress during fertilisation may not only affect the number of zygotes that are produced but also may alter the performance of those produced zygotes (Ritchie and Marshall, 2013).

Free calcium ions are essential secondary messengers in cells from their origin at fertilisation throughout their entire lifespan (Carafoli, 2002). At fertilisation, oocytes undergo an increase in intracellular Ca^{2+} beginning at the point of sperm-oocyte fusion and crossing the oocyte to the antipode in a wave-like fusion (Santella et al., 2004, Whitaker, 2006). This calcium wave is the first fertilisation event triggering the quiescent oocyte into metabolic activity by posttranslational activation of enzymes, exocytosis of cortical granules for the formation of the fertilisation membrane and resumption of the cell cycle (Schäfer et al., 2009, Santella et al., 2004). Therefore, even a slight alteration to Ca^{2+} signalling may affect reproductive success of marine organisms. Copper ions have been shown to alter Ca^{2+} signals in developing embryos of the macroalgae *Fucus serratus* (Zorita et al., 2006). Evidence also exists that incubation of oocytes of *P. miliaris* in 6.3µM copper sulphate before fertilisation increases the fertilisation calcium wave that indicates disturbance of Ca^{2+} homeostasis in the oocyte that resulted in decreased fertilisation (Schäfer et al., 2009).

Further evidence by Schäfer et al. (2009) showed a concentration-dependent effect on the fertilisation rates of copper on *P. miliaris* significantly inhibiting fertilisation at 6.3µM. This is supported by Arizza et al. (2009) who found that gametes of the sea urchin *Paracentrotus lividus* that were exposed to copper and cadmium exhibited decreased fertilisation; furthermore, sperm pre-incubated before fertilisation were found to be most sensitive to treatment. Experimental work from Watson et al. (2008) also indicated that incubation of gametes of the polychaete *Nereis virens* in 500µg/l and 1000µg/l of copper for ten minutes resulted in complete fertilisation failure (Watson et al., 2008). Cytological alterations with increased ionic permeability of organelles to calcium and phosphorus, oxidative stress (Earnshaw et al., 1986), inhibition of gamete respiration (Akberali et al., 1985) and swimming speed of sperm (Earnshaw et al., 1986, Kime et al., 1996) were among the effects of copper and other metals to gametes of marine invertebrates. Any of these factors may be implicated in the reduction in sperm motility and fertilisation success observed in the present study. As biochemical and cytological analysis of the exposed sperm were not undertaken in the

present study the molecular mechanisms behind the observed changes in motility remain speculative.

3.5 Conclusions

Copper and cadmium exposure significantly reduced sperm motility and fertilisation success of *A. rubens, P. miliaris*, and *A. marina* when exposed to $\geq 10.0\mu g/l$ of copper with increasing time and $\geq 100.0\mu g/l$ of cadmium concentration with increasing time. Copper was more toxic than cadmium. In terms of gamete sensitivity to copper, *P. miliaris* was most affected (60 minutes $EC_{50} = 275.73\mu g/L$), then *A. rubens* (60 minutes $EC_{50} = 378.35\mu g/L$) and *A. marina* (120 minutes $EC_{50} = 541.76\mu g/L$) was less sensitive to copper. Exposure to cadmium revealed that the gametes of *P. miliaris* (60 minutes $EC_{50} = 1932.81\mu g/L$) were most sensitive, followed by *A. rubens* (60 minutes $EC_{50} = 3178.841\mu g/L$) and *A. marina* was least sensitive (120 minutes $= EC_{50}$ $4020.33\mu g/L$). Copper and cadmium toxicity EC_{50} values obtained in this study were higher than the measured concentrations of copper (0.05-0.25μg/L) and cadmium (0.08-0.25μg/L) in seawater.

The present findings are in agreement with the proposition that a decrease in fertilisation capacity of *A. rubens*, *P. miliaris*, and *A. marina* sperm may be related to toxic effects of metals on sperm motility. Further studies will be necessary to determine the effects in subsequent stages of development and to evaluate the levels of embryo, larval and adult toxicity of metals. This is particularly the case when considering complex mixtures and that metal-induced developmental toxicity may be cumulative.

Chapter 4: Effects of Ocean Acidification on Sperm Motility and Fertilisation Success of Marine Invertebrates.

Abstract

The oceans face serious changes to their natural geochemical cycles due to uptake of carbon dioxide (CO_2) from anthropogenic activities. Ocean acidification is the term used to describe the decline of seawater pH caused by this absorption. This chapter evaluates the effects of ocean acidification on sperm swimming behaviour and fertilisation success using *Asterias rubens*, *Psammechinus miliaris*, and *Arenicola marina* as study species. Treatment conditions were pH 8.1 (ambient control), and two predicted near future pH levels; pH 7.9 and 7.7. Sperm swimming behaviour was determined by a Computer Assisted Sperm Analyser (CASA) and showed a reduction in sperm swimming speed and percentage of sperm motility in all three species. Fertilisation success was determined by incubating gametes in seawater of modified pH and assessed by embryonic cleavage 60 minutes after initial fertilisation for echinoderms and 90 minutes for the polychaete. Fertilisation inhibition was observed at reduced pH and in combination with the length of exposure. The reduction of sperm motility resulted in decreased fertilisation success in a simulated high CO_2 future ocean; therefore, resultant changes in offspring production could affect recruitment success and population fitness.

4.1 Introduction

The concentration of atmospheric carbon dioxide (CO_2) is rising consistently with progressing industrialisation (Steffen et al., 2007). The major sources of anthropogenic CO_2 are combustion of fossil fuels for transport, power generation, and in industrial processes such as fertiliser manufacture (IPCC, 2005). The oceans play an important role in absorbing CO_2 from the atmosphere and this process leads to a decrease in seawater pH, causing seawater chemistry to change to the extent that has the potential to affect the marine environment and all organisms within it.

The ocean surface is slightly alkaline with pH around 8.1 (Wormworth and Sekercioglu, 2011). Acidification of the oceans is a process in which the pH value of seawater is reduced by increases in the partial pressure of CO_2 (pCO_2) in the seawater. The pCO_2 in seawater is now higher than it has been for at least 800,000 years and has already reduced global mean seawater pH by 0.1 units (Orr et al., 2005), representing an increase of 30% in seawater hydrogen ion concentration. If anthropogenic CO_2 emissions continue to rise at current rates, pCO_2 will increase to two or three times pre-industrial values towards the end of this century, reducing global mean seawater pH by up to 0.3 units which equates to an increase of 150% in hydrogen ion concentration and halving the carbonate ion concentration (Caldeira and Wickett, 2003).

Ocean acidification is increasingly recognised as a serious and growing threat to marine ecosystems (Orr et al., 2005). The adverse effects on marine organisms, communities, and ecosystems are expected to occur through changes of carbonate chemistry in seawater (Doney et al., 2009b). The projected increase in seawater pCO_2 may have critical negative impacts on the majority of marine calcifiers and on a range of physiological processes including calcification across numerous marine taxa (Orr et al., 2005, Raven et al., 2005, Havenhand et al., 2008a, Kurihara, 2008a, Parker et al., 2009). Yet, calcification responses are highly variable (Kroeker et al., 2010, Wernberg et al., 2012). The morphological differences of organisms are one of the reasons that lead to differences in species sensitivity (Kroeker et al., 2010, Kroeker et al., 2011, Pandolfi et al., 2011). The effect of elevated of pCO_2 also depends on other physiological processes which are correlated to different components of the carbonate system such as photosynthesis, acid-base metabolic physiology, and calcification processes (Doney et al., 2009a, Hofmann and Todgham, 2010, Veron, 2011, Andersson and Mackenzie, 2012). Differences of methodology in experimental conditions also contribute to

variable responses (Fabry, 2008, Pandolfi et al., 2011). Calcification responses in marine calcifiers vary depending on the mineral form of calcium carbonate used. Organisms that use high-magnesium calcite (more soluble forms) to build the shell, skeleton, or other parts of the body can be more resistant to ocean acidification compared to organisms that use calcium and/ or aragonite (less soluble forms). Moreover, the sensitivity to ocean acidification varies depending on different developmental stages and taxonomic groups (Kroeker et al., 2013).

It is vital to study and assess the effects of ocean acidification on the early life stages of marine invertebrates such as fertilisation, embryogenesis, and larval development as these species are typically sensitive to environmental factors (Harley et al., 2006, Havenhand et al., 2008a, Ross et al., 2011). Laboratory experiments have suggested that ocean acidification has negative impacts on fertilisation, cleavage, larval development, settlement, and reproductive maturation of marine calcifiers, including echinoderms, bivalve molluscs, corals, and crustacean species (Havenhand et al., 2008a, Kurihara, 2008b). Several studies on ocean acidification reported different effects on different marine invertebrate taxa and different pCO_2 levels (Kurihara and Shirayama, 2004b, Havenhand et al., 2008a, Havenhand and Schlegel, 2009, Parker et al., 2009, Byrne et al., 2010b, Byrne et al., 2010c, Gonzalez-Bernat et al., 2013, Sung et al., 2014)

In echinoderms, no effects on fertilisation success were observed when gametes were exposed to elevated pCO_2 predicted to occur by 2100 (Byrne et al., 2009, Byrne et al., 2010b, Byrne et al., 2010c, Dupont et al., 2010b) using optimum sperm densities (sperm concentration not limited) (Byrne et al., 2010c, Reuter et al., 2011b). However, gametes were sensitive and resulted in reduced fertilisation success when exposed to elevated pCO_2 predicted for the year 2300 (Kurihara and Shirayama, 2004b, Kurihara and Shirayama, 2004a). Fertilisation success of *Echinometra mathaei* and *Hemicentrotus pulcherrimus* only declined significantly at pH~7.1 (5000ppmv) (Kurihara and Shirayama, 2004b); however, *Heliocidaris erythrogramma* showed greater sensitivity as fertilisation success reduced significantly at pH ~7.7 (1000ppmv) (Havenhand et al., 2008a, Byrne et al., 2009, Byrne et al., 2010b). These findings indicate that effect of ocean acidification on fertilisation success may vary widely even between closely related species.

Further, studies on species from the same geographic region reported contrasting results. This was exemplified by Byrne et al. (2010b) and Byrne et al. (2009) where no

significant reduction on fertilisation success was observed for four sea urchins (*Heliocidaris erythrogramma, H. tuberculata, Centrostephanus rodgersii,* and *Tripnuestes gratilla*), an abalone (*Haliotis coccoradiata*), and a seastar (*Pateriella regularis*) exposed to pH7.6-8.2 and elevated temperature.

It is important to examine the effect of elevated pCO$_2$ on marine organisms and ecosystems and enlarge bioassays to include diverse species to identify which species are affected. The objective of this chapter is to examine the effects of reduced seawater pH on sperm motility and fertilisation success in *Asterias rubens*, *Psammechinus miliaris*, and *Arenicola marina*. These species reproduce by broadcast spawning, whereby they release sperm and eggs into the water column and fertilisation occurs externally. The gametes released directly into seawater during spawning are unprotected and therefore exposed to a 'changed environment'; thus, there is the potential for such altered environmental conditions to disrupt the fertilisation process. If sperm lose their ability to find oocytes in the seawater, the life of marine organisms is potentially limited. Thus, as the effects of ocean acidification on sperm motility and fertilisation success are assessed in this chapter, some light may be shed as to how these species may fare with respect to predicted future pH conditions.

4.2   Materials and methods

All material and methods in this chapter were as described in chapter 2 with the exception of CO$_2$ treatment to produce the ocean acidification scenarios. Unlike chapter 2 there are no co-occurring chemical treatments in this chapter. Statistical analysis for incubation of sperm and oocytes used one-way ANOVA with Tukey post-hoc tests using arcsine transformed data to meet assumptions for normality and homoscedasticity.
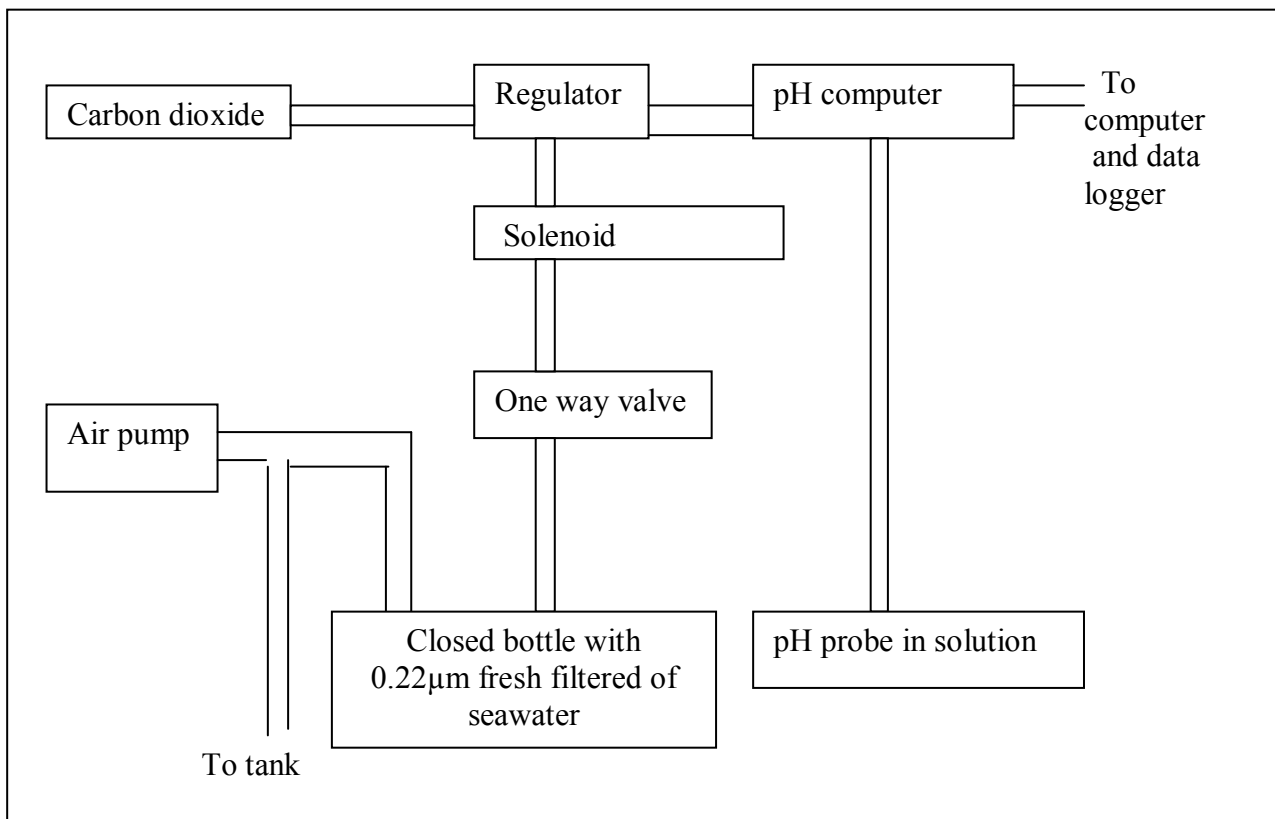
Figure 4.1. Ocean acidification methodology used during experimental work. The experiment was setup in a small aquaria housed in the Ridley Building, Newcastle University.

Reduced seawater pH was produced by gently bubbling 0.22μm fresh filtered seawater pH in a bottle with CO_2 from a cylinder. A smooth and continuous flow of CO_2 was controlled by an Aqua-medic^{TM} pH computer attached to a solenoid valve (Figure 4.1). The pH computer was pre-programmed to pH 7.7 and pH 7.9 and it automatically triggered the stop or start injection of CO_2 when needed. All reduced pH seawater was used immediately upon reaching the pre-set target.

4.3   Results

4.3.1   Sperm motility and sperm velocity

Percentage sperm motility of the control was in the range of 90-82% (Figure 4.2 (A)). A significant decrease of sperm motility was observed when exposed to pH 7.9 wherein only 77-62% of sperm were motile; while 59-67% of sperm were motile at pH 7.7. Seawater pH and time had a significant interaction (F=3.586, p=0.004; Table 4.1). For *A. rubens*, the swimming speed (VCL) of motile sperm ranged from 69.7-183.7µm/s across experimental treatments (Figure 4.2(B)). There was a significant reduction in VCL with time when exposed to pH 7.7 and 7.9 compared to control seawater pH 8.1 (F=2.354, p=0.042; Table 4.1).
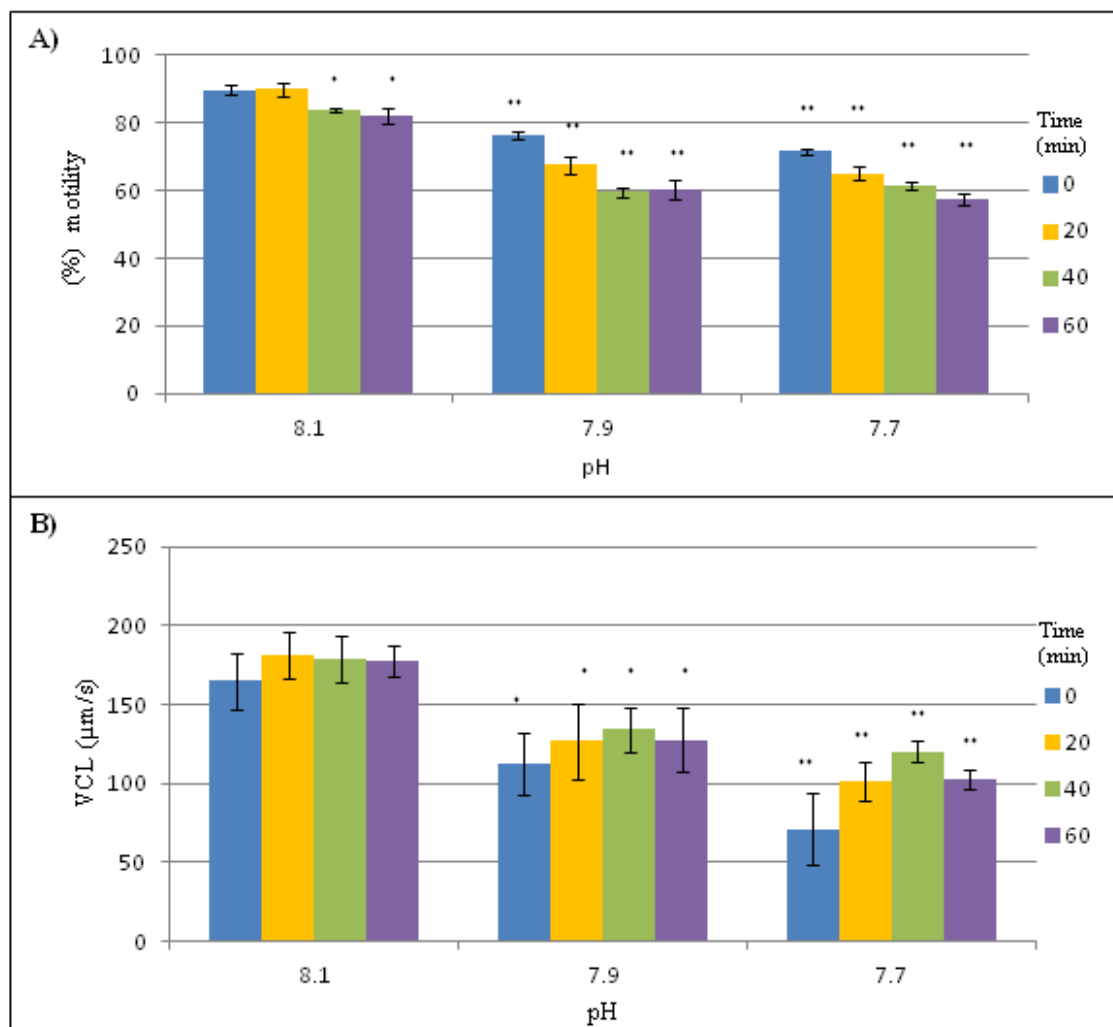
Figure 4.2. Effect of seawater pH with increasing exposure time on motility and swimming velocity of *Asterias rubens* sperm. Treatments significantly different from the controls within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Results of sperm motility and sperm swimming speed for *P. miliaris* sperm are shown in Figure 4.3. Significantly reduced swimming speed and sperm motility compared to pH 8.1 were observed. A significant interaction between pH and time was seen in both experiments (Tables 4.1). Exposure of sperm to control seawater pH resulted in a VCL range of 140-67µm/s. Swimming velocity of sperm exposed to pH 7.7 was 101-45µm/s and 110-44µm/s on exposure to pH 7.9. There was an almost 22% reduction of mean sperm motility at pH 7.7 compared to pH 8.1 and a 16% drop for pH 7.9.
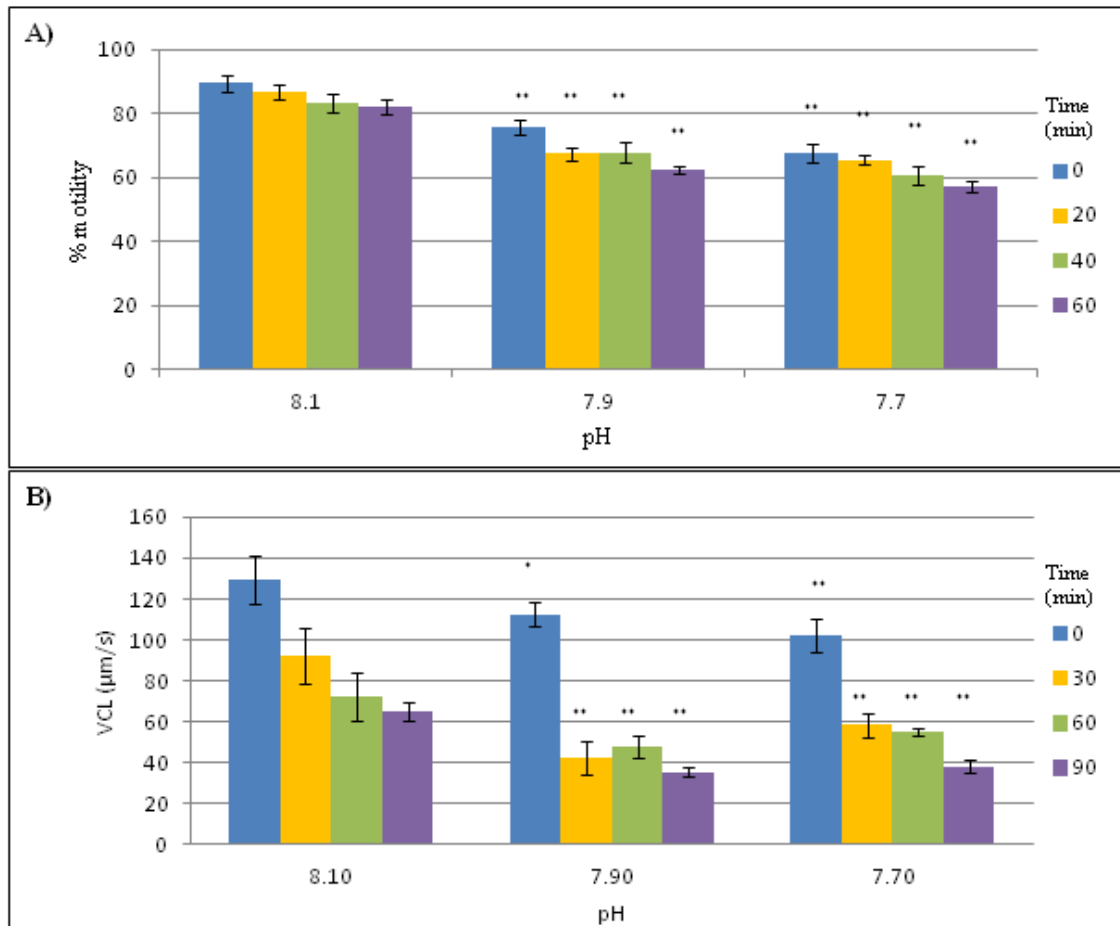
Figure 4.3. Effect of seawater pH with increasing exposure time on motility and swimming velocity of *Psammechinus miliaris* sperm. Treatments significantly different from controls within the same time intervals are indicated by asterisks *$p<0.05$; **$p<0.01$.

Figure 4.3 shows the results for swimming velocity (VCL) and motility trials of *A. marina* sperm. VCL was significantly reduced at pH 7.7 and 7.9 compared to the control pH. VCL ranged from 150-152μm/s, 125-90μm/s and 78-80μm/s for pH 8.1, 7.9 and 7.7, respectively. pH and time had a significant interaction (F=3.164, p=0.006; Table 4.1). A significant decrease was also noticed for percentage of sperm motility. Between 82-85% of sperm were motile at pH 8.1. The percentage motility reduction was slightly higher at pH 7.7 (68-52%). Seawater pH and time had significant interactions (F=9.824, p=<0.001; Table 4.1).
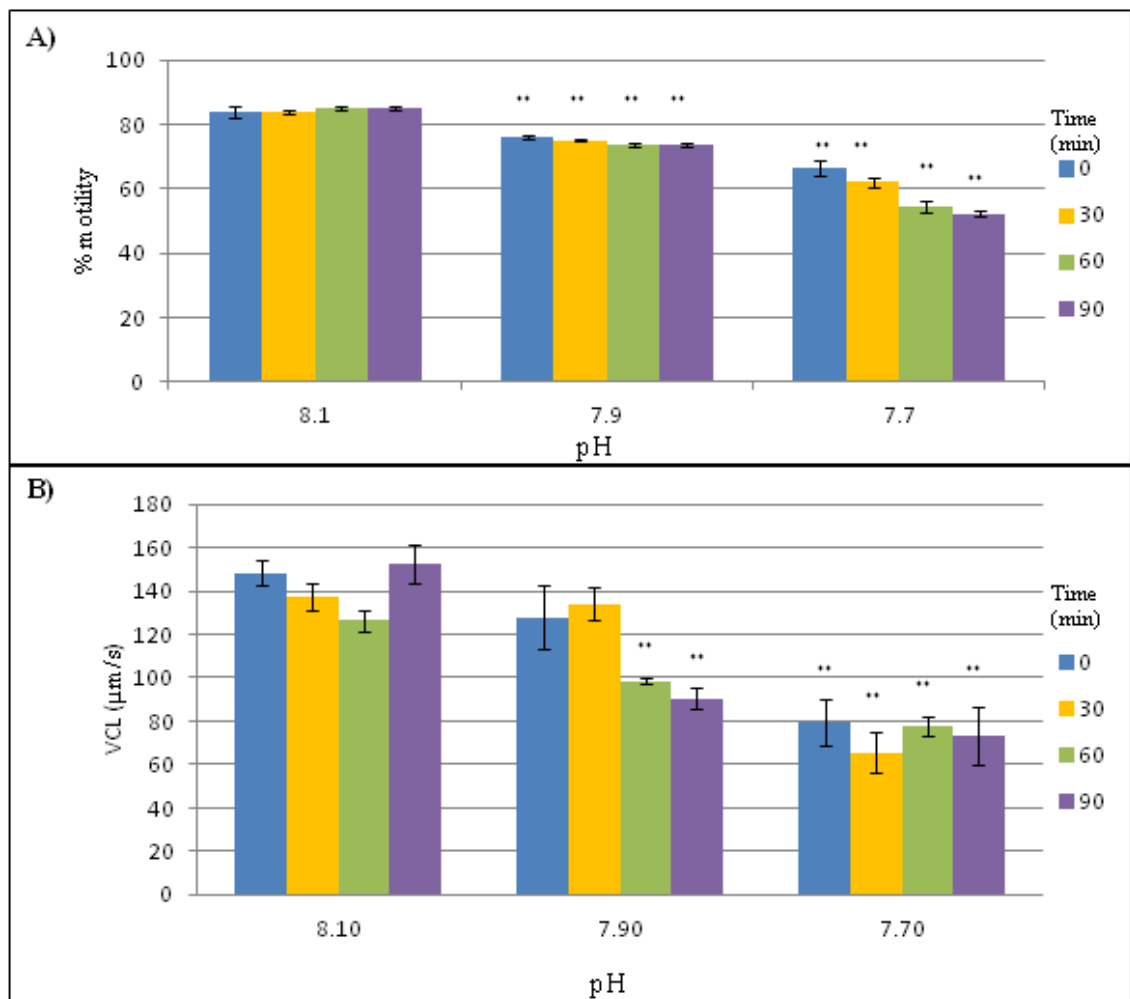
Figure 4.4. Effect of seawater pH with increasing exposure time on motility and swimming velocity of *Arenicola marina* sperm. Treatments significantly different from controls within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Table 4.1. Two-way ANOVA for sperm motility and curvilinear velocity (VCL) of *Asterias rubens. Psammechinus miliaris* and *Arenicola marina* across time treatments and seawater pH treatments.

Species	Parameter	Source	SS	df	MS	F	Sig.
Asterias rubens	Sperm motility	pH	7629.78	2	3814.89	199.269	<0.001
		Time	1869.61	3	623.204	32.553	<0.001
		pH * Time	411.889	6	68.648	3.586	0.004
		Error	1148.67	60	19.144		
	Curvilinear velocity	Time	9460.58	3	3153.53	1.997	0.024
		pH	48933.9	2	24467	15.493	<0.001
		Time * pH	22307.7	6	3717.94	2.354	0.042
		Error	94753.4	60	1579.22		
Psammechinus miliaris	Sperm motility	pH	8254.95	2	4127.48	357.063	<0.001
		Time	971.083	3	323.694	28.002	<0.001
		pH * Time	274.381	6	45.73	3.956	0.002
		Error	832.286	72	11.56		
	Curvilinear velocity	Time	0.955	3	0.318	8.828	<0.001
		pH	0.931	2	0.466	12.915	<0.001
		Time * pH	0.724	6	0.121	3.345	0.006
		Error	2.596	72	0.036		
Arenicola marina	Sperm motility	pH	8065.19	2	4032.6	462.631	<0.001
		Time	311.278	3	103.759	11.904	<0.001
		pH * Time	513.806	6	85.634	9.824	<0.001
		Error	523	60	8.717		
	Curvilinear velocity	Time	3299.49	3	1099.83	2.561	0.063
		pH	54279.6	2	27139.8	63.184	<0.001
		Time * pH	8154.76	6	1359.13	3.164	0.009
		Error	25772.2	60	429.537		

4.3.2 Pre-incubation of sperm in seawater of different pH before fertilisation

Pre-incubation of sperm before fertilisation at different seawater pH treatments for increasing periods of time had a negative effect on fertilisation success of *A. rubens* (Figure 4.5(A)). Sperm exposed to control pH seawater maintained a fertilisation rate of 96.00±0.89% to 87.00±1.12%. Fertilisation success for sperm incubated in pH 7.7 declined to 72.67±1.2% after 20 minutes but the inhibition rate subsequently declined with time. After 60 minutes only 47.00±1.43% of gametes were fertilised. A similar pattern was recorded for *P. miliaris* sperm (Figure 4.5(B)). A pronounced decline in fertilisation success was observed at pH 7.7 and 7.9. Sperm incubated for 60 minutes at

pH 7.7 and 7.9 had a mean fertilisation success of 48.28±2.11% and 62.29±3.97% respectively, whereas controls had a mean fertilisation success of 84.57±0.57%. The effect of pre-incubation of *A. marina* sperm is shown in Figure 4.5(C). Control seawater pH exposed sperm maintained a fertilisation rate of 96.33±0.33 to 97.33±0.66%. At 20 minutes fertilisation significantly reduced at pH 7.9 and decreased further at pH 7.7. pH and time significantly interacted (F=40.32, p=<0.001; Table 4.2).
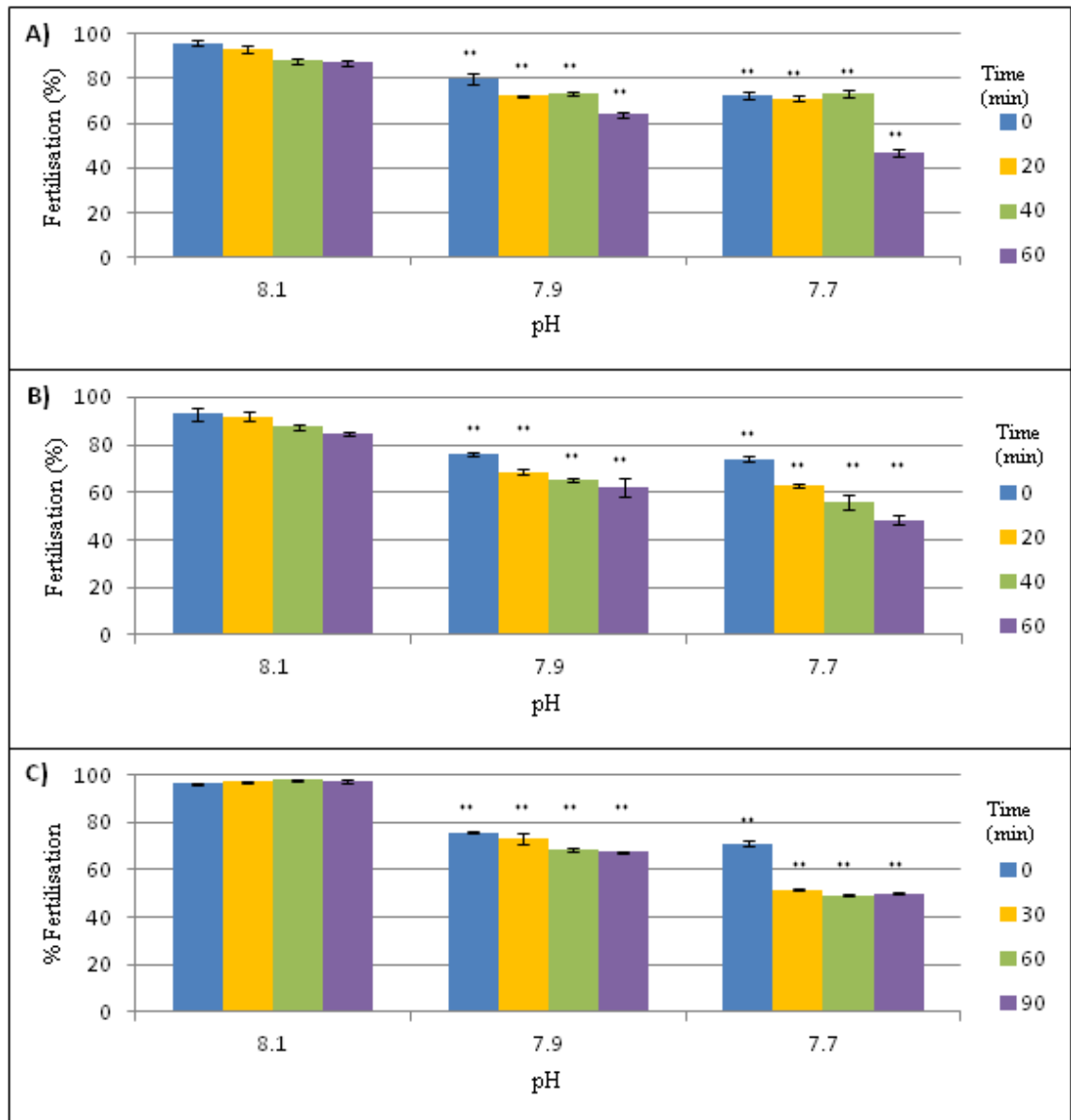
Figure 4.5. Percent fertilisation success of (A) *Asterias rubens*, (B) *Psammechinus miliaris* and (C) *Arenicola marina* after incubation of sperm with different seawater pH and times prior to fertilisation. Treatments significantly different from controls within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

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Table 4.2. Two-way ANOVA for percentage of fertilisation success of *Asterias rubens*, *Psammechinus miliaris*, and *Arenicola marina* across time and pH treatments.

Source	SS	df	MS	F ratio	P value
Asterias rubens					
pH	8166.778	2	4083.389	345.075	<0.001
Time	2849.944	3	949.981	80.280	<0.001
pH * Time	1133.222	6	188.870	15.961	<0.001
Error	710.000	60	11.833		
Psammechinus miliaris					
pH	12597.714	2	6298.857	238.335	<0.001
Time	2990.857	3	996.952	37.723	<0.001
pH * Time	601.143	6	100.190	3.791	0.002
Error	1902.857	72	26.429		
Arenicola marina					
pH	21156.778	2	10578.389	2173.642	<0.001
Time	1042.889	3	347.630	71.431	<0.001
pH * Time	1177.444	6	196.241	40.323	<0.001
Error	292.000	60	4.867		

4.3.3 Pre-incubation of oocytes in seawater of different pH before fertilisation

The results for the pre-incubation of *Asterias rubens* oocytes are presented in Figure 4.6(A). Two-way ANOVA revealed a significant reduction in fertilisation success when exposed to pH 7.7 and 7.9 at 20 minutes with the reduction in fertilisation success increasing with time. Control oocytes maintained a fertilisation success of 97.67±0.95% to 90.0±0.1.03%. After 60 minutes only 47.00±1.43% and 64.00±1.37% of gametes were fertilised in pH 7.7 and 7.9 respectively. Incubation of oocytes in different seawater pH also had negative effects on fertilisation success of *P. miliaris* (Figure 4.6(B)). A significant decline in fertilisation success was observed at both pH 7.9 and 7.7. Oocytes incubated for 60 minutes at pH 7.7 had a mean fertilisation success of 72.85±1.07%, whereas controls had a mean fertilisation success of 88.44±0.92%. Figure 4.6(C) shows the effect of pre-incubation of oocytes of *A. marina*. A significant reduction in fertilisation success was observed at pH 7.9 and 7.7 with increasing time at ≥30 minutes. After 90 minutes incubation in pH 7.7 less than 50% of oocytes were fertilised.
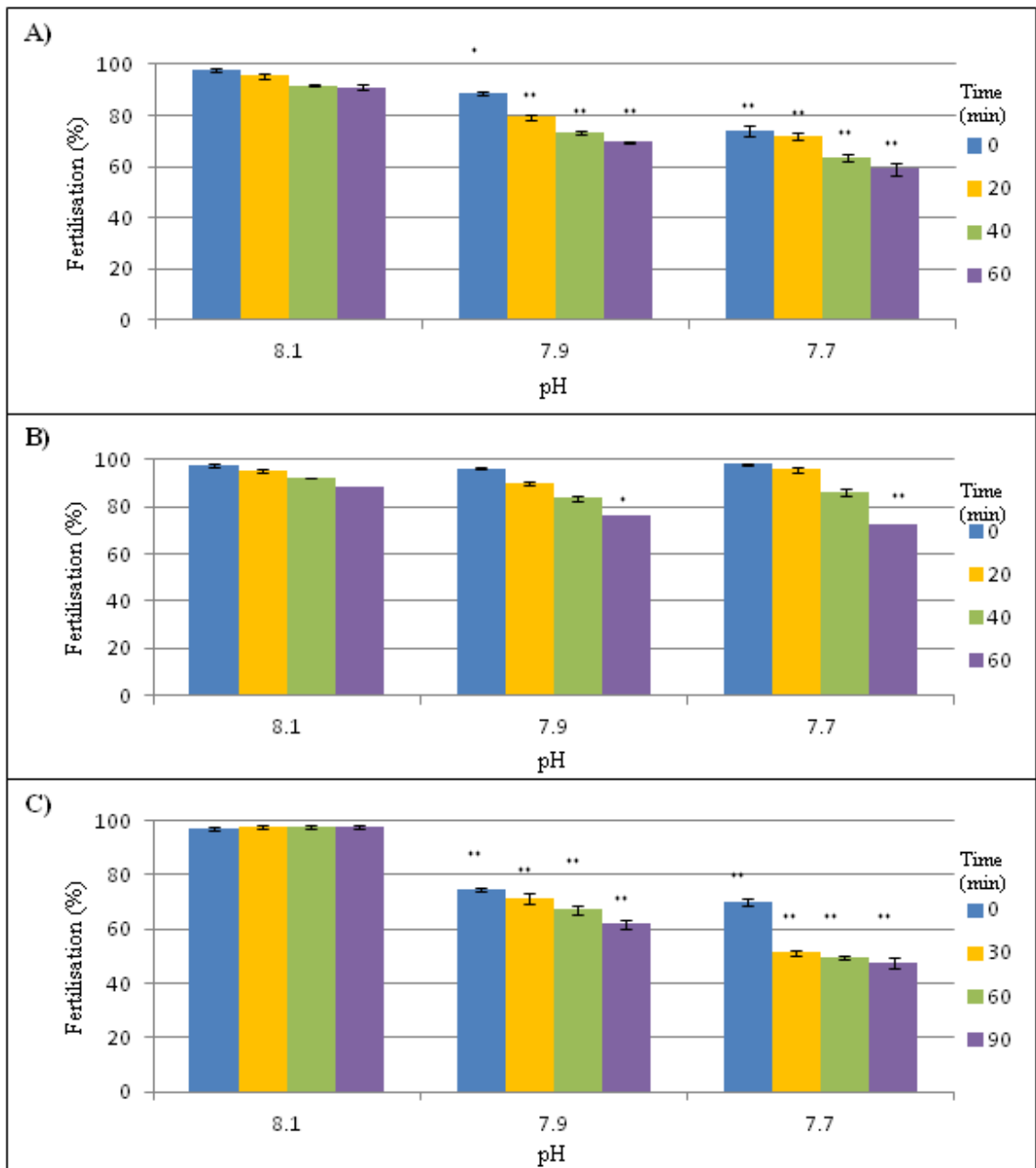
Figure 4.6. Percent fertilisation success of (A) *Asterias rubens*, (B) *Psammechinus miliaris,* and (C) *Arenicola marina* after incubation of oocytes with different seawater pH and times prior to fertilisation. Treatments significantly different from controls within the same time intervals are indicated by asterisks *$p<0.05$; **$p<0.01$.

Table 4.3. Two-way ANOVA for percentage fertilisation success of *Asterias rubens, Psammechinus miliaris,* and *Arenicola marina* across time and pH treatments.

Source	SS	df	MS	F ratio	P value
Asterias rubens					
pH	7320.444	2	3660.222	335.458	<0.001
Time	1850.000	3	616.667	56.517	<0.001
pH * time	521.333	6	86.889	7.963	<0.001
Error	654.667	60	10.911		
Psammechinus miliaris					
pH	717.810	2	358.905	72.010	<0.001
Time	3995.810	3	1331.937	267.236	<0.001
pH * time	658.190	6	109.698	22.010	<0.001
Error	358.857	72	4.984		
Arenicola marina					
pH	22933.444	2	11466.722	1269.379	<0.001
Time	1317.500	3	439.167	48.616	<0.001
pH * time	1161.667	6	193.611	21.433	<0.001
Error	542.000	60	9.033		

4.3.4   Fertilisation of gametes in reduced pH seawater

Figure 4.7 shows the results of the effects of pre-incubation of both sperm and oocytes in decreasing seawater pH after 60 minutes of fertilisation. There was reduced fertilisation success in all species. For *A. rubens*, fertilisation success was 98.00±0.52% for pH 8.1, 63.667±1.49% for pH 7.9 and 35.67±1.50% for pH 7.7. Results for *P. miliaris* had a significant decrease in fertilisation success at reduced pH. Fertilisation success for controls was 93.14±2.69%. A significant decline of 44.86% and 30.85% was observed at pH 7.7 and 7.9, respectively. *A. marina* also showed a significant decrease in fertilisation success; ($F=141.72$, $<p=0.001$; Table 4.4) from of 97.67±1.20% for controls, 62.33±1.20% at pH 7.9 and 47.00±3.29% for pH 7.7.
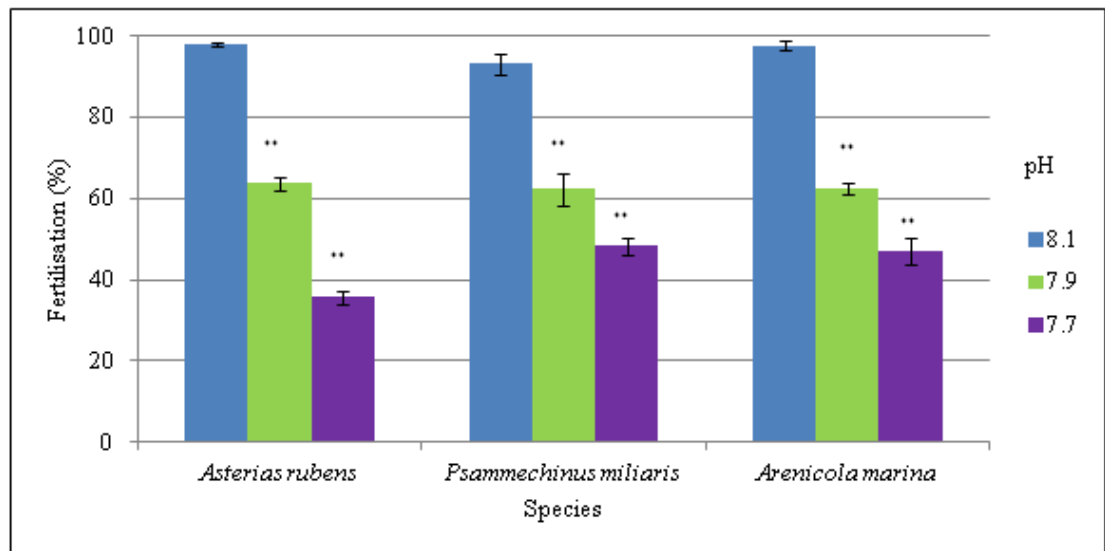
Figure 4.7. Percentage fertilisation success for *Asterias rubens, Psammechinus miliaris*, and *Arenicola marina* after both sperm and oocytes were pre-exposed to pH conditions. Treatments significantly different from controls are indicated by asterisks *p<0.05; **p<0.01.

Table 4.4. ANOVA for percentage fertilisation success for all three test species.

Source	SS	df	MS	F ratio	P value
Psammechinus miliaris					
Sperm oocyte	7374.095	2	3687.048	59.377	<0.001
Error	1117.714	18	62.095		
Asterias rubens					
Sperm oocyte	8483.111	2	4241.556	445.958	<0.001
Error	142.667	15	9.511		
Arenicola marina					
Sperm oocyte	8101.333	2	4050.667	141.742	<0.001
Error	428.667	15	28.578		

4.4 Discussion

4.4.1 Sperm motility and swimming speed

The motile and swimming speed of sperm are crucial characteristics in determining fertilisation success for broadcast spawners, especially when sperm concentration is low (diluted with seawater) (Levitan, 2000b, Hollows et al., 2007). In this chapter, it was observed that swimming speed and percentage motility were influenced by a reduction of seawater pH. Lowered pH significantly reduced the

swimming speed (VCL) and percentage motility of all three test species. This finding is supported by the majority of previous studies that reported the effects on pH reduction on sperm motility (Havenhand et al., 2008b, Morita et al., 2010b, Nakamura and Morita, 2012, Schlegel et al., 2012b, Vihtakari et al., 2013).

Schlegel et al. (2012b) reported that sperm swimming speed of *Heliocidaris erythrogramma* was significantly reduced at pH 7.8 and 7.6. A similar study on *Mytilus galloprovincialis* found inhibitory effects at pH 7.6 (Vihtakari et al., 2013). However, using *Psammechinus miliaris*, Caldwell et al. (2011a) recorded an increased percentage of motile sperm and swimming speed when exposed to ~pH 7.9 which then decreased at ~pH 7.7. The differences in experimental design maybe one of the causes of dissimilar results obtained. In addition, these differences may be caused from responses influenced by gamete quality and parental experience (Crean et al., 2013, Jensen et al., 2014); these authors explained that differences in parental genotypes and environments can directly affect performance and plasticity of gametes.

Morita et al. (2009) showed a significant decrease in sperm flagellum motility of *Acropora digitifera* with 69% of sperm motile at pH 8.0 , pH 7.8 was 46% and at ≤7.7 it was less than 20%. A similar finding in *Holothuria spp*, noted a significant reduction of motility of the flagellum with 73% at pH 8.0, 72% at pH 7.8 and < 30% at pH ≤ 7.7. Havenhand et al. (2008b) also found that low pH or increase pCO$_2$ resulted in inhibitory effects on sea urchin sperm motility. Species utilising free-spawning sperm store the immotile sperm in an acidic environment inside the testis after maturation. Reduction in seawater pH can alter sperm swimming capacity both negatively (Havenhand et al., 2008b, Morita et al., 2010a) and positively (Caldwell et al., 2011a), with reduction attributed to changes in the intracellular pH (pH$_i$) (Christen et al., 1982, Morita et al., 2010a). Sperm flagellum motility is regulated by an increase of intracellular sperm pH (pH$_i$) (Christen et al., 1982, Lee et al., 1983, Nakajima et al., 2005). Increasing external CO$_2$ results in acidification of body fluids in marine organisms and changes in ion balances (Raven et al., 2005). The acidic conditions prevent respiration and metabolic processes prior to release (Christen et al., 1982, Christen et al., 1983, Lee et al., 1983, Hamamah and Gatti, 1998). Immotile sperm of broadcast spawners require changes in pH$_i$ to activate the sperm flagellum. Upon spawning, the difference between the pH$_i$ in the testis and the extracellular pH in the seawater triggers mitochondrial activity resulting in sperm motility (Johnson et al., 1983, Cosson, 2004). Changes of seawater pH caused by ocean acidification may decrease the pH gradient upon spawning to a

point where, at worst, it is deficient to activate the sperm mitochondria, but perhaps more realistically, to reduce the extent of sperm activation.

The flagellum is a vital part of the sperm cell that drives the capacity to be motile. Any factor that affects the sperm flagellum will eventually reduce sperm motility and curvilinear velocity. Broadcast spawners release their gametes into seawater to facilitate fertilisation; however, upon releasing to seawater the sperm concentration is rapidly diluted (Levitan and Petersen, 1995a, Hollows et al., 2007). Therefore, a slight decrease in sperm motility and disruption in sperm swimming speed could seriously threaten fertilisation success.

4.4.2 Fertilisation success

In the present study, both sets of gametes were pre-exposed to each treatment. This allowed for the determination of the differential and potential effects of exposure of male and female reproductive cells to acidified conditions. Oocytes exposed to reduced pH seawater had significantly reduced fertilisation success for all species. Even though there were no clear reasons how or why this happened it may be due to internal pH modification. According to Bögner et al. (2014), finely tuned internal pH modifications are important for cell activation after fertilisation. Therefore, the limited buffering capacity of a stressed cell due to external acidified conditions might influence the cellular ability to respond to activation mechanisms. This may result in fertilisation failure.

The percentage of sperm motility reflects the number of sperm available for fertilisation. The results for sperm motility and pre-incubation of sperm in reduced seawater pH were closely related. Reduction in motile sperm and sperm swimming speed eventually affected the fertilisation success. Decreased fertilisation success was observed with all three species under elevated pCO_2 relative to controls. This may be due to fewer collision between gametes (sperm/eggs) as a consequence of reduced sperm motility and swimming speed.

A few studies recorded changes in sperm swimming behaviour that affect fertilisation success (Vogel et al., 1982, Styan and Butler, 2000). A number of studies have shown a positive relationship between fertilisation success and sperm concentration which is influenced by sperm motility as well as swimming speed for the polychaete *Galeolaria caespitose* (Kupriyanova and Havenhand, 2002, Kupriyanova and

Havenhand, 2005). Kupriyanova and Havenhand (2005) also reported an increase of water temperature may enhance sperm swimming speed, and therefore future ocean warming could modify acidification related reductions in sperm swimming speeds, particularly during warmer summer temperatures (Hobday and Lough, 2011).

Barros et al. (2013) reported fertilisation success in the oyster, *Crassostrea gigas* was adversely affected by up to 50% when exposed to pH 7.4. They claimed that sperm were not functional to penetrate the egg, or the egg was not healthy for fertilisation, or both (Barros et al., 2013). The negative effects have previously been demonstrated for the oyster *Saccostrea glomerata* at pH 7.6 and 18-30ºC (Parker et al., 2009). Reuter et al. (2011) indicated that increasing pCO$_2$ can reduce fertilisation success by slowing the fast block to polyspermy. Even though the mechanisms are still not fully understood, it is suggested that an increase of pH$_i$ and extracellular pH in fertilised eggs interferes with Na$^+$/H$^+$ exchange associated with the fast block process which prevents the fertilisation membrane being raised (Reuter et al., 2011a). Increases in polyspermy are accentuated at higher sperm concentrations which means that pCO$_2$ effectively narrows the window of optimal sperm concentrations (Reuter et al., 2011a).

Another reason for inhibition of fertilisation success may be due to impairment of the formation of the fertilisation envelope and sperm:egg contact. This may be related to a disorder in the extracellular matrix (ECM). Ocean acidification may disrupt gamete interactions through elements of the ECM in the egg coat. Fucose sulphate glycoconjugates (FSG) and sperm-activating peptides (SAP) are both main components of the ECM involved in the Ca^{2+} and pH$_i$ dependent activation at fertilisation (Bögner et al., 2014). Hishino et al (1992) indicated effects of two extracellular pH conditions (pH 8 and 6.6) on FSG and SAP and its consequences on intracellular levels of Ca^{2+} (Ca$_i^{2+}$) and pH$_i$. They found that at pH 6.6 these ECM elements do not induce the expected increase in Ca^{2+}. Moreover, at these levels of extracellular pH, an increase in pH$_i$ is only induced by exposure to SAP. Based on these results, even SAP seems not to be affected by acidic conditions, FSG will putatively be impaired leading to incomplete activation of the Ca$_i^{2+}$ response. This affects the synthesis of cyclic nucleotides necessary during the acrosome reaction impairing gametes interaction (Hishino et al., 1992).

Numerous researchers have examined the effects of ocean acidification on fertilisation success using diverse marine species; often with conflicting results reported both within and between species. Havenhand et al. (2008b) found a significant reduction

of fertilisation success in the sea urchin *Heliocidaris erythrogramma*, and Parker et al. (2009) reported the same result using the Sydney rock oyster *Saccostrea glomerata* and reduction of fertilisation success of *S. glomerata* and the Pacific oyster, *Crassostrea gigas* (Parker et al., 2010). Other studies recorded fertilisation was not affected by the elevated seawater of pCO$_2$ (Byrne et al., 2009, Byrne et al., 2010c, Byrne et al., 2010b, Ericson et al., 2010c) even though there may be variations in fertilisation response between individual mating pairs (Sewell et al., 2014). These variations might be because of true intraspecific differences in ocean acidification response, driven by the strong intermale and interspecies variation in sperm functional response to ocean acidification being observed (Schlegel et al., 2012b, Schlegel et al., 2014). However, weaknesses in methodology or experimental design have also been recommended as a possible source of these differences in fertilisation success response to reduced seawater pH (Ross et al., 2011, Byrne, 2012). In addition to other studies, fertilisation success is also correlated to gamete concentrations, ratio of sperm to egg, time contact of sperm and egg and size of egg (Levitan et al., 1991, Lera et al., 2006, Byrne et al., 2010a).

Moreover, evidence reported that ocean acidification may not affect fertilisation success of marine invertebrates, especially in studies using sea urchins species (Bay et al., 1993, Kurihara and Shirayama, 2004a, Carr et al., 2006). They found that fertilisation still occurred at about 80-90% when exposed to pH 7.3 and higher, only at seawater pH lower than 7.0 was fertilisation reduced up to less than 60-70%. Byrne et al. (2009) suggested these results might be due to the low pH naturally associated with echinoderm reproduction; the internal pH of activated sperm initially at pH 7.6 and acid is released by echinoderm eggs at fertilisation (Peaucellier and Doree, 1981, Holland and Gould-Somero, 1982) – a phenomena characteristic of marine invertebrate reproduction (Holland et al., 1984). Moreover, the early life history of *H. erythrogramma* and other intertidal invertebrates is likely to be adapted to the broad pH range (e.g. pH 7.4-8.9) characteristic of this habitat due to photosynthetic activity (Wootton et al., 2008).

4.5   Conclusions

This chapter presented an evaluation of the effects of ocean acidification on a range of species looking at sperm motility and fertilisation success. The results obtained demonstrate that *A. rubens*, *P. miliaris*, and *A. marina* were influenced by CO$_2$ driven acidification as projected to occur in the surface ocean by year 2100 and beyond as sperm motility and fertilisation success progressively decreased. Seawater of pH $\leq$7.9

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also reduced motile sperm and decreased sperm swimming speed. Pre-incubation of sperm and oocytes before fertilisation and incubation of sperm and oocytes in reduced seawater pH showed a reduction in fertilisation success. This study presents evidence that both sperm and oocytes are affected by seawater $\leq$ pH 7.9. Adaptation could be possible but given the accelerating rate of the ocean acidification it seems questionable if there will be enough time for most species to adapt. If pH sensitivity of sperm motility and fertilisation success of these study species cannot adapt over the next several decades, the percentage of motility and fertilisation will eventually drop, which may affect the viability of the species.

Chapter 5: The effects of nonylphenol and ocean acidification on sperm motility and fertilisation success of marine invertebrates

Abstract

The gametes of marine invertebrates are being released into oceans that are simultaneously decreasing in pH and increasing in the levels of many manmade contaminants. In chapter 4 it was demonstrated that reproductive success can be highly sensitive to ocean acidification, yet little is known of the potential impacts of combining pollutants (such as nonylphenol) with ocean acidification. The effects of nonylphenol on sperm motility and fertilisation success, at the concentrations tested, were assessed using *Asterias rubens*, *Psammechinus miliaris* and *Arenicola marina*. The combined effects of nonylphenol and decreasing pH (0.2-0.4 pH units relative to ambient seawater pH) were also examined using *P. miliaris* and *A. rubens*. Sperm from all test species were severely affected by nonylphenol concentration with reduced motility and fertilisation success; however, oocytes were unaffected by nonylphenol. In addition, sperm motility and fertilisation success were negatively affected by reduced pH, nonylphenol concentration and exposure time with the exception of *P. miliaris* oocytes, where pH did not affect fertilisation success.

5.1   Introduction

Nonylphenol is an alkylphenol used in the manufacture of antioxidants, lubricating oil additives, and the production of ethoxylate surfactants (USEPA, 1990). Alkylphenols are released to the environment as a result of microbial breakdown of alkylphenol polyethoxylates (APEs) during sewage treatment processes (Ashfield et al., 1998b). Approximately 650,000 metric tons of APEs are produced yearly worldwide, of which 60% accumulates in aquatic systems (Guenther et al., 2002). Nonylphenol concentrations in surface waters of Italy, England and Canada have been measured at 0.084 µg/l, 45-71 µg/l and 0.15-13.7 mg/l, respectively (Ahel et al., 1994a, Bennie et al., 1997, Marcomini et al., 1990).

Nonylphenol is a recognised endocrine disruptor with oestrogenic (feminising) activities (Soares et al., 2008). It stays in a biologically active state in the body longer than natural oestrogen (Tapiero et al., 2002). The environmental effects of nonylphenol are mostly felt in aquatic ecosystems (Arslan et al., 2007, Arslan and Parlak, 2007, Hara et al., 2007). Despite the wide environmental distribution of nonylphenol and its hazards to reproductive health, the detailed toxicity mechanism has not yet been fully elucidated. Particularly, there is a lack of evidence about the effects of nonylphenol on sperm motility and fertilisation success of marine invertebrates.

The elevated carbon dioxide in seawater which is resulting in changes in ocean chemistry is not happening in isolation. There is now an increasing need to understand the impacts of multiple stressors in combination with ocean acidification on marine organism health (Denman et al., 2011b). The oceans are currently changing at an unprecedented rate in terms of sea surface temperature, sea ice cover, salinity, alkalinity and ocean circulation as well as pH (Bulling et al., 2010, Hönisch et al., 2012). Anthropogenic pollutants are also increasing and pose a threat to the marine environment.

Recent studies have begun to take multi-stressor interactions with ocean acidification into consideration (De Bodt et al., 2010, Holcomb et al., 2010). An understanding of the combined effects of ocean acidification with increasing temperature is starting to emerge (Bulling et al., 2010, Byrne et al., 2013, Ho et al., 2013, Hardy and Byrne, 2014) with increasing evidence that warming and high pCO$_2$ negatively affects

marine organisms (Nowicki et al., 2012, Torstensson et al., 2012). Increases in temperature and pCO$_2$ can have synergistic (Zittier et al., 2013) or additive effects (Talmage and Gobler, 2010) on key physiological processes such as growth or survival. Moreover, a combination of changes in seawater temperature and pH may affect marine biodiversity and the geographical distribution of some species (Findlay et al., 2010, Hale et al., 2011). Studies on the interactions of pollutants and ocean acidification are relatively few, and mainly focus on combination with metals (Campbell et al., 2014, Götze et al., 2014, Lewis et al., 2013, Fitzer et al., 2013, Pascal et al., 2010b). However, the potential effects of ocean acidification and organic pollutants, especially endocrine disruptor compounds have received no attention.

In this chapter, I look to address a key knowledge gap in our understanding of ocean acidification effects in echinoderms and polychaetes by investigating the potential for environmentally realistic levels of nonylphenol, combined with ocean acidification, to influence reproductive success.

5.2   Material and methods

All materials and methods were as reported in chapter 2 with the exception of chemical preparation and statistical analysis.

5.2.1   Chemical preparation

4-nonylphenol with a chemical purity of 94% (CAS no. 84852-15-3) was purchased from Sigma-Aldrich. Due to its low solubility in water, it was dissolved in dimethyl sulfoxide (DMSO). DMSO concentrations did not exceed 0.001% v/v in any of the experiments. Each nonylphenol concentration for sperm motility tests for *A. rubens* were prepared separately without serial dilution; however, for all other tests nonylphenol was prepared by serial dilution. Seawater pH adjustments were made according to chapter 4.

5.2.2   Statistical analysis

All statistical analyses were performed using SPSS (V17). Percentage data (%) were arcsine transformed while curvilinear velocity (VCL) data were log transformed. Data were subjected to parametric tests including analysis of variance (ANOVA) when

assumptions for normality and homoscedasticity were met (Shapiro-Wilk and Levene test, respectively). The significance level was set at $\alpha=0.05$. Significant ANOVAs were followed by a Tukey test to identify differences among groups. All figures and tables shown represent the mean $\pm$ standard error (SE). In this chapter all data met the parametric test assumptions. The No Observed Effect Concentration (NOEC) and the Effective Concentration (EC_{50}) of nonylphenol were obtained from statistical analysis described in chapter 2.

Stepwise multiple regression analysis was employed to determine the relationship between variables and also to make prediction of the effects of decreased pH, increasing nonylphenol concentration and time on sperm motility and fertilisation success. A stepwise method was used because the method was calculated using a combination of forward and backwards methods and this method utilised criteria that allowed us to enter and remove an independent variable (pH, concentration of nonylphenol, and time) in order to obtain the best regression equation. In order to use multiple regression analysis, test assumptions had to be met; normality of distribution, independence of residuals, outliers, linearity, homoscedasticity and multicollinearity.

5.3 Results

5.3.1 The effects of nonylphenol

5.3.1.1 Sperm motility and sperm swimming velocity experiments

Data on the effects on sperm motility and curvilinear velocity of *A. rubens* sperm exposed to different concentration of nonylphenol with increasing time are presented in Figure 5.1. There were significant decreases in percentage sperm motility and curvilinear velocity when exposed to $\geq 0.1 \mu g/l$ at ≥ 20 minutes exposure time with sperm motility declined in a concentration- and time- dependent manner (F=3.99, p=<0.; (Table 5.1))
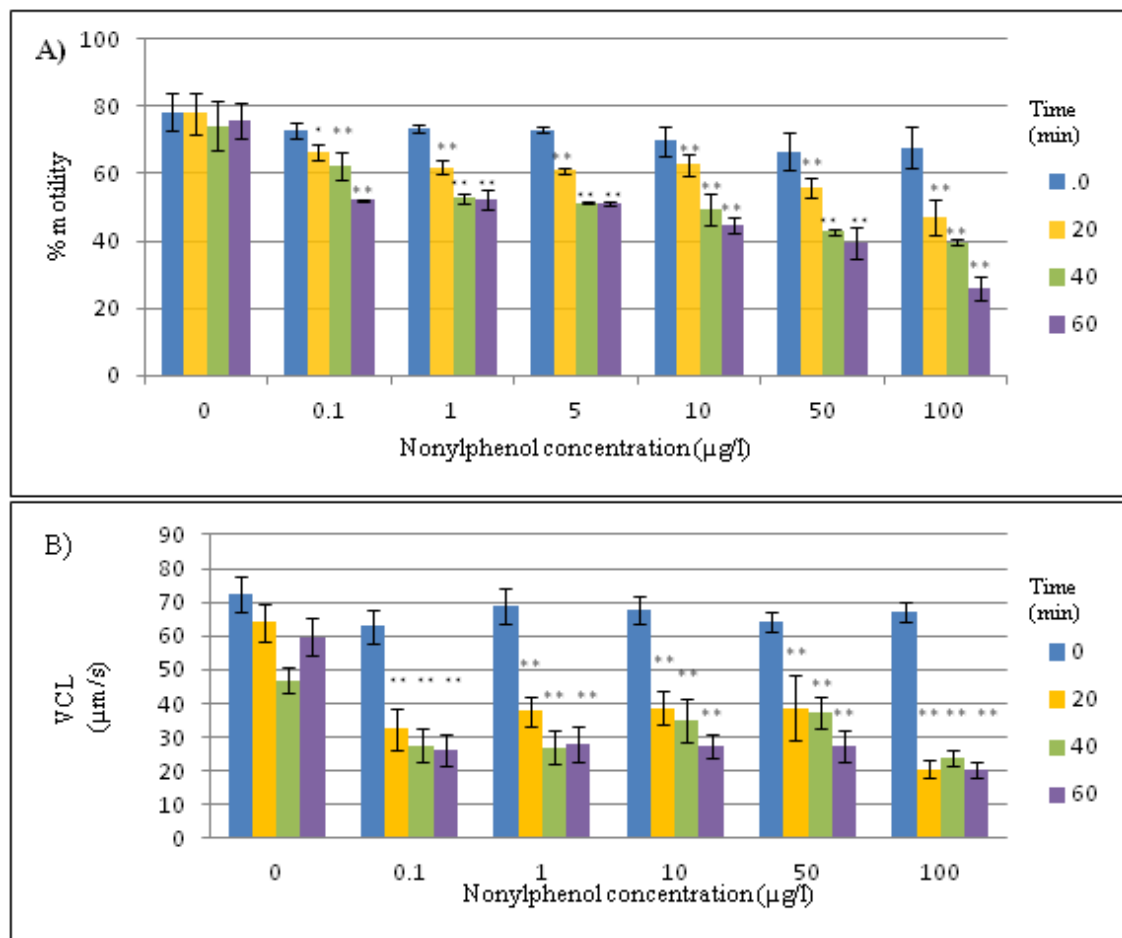
Figure 5.1. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Asterias rubens* spermatozoa after exposure to nonylphenol for up to 60 minutes. Treatments significantly different from controls within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Figure 5.2 presents the sperm motility and sperm curvilinear velocity of *P. miliaris*. Similar to *A. rubens*, there was a significant decrease in percentage sperm motility and VCL when exposed to ≥0.1µg/l for ≥20 minutes. Sperm motility and VCL also declined in a concentration- and time- dependent manner (Table 5.1)
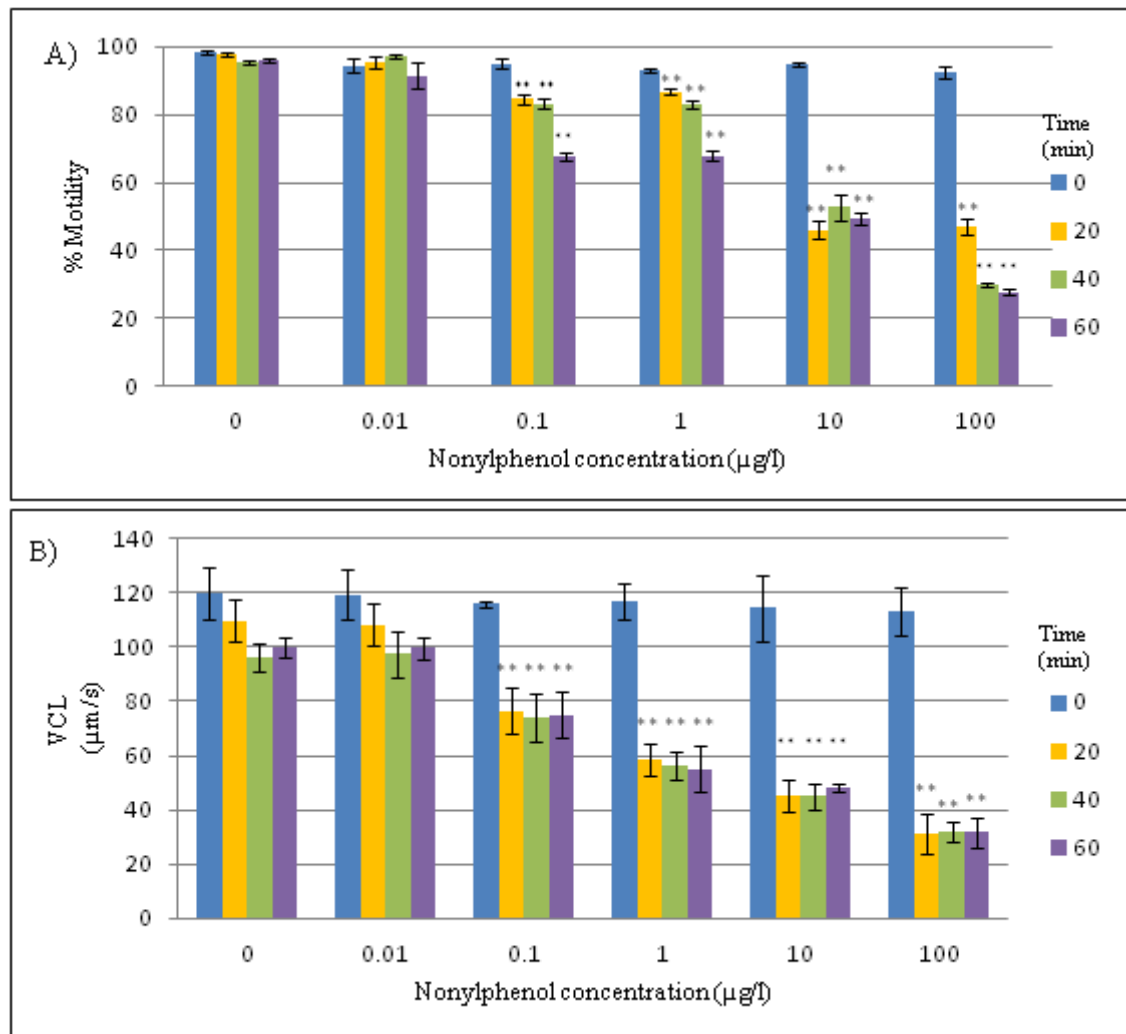
Figure 5.2. A) Percentage of sperm motility and B) curvilinear velocity (VCL) of *Psammechinus miliaris* spermatozoa after exposure to nonylphenol for up to 60 minutes. Treatments significantly different from controls within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Figure 5.3 present the nonylphenol data for *A. marina* sperm. Results show that ≥0.1µg/l at ≥20 minutes exposure adversely affected sperm motility and curvilinear velocity. Sperm motility in the control was in the range 90.2±1.3% to 93.25±3.24%. A reduction of 68% in motility was recorded at 60 minutes for 100 µg/l compared to the control (Table 5.1).
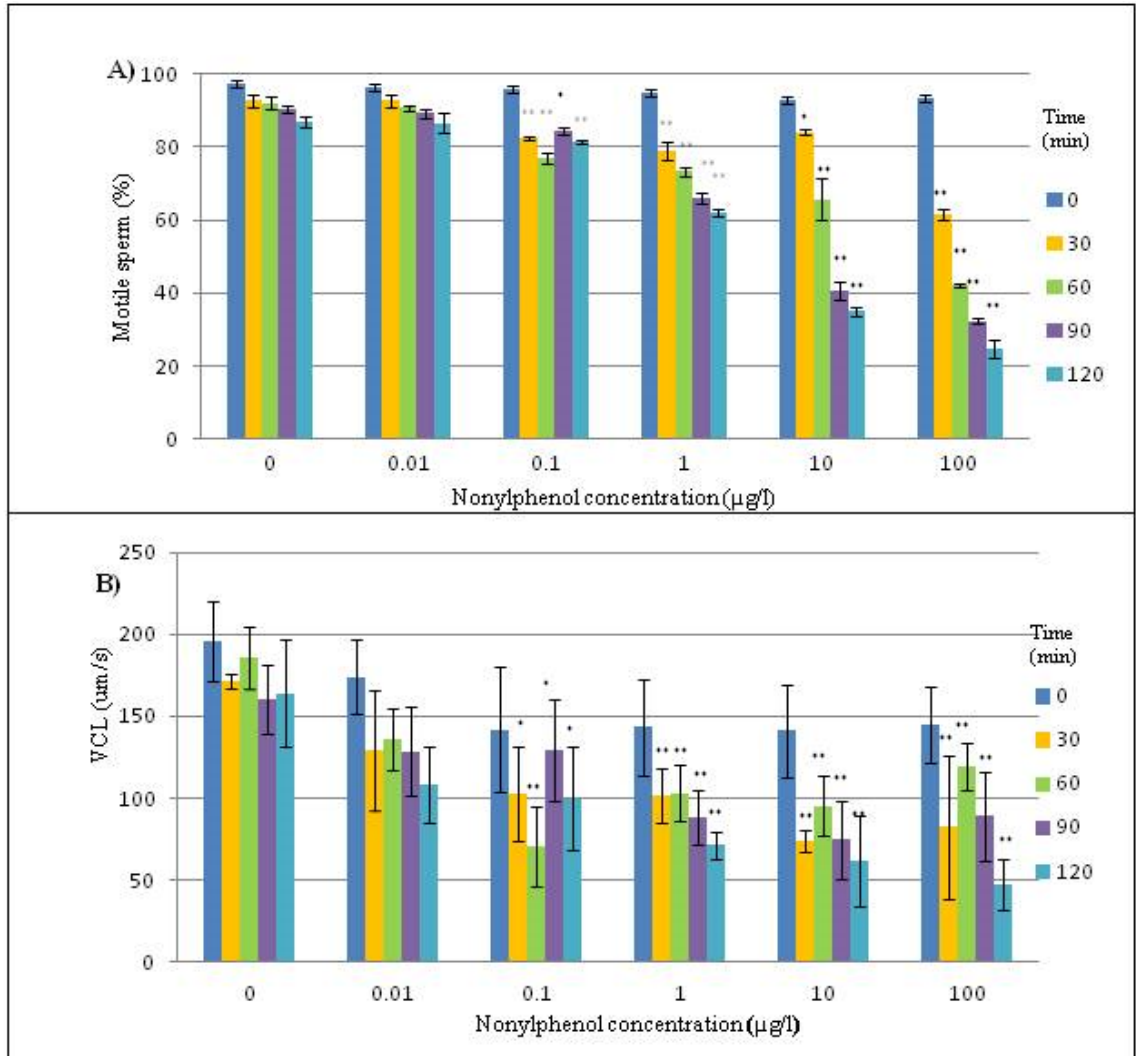
Figure 5.3. A) Percentage sperm motility and B) curvilinear velocity (VCL) of *Arenicola marina* spermatozoa after exposure to nonylphenol for up to 120 minutes. Treatments significantly different from controls within the same time intervals are indicated by asterisks *p<0.05; **p<0.01.

Table 5.1. Analysis of variance on arcsine transformed data for sperm motility and log transformed for VCL for *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* sperm, with time and nonylphenol concentration as the main effects.

Species	Parameter	Source	SS	df	MS	F	Sig.
Asterias rubens	Sperm motility	Time	15420.9	3	5140.3	74.516	<0.001
		Concentration	33704.3	6	5617.38	81.432	<0.001
		Time*Concentration	4954.53	18	275.251	3.99	<0.001
		Error	15452.1	224	68.983		
	Curvilinear velocity	Time	0.248	3	0.083	2.219	0.027
		Concentration	4.129	6	0.688	18.491	<.001
		Time*Concentration	0.892	18	0.05	1.331	0.017
		Error	8.336	224	0.037		
Psammechinus miliaris	Sperm motility	Time	9651.81	3	3217.27	121.758	<0.001
		Concentration	82719	5	16543.8	626.1	<0.001
		Time*Concentration	12591.2	15	839.416	31.768	<0.001
		Error	5073.32	192	26.424		
	Curvilinear velocity	Time	60.174	3	20.058	64.902	<0.001
		Concentration	4.32	5	78.338	23.065	<0.001
		Time*Concentration	12.504	15	0.834	2.697	<0.001
		Error	7.193	192	309		
Arenicola marina	Sperm motility	Time	23672.2	4	8157.72	456.784	<0.001
		Concentration	40788.6	5	5918.04	331.375	<0.001
		Time*Concentration	16078.3	20	803.945	45.016	<0.001
		Error	2696.71	151	17.859		
	Curvilinear velocity	Time	132.258	4	33.065	3.824	0.005
		Concentration	340.106	5	68.021	7.867	<0.001
		Time*Concentration	396.452	20	19.823	2.292	0.003
		Error	1297.02	150	8.647		

5.3.1.2 Pre-incubation of sperm with nonylphenol

Conducting fertilisation success experiments whereby both oocytes and sperm are simultaneously exposed to toxicants reveals little about the sensitivity of either germ cell to the toxin. Therefore, oocytes and sperm were incubated independently in nonylphenol and fertilised with germ cells that were not toxin exposed (Caldwell et al., 2004). Results for mean percentage fertilisation success of *A. rubens* and *P. miliaris* after the pre-incubation of sperm for 0, 20, 40 and 60 minutes in increasing concentrations of nonylphenol are presented in Figure 5.4. Mean fertilisation success for *A. rubens* controls was maintained in the range of 93.6±0.8% to 97.3±0.8%. There was a significant decline in fertilisation success when sperm were exposed to 1µg/l nonylphenol and with increased concentrations from that point; percentage fertilisation success dropped from 92.5±2.0% to 82.2±1.9%. Both nonylphenol concentration and incubation period had significant effects (Table 5.2). The incubation of sperm in nonylphenol affected *P. miliaris* fertilisation success. Control sperm maintained a fertilisation success of 96.0±0.47 to 98.4±0.56%. There were significant effects of both nonylphenol concentration and incubation period (Table 5.2). Fertilisation success for sperm incubated in 100µg/l declined to 40.4±1.7% after 20 minutes but the inhibition effect subsequently declined with time. After 60 minutes only 35.9±1.6% of oocytes were fertilised. Figure 5.4 shows the percentage fertilisation success of *A. marina*. A significant decrease in fertilisation success was observed when exposed to concentrations of 0.1µg/l and above. Generally, a similar pattern was observed for *A. rubens*, *P. miliaris* and *A. marina* characterised by a general decline in fertilisation success with time and concentration.

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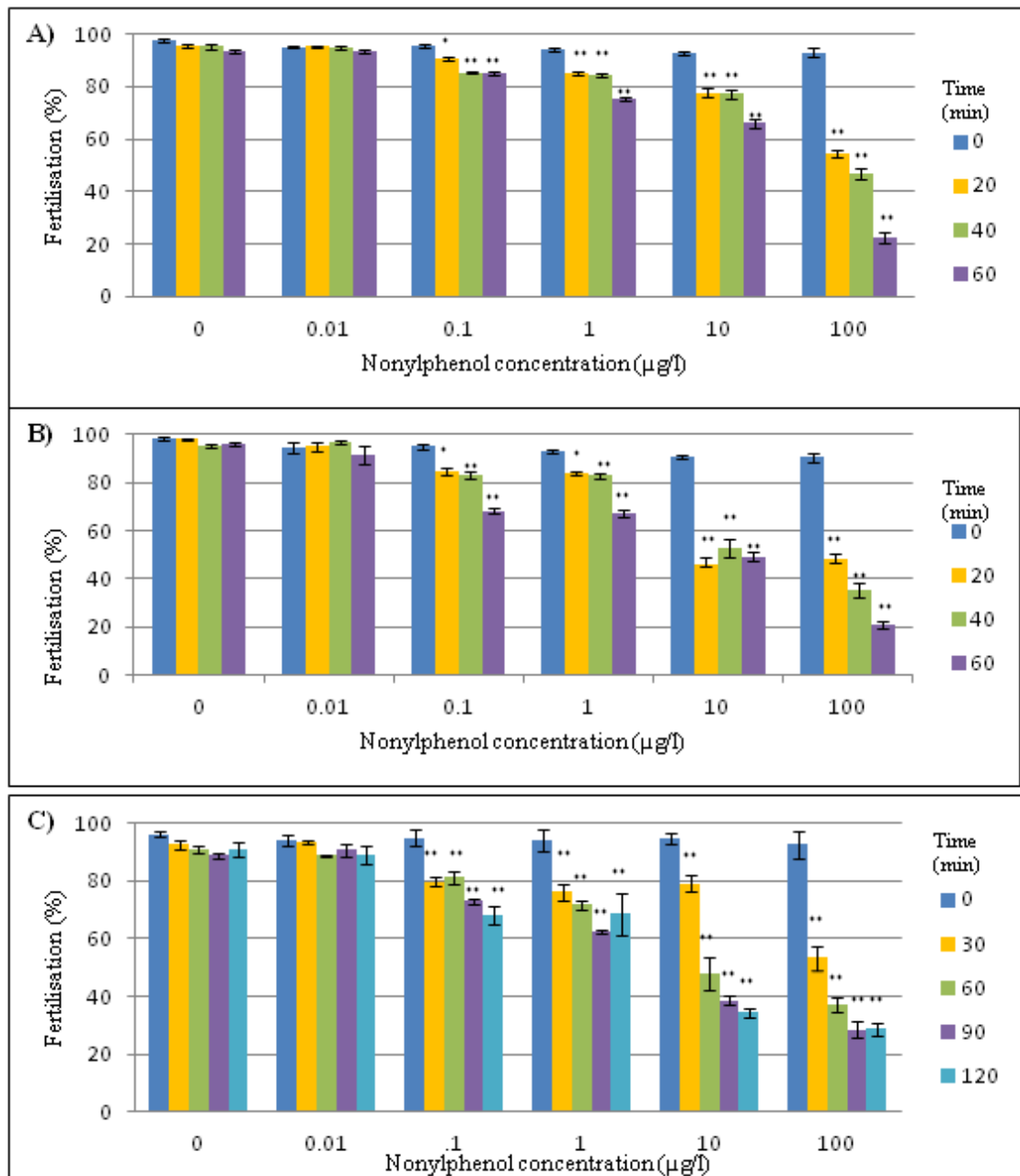
Figure 5.4. Percent fertilisation of A) *Asterias rubens*, B) *Psammechinus miliaris* and C) *Arenicola marina* oocytes after incubation of sperm with nonylphenol at different concentrations and times prior to fertilisation. Treatments significantly different from controls within the same time intervals are indicated by asterisks *$p<0.05$; **$p<0.01$.

Table 5.2. *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Analysis of variance on arcsine-transformed fertilisation success, with time and nonylphenol concentration as main effects.

Source	SS	df	MS	F ratio	P value
Asterias rubens					
Concentration	65189.200	5	13037.840	776.032	<0.001
Time	1573.274	3	524.425	31.215	<0.001
Time * Concentration	831.740	15	55.449	3.300	<0.001
Error	3225.725	192	16.801		
Psammechinus miliaris					
Concentration	49250.825	5	9850.165	407.637	<0.001
Time	4309.574	3	1436.525	59.449	<0.001
Time * Concentration	5102.121	15	340.141	14.076	<0.001
Error	4639.497	192	24.164		
Arenicola marina					
Concentration	13413.161	5	3353.290	37.730	<0.001
Time	54519.929	4	10903.986	220.240	<0.001
Time * Concentration	14913.336	20	745.667		<0.001
Error	7475.929	151			

5.3.1.3 Pre-incubation of oocytes with nonylphenol

Figure 5.5 (A) does not indicate any effect of pre-incubation of oocytes of *A. rubens* in nonylphenol. Control oocytes maintained a fertilisation success of 93.8±0.52 to 97.8±0.7%. There were no significant effects of either nonylphenol concentration or incubation period, and no significant interaction between the factors of concentration and time. A similar pattern was recorded for *P. miliaris* oocytes (Figure 5.5 (B)) with no significant effect between treatment groups. The effect of pre-incubation with increasing nonylphenol concentration on *A. marina* is shown in Figure 5.5 (C). Once again, there were no significant differences between time, concentration and combinations of time and concentration (Table 5.3).
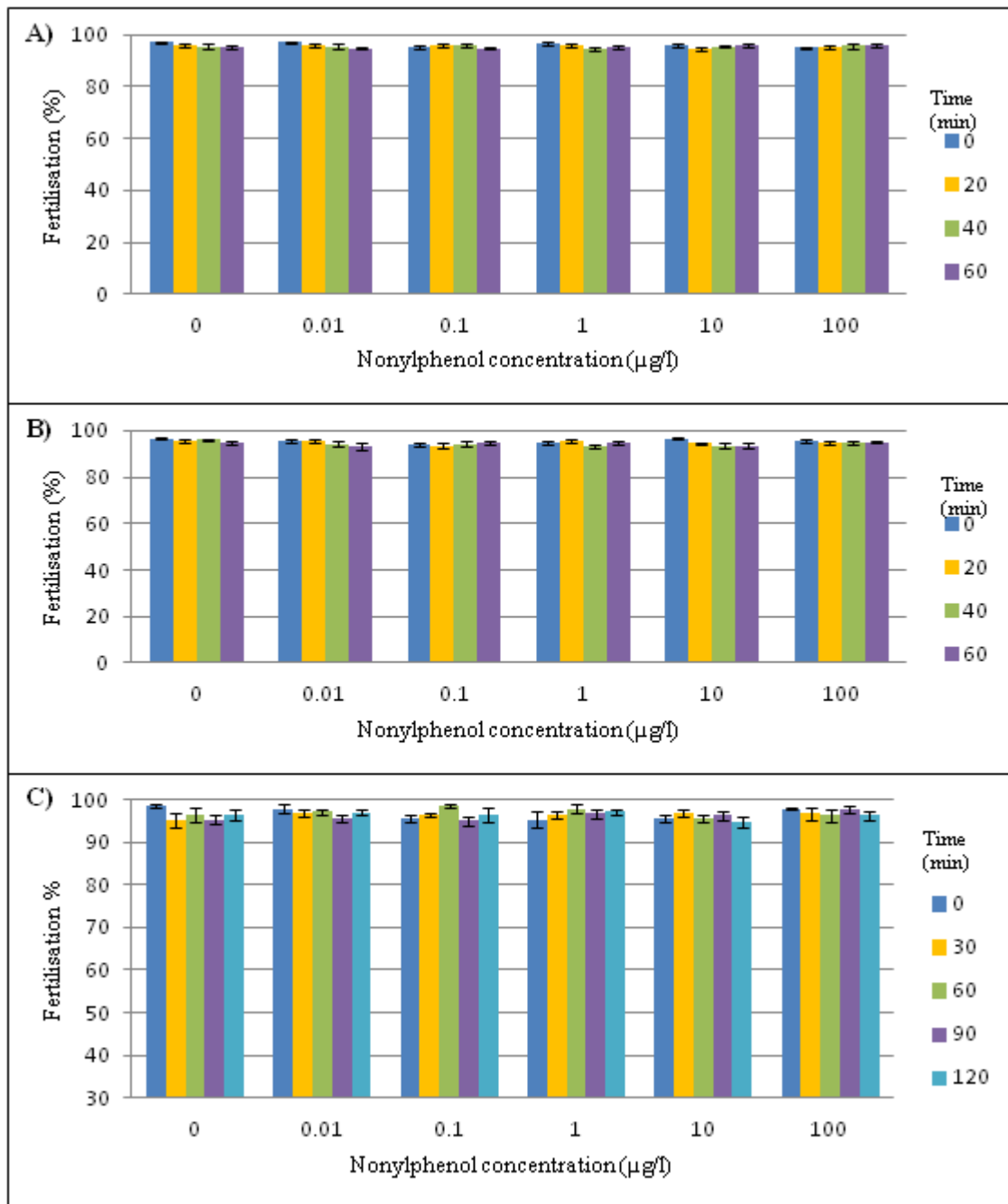
Figure 5.5. Percent fertilisation of A) *Asterias rubens*, B) *Psammechinus miliaris* and C) *Arenicola marina* oocytes after incubation with nonylphenol at different concentrations and times prior to fertilisation.

Table 5.3. *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Analysis of variance on arcsine-transformed fertilisation levels, with time and nonylphenol concentration as main effects.

Source	SS	df	MS	F ratio	P value
Asterias rubens					
Time	28.648	3	9.549	2.347	0.740
Concentration	6.981	5	1.369	.343	0.886
Time * Concentration	62.574	15	4.172	1.025	0.431
Error	781.333	192	4.069		
Psammechinus miliaris					
Time	49.259	3	16.420	2.468	0.063
Concentration	53.704	5	10.741	1.614	0.158
Time * Concentration	95.185	15	6.346	0.954	0.506
Error	1277.333	192	6.653		
Arenicola marina					
Time	21.94	4	5.476	0.780	0.540
Concentration	27.194	5	7.363	0.774	0.570
Time * Concentration	147.250	20	7.363	1.048	0.411
Error	3168.929	151	7.025		

5.3.1.4 Incubation of sperm and oocytes in nonylphenol

The incubation of gametes with nonylphenol resulted in reduced fertilisation success (Figure 5.6). Fertilisation was still evident at 100µg/l. Fertilisation success declined rapidly in *P. miliaris*, dropping from a mean of 97±0.67% for controls to 8±1.8% at 100µg/l ($F=31.215$, $p= <0.001$; Table 5.4). A significant decline in fertilisation occurred when exposed to 0.10µg/l and above. Significant fertilisation failure was recorded for *A. rubens*, with mean fertilisation success of 89.1±3% at 0.1µg/l, which steadily declined to 22.2±3.3% ($F=119.527$, $p =<0.001$; Table 5.4) at 100µg/l. There was a significant reduction in fertilisation success when exposed to 0.1µg/l and higher.
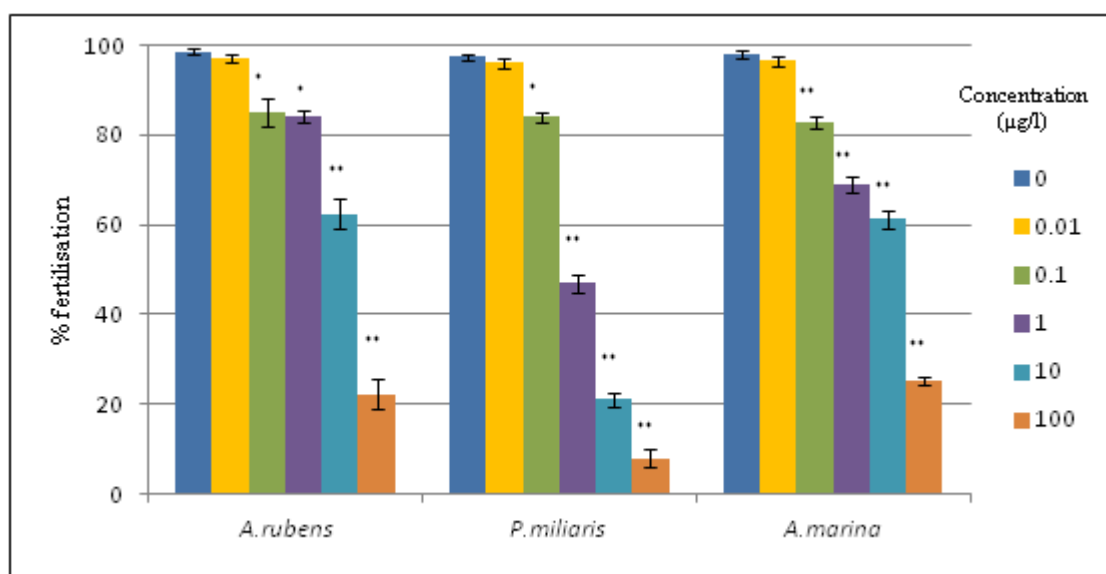
Figure 5.6. Percent fertilisation of *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* oocytes after incubation of sperm and oocytes with nonylphenol for 60 minutes at different concentrations. Treatments significantly different from controls are indicated by asterisks *$p<0.05$; **$p<0.01$.

Table 5.4. *Asterias rubens, Psammechinus miliaris* and *Arenicola marina*. Analysis of variance on arcsine-transformed fertilisation levels with nonylphenol concentration as main effects.

Source	SS	df	MS	F ratio	P value
Psammechinus miliaris					
Concentration	1573.274	3	524.425	31.215	<0.001
Error	3225.725	192	16.801		
Asterias rubens					
Concentration	19876.071	5	3975.214	119.527	<0.001
Error	1596.375	48	33.258		
Arenicola marina					
Concentration	22164.000	5	4432.800	350.881	<0.001
Error	379.000	30	12.633		

5.3.1.5 NOEC and EC_{50} value for nonylphenol

Table 5.6. No observe effects (NOEC) or lowest observe effect concentration (LOEC), half minimal effective concentration (EC_{50}) value of *Asterias rubens, Psammechinus miliaris* and *Arenicola marina* for nonylphenol. EC_{50} for *A. rubens* and *P. miliaris* = 60 minutes, EC_{50} for *A. marina* = 120 minutes.

Species	Test	NOEC or LOEC (µg/l)	EC_{50} (µg/l)
A. rubens	sperm motility	LOEC = 0.1	1211.3
	fertilization success, sperm pre-incubation	NOEC = 0.01	1112.2
	fertilization success, oocyte pre-incubation	NOEC = 100	N/A
	fertilisation success, sperm and oocyte exposure	NOEC = 0.01	1118.2
P. miliaris	sperm motility	NOEC = 0.01	419.5
	fertilization success, sperm pre-incubation	NOEC = 0.01	298.08
	fertilization success, oocyte pre-incubation	NOEC = 100	N/A
	fertilisation success, sperm and oocyte exposure	NOEC = 0.01	377.2
A. marina	sperm motility	NOEC = 0.01	2335.8
	fertilization success, sperm pre-incubation	NOEC = 0.01	2610.1
	fertilization success, oocyte pre-incubation	NOEC = 100	N/A
	fertilisation success, sperm and oocyte exposure	NOEC = 0.01	1516.48

According to Table 5.6, the 60 minutes LOEC sperm motility for *A. rubens* sperm is 0.10µg/l, while the NOEC for *P. miliaris* sperm is 0.01µg/l. The 120 minute NOEC for *A. marina* sperm is 0.01µg/l. Regression log-concentration probit was obtained for nonylphenol to estimate acute effects of the effective concentration (EC_{50}) at 95% significance causing the concentration of nonylphenol in this study to reduce by 50% in each test species (Table 5.7). For the 60 minutes EC_{50}, *A. rubens* sperm motility was 1211.30µg/l and 419.5µg/l for *P. miliaris*. The 120 minute EC_{50} nonylphenol for *A. marina* sperm motility was 2335.8µg/l. For fertilisation success of pre-incubated sperm the 60 minute NOEC for *A. rubens* and *P. miliaris* was 0.01µg/l whereas the 120 minute NOEC for *A. marina* was 0.01µg/l. The 60 minute EC_{50} effect on fertilisation success of sperm pre-incubated in nonylphenol for *A. rubens* and *P. miliaris* were 1112.20µg/l and 298.08µg/l respectively and the 120 minutes EC_{50} for *A. marina* was 2610.1µg/l. The NOEC for the oocytes of the three test species exposed to nonylphenol was 100µg/l as there was no significant effect on fertilisation success. The NOEC for fertilisation success of the incubation of both sperm and oocytes in nonylphenol was 0.01µg/l for all test species. The 60 minute acute effect for *A. rubens, P. miliaris* and *A. marina* was 1118.20, 377.20 and 1516.48µg/l respectively.

5.3.2   Combination of nonylphenol and reduce pH of seawater

5.3.2.1 Sperm curvilinear velocity

A stepwise multiple regression was conducted to predict the effects on swimming speed of *A. rubens* and *P. miliaris* sperm with factors of exposure time, nonylphenol concentration and pH. For *A. rubens*, the overall model was significant F (3,500) = 1.430, p <0.001 (Table 5.8) and accounted for 6.4% of the variance. This indicated that the model could predict statistically the swimming speed of *A. rubens* sperm based on the following regression equation:

Log sperm swimming speed (VCL) = -211.987 – 0.277*exposure time + 36.654*pH seawater – 0.088*concentration of nonylphenol (Table 5.9)

The reduction of swimming speed was associated with exposure time (Beta = -0.171, p <0.001) (Table 5.9). As pH increased, the swimming speed also increased (Beta = 0.165, p <0.001) while an increase of nonylphenol resulted in a decreased swimming speed (Beta = -0.088, p = 0.041). For *P. miliaris,* exposure time, pH, and nonylphenol concentration significantly affected swimming speed, F (3,500) = 17.083, p < 0.001 explaining 9.3% of the variance. The regression equation was derived as:

Log sperm swimming speed (VCL) = 275.920 – 0.385*exposure time + 26.471*pH seawater – 0.110*concentration nonylphenol (Table 5.9)

Reduced exposure time (Beta = -0.251, p < 0.001) and nonylphenol concentration (Beta = -0.118, p = 0.006) were associated with increased swimming speed. As pH increased, motility and swimming speed also increased (Beta = 0.126, p < 0.003). Time was the biggest influence as a predictor of sperm motility and swimming speed.

Table 5.7. Correlation coefficient for time, pH and nonylphenol concentration for log transformed curvilinear velocity data.

Asterias rubens				
Predictors	R	R Square	Adjusted R Square	Std. Error
(Constant), Time	0.171	0.029	0.027	35.82624
(Constant), Time, pH	0.237	0.056	0.053	35.35590
(Constant), Time, pH, Concentration	0.253	0.064	0.059	35.24349
Psammechinus miliaris				
Predictors	R	R Square	Adjusted R Square	Std. Error of
(Constant), Time	0.251	.063	.061	33.18748
(Constant), Time, pH	0.281	.079	.075	32.93646
(Constant), Time, pH, Concentration	0.305	.093	.088	32.72003

Table 5.8. Analysis of variance (ANOVA) for the regression of log transformed curvilinear velocity data.

Asterias rubens						
Predictors	Source	Sum of Squares	df	Mean Square	F	Sig.
(Constant), Time	Regression	19317.592	1	19317.592	15.050	<0.001
	Residual	644326.598	502	1283.519		
	Total	663644.190	503			
(Constant), Time, pH	Regression	37374.491	2	18687.246	14.949	<0.001
	Residual	626269.698	501	1250.039		
	Total	663644.190	503			
(Constant), Time, pH, Concentration	Regression	42592.523	3	14197.508	11.430	<0.001
	Residual	621051.667	500	1242.103		
	Total	663644.190	503			
Psammechinus miliaris						
(Constant), Time	Regression	37259.280	1	37259.280	33.829	<0.001
	Residual	552907.268	502	1101.409		
	Total	590166.549	503			
(Constant), Time, pH	Regression	46676.650	2	23338.325	21.514	<0.001
	Residual	543489.899	501	1084.810		
	Total	590166.549	503			
(Constant), Time, pH, Concentration	Regression	54866.256	3	18288.752	17.083	<0.001
	Residual	535300.293	500	1070.601		
	Total	590166.549	503			

Table 5.9. Coefficients of regression for log transformed curvilinear velocity data.

Asterias rubens					
Predictors	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	75.952	2.670		28.443	<0.001
Time	-.277	.071	-.171	-3.879	<0.001
(Constant)	-213.615	76.234		-2.802	0.005
Time	-.277	.070	-.171	-3.931	<0.001
pH	36.654	9.644	.165	3.801	<0.001
(Constant)	-211.987	75.996		-2.789	0.005
Time	-.277	.070	-.171	-3.944	<0.001
pH	36.654	9.613	.165	3.813	<0.001
Concentration	-.088	.043	-.089	-2.050	0.041
Psammechinus miliaris					
(Constant)	64.763	2.474		26.181	<0.001
Time	-.385	.066	-.251	-5.816	<0.001
(Constant)	273.881	71.017		3.857	<0.001
Time	-.385	.066	-.251	-5.861	<0.001
pH	26.471	8.984	.126	2.946	0.003
(Constant)	275.920	70.554		3.911	<0.001
Time	-.385	.065	-.251	-5.899	<0.001
pH	26.471	8.925	.126	2.966	0.003
Concentration	-.110	.040	-.118	-2.766	0.006

Sperm motility

A stepwise multiple regression was conducted to predict the percentage of motile sperm of *A. rubens* and *P. miliaris* on exposure time, nonylphenol concentration and seawater pH. All predictors tested had a significant influence on *A. rubens* sperm motility, $F_{(3,500)} = 411.161$, $p < 0.001$ (Table 5.11), with 71.2 % variance. The regression equation to predict the percentage of motile sperm was derived as:

Arcsine motile sperm (%) = -348.565 – 0.480*exposure time + 54.702*pH seawater – 0.12*nonylphenol concentration (Table 5.12).

A decrease of exposure time (Beta= -0.618, $p < 0.001$) and nonylphenol concentration (Beta = -0.254, $p < 0.001$) were associated with an increase of sperm motility; when pH increased (Beta = 0.515, $p < 0.001$) so too did the percentage of motile sperm. The biggest predictor was time, followed by pH, then nonylphenol concentration. All predictors significantly influenced *P. miliaris* sperm motility, $F_{(3,300)} = 307.764$, $p <$

0.001 (Table 5.11) and explained 65% of the variance. The regression equation for this model was:

Arcsine motile sperm (%) = -197.914 – 0.260*exposure time + 34.554*pH seawater - 0.052*nonylphenol concentration (Table 5.12).

A reduction in exposure time (Beta = -0.563, p < 0.001) and nonylphenol concentration (Beta = -0.183, p < 0.001) were associated with increases in sperm motility. Sperm motility also increased with increased pH (Beta = 0.546, p < 0.001). Time as the biggest influence, then pH, followed by nonylphenol concentration.

Table 5.10. Correlation coefficient for time, pH and nonylphenol concentration of arcsine transformed data percentage of motile sperm.

Asterias rubens				
Predictors	R	R Square	Adjusted R Square	Std. Error
(Constant), Time	.618	.382	.381	13.66750
(Constant), Time, pH	.804	.647	.646	10.33922
(Constant), Time, pH, Concentration	.844	.712	.710	9.35537
Psammechinus miliaris				
Predictors	R	R Square	Adjusted R Square	Std. Error
(Constant), Time	.563	.317	.316	8.55702
(Constant), Time, pH	.784	.615	.614	6.42957
(Constant), Time, pH, Concentration	.805	.649	.647	6.15012

Table 5.11. Analysis of variance (ANOVA) for arcsine transformed data for the percentage of motile sperm.

Predictors	Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens						
(Constant), Time	Regression	57945.657	1	57945.657	310.201	<0.001
	Residual	93773.835	502	186.800		
	Total	151719.492	503			
(Constant), Time, pH	Regression	98162.848	2	49081.424	459.136	<0.001
	Residual	53556.644	501	106.899		
	Total	151719.492	503			
(Constant), Time, pH, Concentration	Regression	107958.040	3	35986.013	411.161	<0.001
	Residual	43761.452	500	87.523		
	Total	151719.492	503			
Psammechinus miliaris						
(Constant), Time	Regression	17076.825	1	17076.825	233.218	<0.001
	Residual	36757.714	502	73.223		
	Total	53834.540	503			
(Constant), Time, pH	Regression	33123.504	2	16561.752	400.629	<0.001
	Residual	20711.036	501	41.339		
	Total	53834.540	503			
(Constant), Time, pH, Concentration	Regression	34922.560	3	11640.853	307.764	<0.001
	Residual	18911.980	500	37.824		
	Total	53834.540	503			

Table 5.12. Coefficients of regression for arcsine transformed data on the percentage of motile sperm.

Asterias rubens					
Predictors	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	81.354	1.019		79.859	<0.001
Time	-.480	.027	-.618	-17.613	<0.001
(Constant)	-350.795	22.293		-15.735	<0.001
Time	-.480	.021	-.618	-23.282	<0.001
pH	54.702	2.820	.515	19.396	<0.001
(Constant)	-348.565	20.173		-17.279	<0.001
Time	-.480	.019	-.618	-25.731	<0.001
pH	54.702	2.552	.515	21.436	<0.001
Concentration	-.120	.011	-.254	-10.579	<0.001
Psammechinus miliaris					
(Constant)	74.103	.638		116.185	<0.001
Time	-.260	.017	-.563	-15.271	<0.001
(Constant)	-198.870	13.863		-14.345	<0.001
Time	-.260	.013	-.563	-20.325	<0.001
pH	34.554	1.754	.546	19.702	<0.001
(Constant)	-197.914	13.262		-14.924	<0.001
Time	-.260	.012	-.563	-21.248	<0.001
pH	34.554	1.678	.546	20.597	<0.001
Concentration	-.052	.007	-.183	-6.897	<0.001

5.3.2.2 Pre-incubation of sperm in nonylphenol and reduce pH of seawater

All predictors (exposure time, pH and nonylphenol concentration) significantly influenced fertilisation success with pre-incubation of sperm for *A. rubens,* $F_{(3,500)}$ = 210.618, $p < 0.001$ (Table 5.14) and explaining 71% of the variance. The regression equation predicting fertilisation success was:

Arcsine fertilisation success (%) = -18.911 – 0.314*exposure time + 0.116*pH seawater - 14.137*nonylphenol concentration (Table 5.15).

Increased seawater pH caused an increase of fertilisation success (Beta = 0.371, $p < 0.001$). Increasing exposure time by one standard deviation caused fertilisation success to decrease by 0.616 standard deviations with a constant pH and nonylphenol concentration (Beta = -0.616, $p < 0.001$). Nonylphenol concentration was associated with declines in fertilisation success (Beta = -0.202, $p < 0.001$). For *P. miliaris*, the overall model was significant, $F_{(3, 500)}$ = 338.216, $p < 0.001$ with 67% of variance explained. The regression equation predicting fertilisation success was:

Arcsine fertilisation success (%) = -294.870 – 0.436*exposure time + 47.798*pH seawater - 0.90* nonylphenol concentration (Table 5.15).

Increasing exposure time (Beta = -0.617, p <0.001) and nonylphenol concentration (Beta = -0.210, p < 0.001) were associated with decreased fertilisation success while an increase of pH also increased fertilisation success (Beta = 0.495, p <0.001).

Table 5.13. Correlation coefficient for time, pH and nonylphenol concentration of arcsine transformed data percentage fertilisation.

Asterias rubens				
Predictors	R	R Square	Adjusted R Square	Std. Error
(Constant), time	0.618	0.382	0.381	13.6675
(Constant), time, pH	0.804	0.647	0.646	10.33922
(Constant), time, pH, Concentration	0.844	0.712	0.71	9.35537
Psammechinus miliaris				
Predictors	R	R Square	Adjusted R Square	Std. Error
(Constant), time	0.617	0.381	0.380	12.43512
(Constant), time, pH	0.791	0.626	0.624	9.67745
(Constant), time, pH, Concentration	0.818	0.670	0.668	9.10014

Table 5.14. Analysis of Variance (ANOVA) for regression arcsine transformed data of percentage fertilisation.

Predictors	Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens						
(Constant), time	Regression	24891.429	1	24891.429	307.257	<0.001
	Residual	40667.929	502	81.012		
	Total	65559.357	503			
(Constant), time, pH	Regression	33912.302	2	16956.151	268.430	<0.001
	Residual	31647.055	501	63.168		
	Total	65559.357	503			
(Constant), time, pH, Concentration	Regression	36598.314	3	12199.438	210.618	<0.001
	Residual	28961.044	500	57.922		
	Total	65559.357	503			
Psammechinus miliaris						
(Constant), time	Regression	47806.578	1	47806.578	309.163	<0.001
	Residual	77625.390	502	154.632		
	Total	125431.968	503			
(Constant), time, pH	Regression	78511.768	2	39255.884	419.163	<0.001
	Residual	46920.200	501	93.653		
	Total	125431.968	503			
(Constant), time, pH, Concentration	Regression	84025.668	3	28008.556	338.216	<0.001
	Residual	41406.300	500	82.813		
	Total	125431.968	503			

Table 5.15. Coefficients of regression for arcsine transformed data percentage fertilisation.

Asterias rubens					
Predictors	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	90.631	0.671		135.095	<0.001
Time	-.314	0.018	-.616	-17.529	<0.001
(Constant)	92.771	0.619		149.905	<0.001
Time	-.314	0.016	-.616	-19.851	<0.001
pH	.116	0.010	.371	11.950	<0.001
(Constant)	-18.911	16.411		-1.152	<0.001
Time	-.314	0.015	-.616	-20.730	<0.001
pH	.116	0.009	.371	12.480	<0.001
Concentration	-14.137	2.076	-.202	-6.810	<0.001
Psammechinus miliaris					
(Constant)	81.059	0.927		87.455	<0.001
time	-.436	0.025	-.617	-17.583	<0.001
(Constant)	-296.542	20.866		-14.211	<0.001
time	-.436	0.019	-.617	-22.593	<0.001
pH	47.798	2.640	.495	18.107	<0.001
(Constant)	-294.870	19.623		-15.027	<0.001
time	-.436	0.018	-.617	-24.027	<0.001
pH	47.798	2.482	.495	19.256	<0.001
Concentration	-.090	0.011	-.210	-8.160	<0.001

5.3.2.3 Pre-incubation of oocytes in nonylphenol and reduce pH of seawater

Stepwise multiple regression was conducted to predict the fertilisation success of pre-incubation oocyte in pH seawater, exposure time and nonylphenol concentration for *A. rubens* and *P. miliaris*. For *A. rubens*, all predictors significantly contributed to fertilisation success, $F_{(3, 500)} = 210.618$, $p < 0.001$ (Table 5.17) explaining 61% of the variance. The regression equation was:

Arcsine fertilisation success (%) = 55.974 – 0.466*exposure time + 0.185*pH seawater - 5.446*nonylphenol concentration (Table 5.19).

Time was the main factor followed by pH and nonylphenol concentration. For *P. miliaris*, the results indicate that only exposure time and nonylphenol concentration were significant predictors, $F_{(2,501)} = 382.54$, $p < 0.001$ (Table 5.17) with a regression equation derived as:

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Arcsine fertilisation success (%) = 99.033 - 0.467*exposure time – 0.18*nonylphenol concentration (Table 5.19).

Increasing exposure time (Beta = -0.652, p < 0.001) and nonylphenol concentration (Beta = -0.425, p < 0.001) were associated with decreased fertilisation success.

Table 5.16. Correlation coefficient for time, pH and nonylphenol concentration of arcsine transformed data percentage fertilisation.

Asterias rubens				
Predictors	R	R Square	Adjusted R Square	Std. Error
(Constant), time	0.0652	0.425	0.424	12.15036
(Constant), time, pH	0.777	0.604	0.603	10.09147
(Constant), time, pH, Concentration	0.779	0.607	0.605	10.06202
Psammechinus miliaris				
Predictors	R	R Square	Adjusted R Square	Std. Error
(Constant), time	0.652	0.425	0.424	12.15939
(Constant), time, concentration	0.777	0.604	0.603	10.09831

Table 5.17. Analysis of Variance (ANOVA) for regression arcsine transformed data of percentage fertilisation.

Predictors	Source	Sum of Squares	df	Mean Square	F	Sig.
Asterias rubens						
(Constant), time	Regression	24891.429	1	24891.429	307.257	<0.001
	Residual	40667.929	502	81.012		
	Total	65559.357	503			
(Constant), time, pH	Regression	33912.302	2	16956.151	268.430	<0.001
	Residual	31647.055	501	63.168		
	Total	65559.357	503			
(Constant), time, pH, Concentration	Regression	36598.314	3	12199.438	210.618	<0.001
	Residual	28961.044	500	57.922		
	Total	65559.357	503			
Psammechinus miliaris						
(Constant), time	Regression	54889.334	1	54889.334	371.248	<0.001
	Residual	74221.093	502	147.851		
	Total	129110.427	503			
(Constant), time, pH	Regression	78020.549	2	3910.275	382.544	<0.001
	Residual	51089.877	501	101.976		
	Total	129110.427	503			

Table 5.18. Excluded variables from analysis of variable (ANOVA) for arcsine-transformed data of percentage of fertilisation.

Predictor	Beta In	t	Sig.
Concentration	.054	1.600	0.110
pH	.423	15.061	0.000
Concentration	.054	1.928	0.054

Table 5.19. Coefficients of regression for arcsine transformed data percentage fertilisation.

Predictors	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
Asterias rubens					
	B	Std. Error	Beta		
(Constant)	95.578	0.906		105.537	<0.001
Time	-0.466	0.024	-0.652	-19.259	<0.001
(Constant)	99.001	0.786		125.991	<0.001
Time	-0.466	0.020	-0.652	-23.188	<0.001
pH	0.185	0.012	0.423	-15.058	<0.001
(Constant)	55.974	21.697		2.580	0.010
Time	-0.466	0.020	-0.652	-23.256	<0.001
pH	0.185	0.012	0.423	15.102	<0.001
Concentration	-5.446	2.745	-0.056	-1.984	0.048
Psammechinus miliaris					
(Constant)	95.606	0.906		105.490	<0.001
time	-0.467	0.024	-0.652	-19.268	<0.001
(Constant)	99.033	0.786		125.945	<0.001
time	-0.467	0.20	-0.652	-23.200	<0.001
pH	0.185	0.012	0.423	15.061	<0.001

5.3.2.4 Incubation of sperm and oocytes in nonylphenol and reduced pH of seawater for 60 minutes

Stepwise multiple regression was applied to predict fertilisation success after incubating sperm and oocytes of *A. rubens* and *P. miliaris* in reduced pH seawater plus nonylphenol. pH and nonylphenol concentration significantly affected fertilisation success, $F_{(2, 123)} = 101.5022$, $p < 0.001$ (Table 5.21) explaining 62% of the variance. The regression equation derived from this model was:

Arcsine fertilisation success (%) = -266.357 – 0.357*nonylphenol concentration + 42.857*pH seawater (Table 5.22).

An increasing nonylphenol concentration was associated with decreased fertilisation success (Beta = -0.357, $p < 0.001$) and as pH increased, so too did fertilisation success (Beta = 0.372, $p < 0.001$). For *P. miliaris*, nonylphenol concentration and pH significantly contributed to fertilisation success, $F_{(2, 123)} = 101.348$, $p < 0.001$ explaining a variance of 69%. The regression equation was:

Arcsine fertilisation success (%) = -267.717 – 0.351*nonylphenol concentration +
43.155*pH seawater (Table 5.22).

Nonylphenol concentration was associated with decreased fertilisation success (Beta = -0.691, $p < 0.001$) as was increased pH (Beta = -0.379, $p < 0.001$).

Table 5.20. Correlation coefficient for pH and nonylphenol concentration of arcsine transformed percentage fertilisation data.

Asterias rubens				
Predictors	R	R Square	Adjusted R Square	Std. Error
(Constant), Concentration	0.696	0.484	0.480	13.60639
(Constant), Concentration, pH	0.789	0.623	0.617	11.68182
Psammechinus miliaris				
(Constant), Concentration	0.692	0.478	0.474	13.52466
(Constant), Concentration, pH	0.689	0.688	0.602	11.55552

Table 5.21. Analysis of Variance (ANOVA) for arcsine transformed data for percentage fertilisation.

Asterias rubens						
Predictors	Source	Sum of Squares	df	Mean Square	F	Sig.
(Constant), Concentration	Regression	21531.552	1	21531.552	116.303	<0.001
	Residual	22956.606	124	185.134		
	Total	44488.159	125			
(Constant), Concentration, pH	Regression	27702.981	2	13851.491	101.502	<0.001
	Residual	16785.178	123	136.465		
	Total	44488.159	125			
Psammechinus miliaris						
(Constant), Concentration	Regression	20808.488	1	20808.488	113.759	<0.001
	Residual	22681.647	124	182.917		
	Total	43490.135	125			
(Constant), Time, Concentration	Regression	27068.928	2	13532.964	101.348	<0.001
	Residual	16424.207	123	133.530		
	Total	43490.135	125			

Table 5.22. Coefficients of regression for arcsine transformed percentage fertilisation data.

Asterias rubens					
Predictors	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	72.215	1.358		53.163	<0.001
Concentration	-0.357	0.033	-0.696	-10.784	<0.001
(Constant)	-266.357	50.360		-5.289	<0.001
Concentration	-0.357	0.028	-0.696	-10.784	<0.001
pH	42.857	6.373	0.372	6.725	<0.001
Psammechinus miliaris					
(Constant)	72.206	1.350		54.218	<0.001
Concentration	-0.351	0.033	-0.692	10.6666	<0.001
(Constant)	-267.717	49.815		-5.374	<0.001
Concentration	-0.351	0.28	-0.692	-12.483	<0.001
pH	43.155	6.304	0.379	6.846	<0.001

5.4   Discussion

5.4.1   Nonylphenol

Nonylphenol was toxic to sperm motility and fertilisation success in *A. rubens, P. miliaris* and *A. marina* in an EC_{50} range of 298.08-1211.3, 377.2-419.5 and 1516.48-2610.1μg/l respectively. *P. miliaris* sperm was more sensitive than *A. rubens*. *A. marina* sperm were the most robust. This may be related to *A. marina* inhabiting areas experiencing greater levels of contaminants (Campbell et al., 2014) which may confer an adaptive advantage, but without a transcriptomic study this remains speculative. The NOEC and LOEC for sperm motility and fertilisation success of the three species was 0.01μg/l. The concentration of nonylphenol in UK surface waters ranges from 0.011 to 53μg/l, therefore nonylphenol is a potential threat to the reproductive cells of these species (Nico et al., 1998, Darbre et al., 2009). However, the EC_{50} values are far higher that the nonylphenol concentration found in aquatic environments.

The NOEC value from this study was far smaller compared to the NOEC of other species in previous reports. The NOEC value for malformations of embryo development for the zebrafish, *Dania rerio* is in the range of 1-15mg/l while the EC_{50} value was in range 6.17-27.1mg/l (Kammann et al., 2009). In *Daphnia magna* the NOEC for

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immobilisation was 0.024mg/l and the 24 and 48 hour EC_{50} was 0.3 and 0.19mg/l respectively (Comber et al., 1993). Using the same species, Zhang et al. (2003) reported a 96 hour EC_{50} for embryo development and deformed neonates to be 738 and 263μg/l respectively. A study on the larval development of the Pacific oyster, *Crassostea gigas* found D-shape formation to be delayed when exposed to ≥1.0μg/l (Nice et al., 2000). In a different study using the same species, sperm motility was reduced by nonylphenol. Exposure to 1.0 and 100.0μg/l during a 72 hour gametogenesis significantly reduced motility and consequently caused a reduction in fertilisation success (Nice, 2005).

The nonylphenol EC_{50} value for embryonic development and reproduction of the sea urchin *Paracentrotus lividus* was reported as 270μg/l (Arslan et al., 2007). In the present study an acute toxicity value was calculated as 377.2-419.5μg/l for sperm motility and fertilisation *P. miliaris*, while the lowest observed effect concentration (LOEC) was 0.937μg/l. However, based on present data, *P. miliaris* has a higher tolerance to nonylphenol in term of EC_{50} for fertilisation success. Arslan et al. (2007) reported that exposure of sperm and oocytes of *P. lividus* to nonylphenol resulted in a 20% reduction in fertilisation success. Further investigations were done on the larval development stages of this species with the authors reported a reduction in growth during early development and deformed skeleton formation. Further investigations using the sea urchin *Arbacia lixula* produced similar results. Exposure to nonylphenol to early life stages at lower concentrations caused skeletal malformations while high exposures prevented mitosis and inhibited embryo growth (Arslan and Parlak, 2007). The authors concluded that nonylphenol at a lower concentration presented a major risk to reproduction and normal development and likely represented an ecological hazard at the population level. Nonylphenol is also reported to be toxic to developmental stages of several other sea urchin species (Roepke et al., 2005, Kiyomoto et al., 2006) as well as other aquatic species including crustaceans, bivalves (Lussier et al., 2000), algae (Graff et al., 2003, Naylor, 1995) and fish (Kelly and Giulio, 2000).

In response to the realisation of the hazardous nature of nonylphenol to aquatic organisms, legislative action has been taken to make sure that the release of nonylphenol and its metabolites reduces. Nonylphenol concentration has been set to not exceeded 1.7μg/l in seawater by the EPA guidelines for ambient water quality (Brooke and Thursby, 2005). Since 2005, according to Directive 2003/53/EC, all 17 European countries have been banned from using and marketing nonylphenol ethoxylates, so concentrations in the environment are expected to reduce (Kammann et al., 2009).

In the present study, curvilinear velocity and the percentage of motile sperm significantly reduced when sperm were exposed to ≥0.1μg/l nonylphenol with increasing time. The pattern of declining sperm motility followed a concentration-response. Uguz et al. (2009) reported that nonylphenol had an adverse effect on progressive motility and mitochondrial membrane potential (MMP) in rats. In sperm, the mitochondria is important to supply energy for sperm movement. Any disruption to mitochondrial integrity will result in motility reduction. Even though there is no data about the effect of nonylphenol on the mitochondrial activity of sperm from invertebrates, this may suggest that mitochondrial damage during nonylphenol exposure could be one of the major reasons for the observed reduction in sperm motility. In addition, Montgomery et al. (2014) reported that 17α-ethinylestradiol (EE2), another oestrogen-like compound, causes a significant decrease in intracellular ATP that reduces sperm swimming velocity. Further evidence that nonylphenol can affect sperm quality was shown by Kawana et al. (2003b) and Hara et al. (2007). They showed that short-term exposure to sperm of the Japanese Medaka, *Oryzias latipes* to 100 μmol/l decreased swimming speed and percentage motility.

In the present study, sperm were pre-incubated with nonylphenol and fertilisation success was reduced. Several studies report that sperm motility and velocity influences successful fertilisation in free spawning invertebrates. It is an essential prerequisite for broadcast spawning species to have motile sperm. According to Berdyshev et al. (1995b), increased sperm motility and velocity resulted in an increased fertilisation capacity in the sea urchin *Strongylocentrotus intermedius*. In fish, fertilisation capacity improved with prolonged and intensified sperm motility (Lahnsteiner et al., 1997). Sperm velocity and percentage motility were adversely affected when *P. miliaris, A. rubens* and *A. marina* was exposed to 0.1μg/l; a concomitant reduction in percentage fertilisation was recorded when the sperm were incubated with 0.10μg/l. The present findings support the proposition that a decrease in fertilisation capacity of *P. miliaris, A. rubens* and *A. marina* sperm may be related to sperm motility. A reduction of sperm speed and motility will result in fewer collisions between gametes and likely result in a reduction in fertilisation success. Au et al. (2001) and Berdyshev et al. (1995a) are among many studies to report that sperm motility influences fertilisation success of broadcast spawners.

The sperm of the echinoderms used in this study have a motile lifespan of several hours (Williams and Bentley, 2002a) and up to three days for *A. marina* (Williams, 1999). Therefore, the rapid inhibition of nonylphenol on motility could provide a potent barrier to fertilisation. Although swimming activity was arrested, the sperm remained viable as evidenced by the prolonged oscillation of the sperm head. Flagellum beating is not always important for sperm incorporation upon contact between oocyte and sperm (Uguz et al., 2009) and this may account for the presence of a basal fertilisation rate of 8-20%, even when the sperm were becoming fully immotile. This suggests that mixing during sperm addition to the oocyte suspension facilitated a degree of passive fertilisation by immotile but otherwise functionally viable sperm. This is likely to be an artefact of the *in vitro* fertilisation technique as argued by (Caldwell et al., 2004).

In this study, the effect of pre-incubating oocytes with nonylphenol was also examined. Fertilisation success was unaffected which indicates that oocytes are tolerant to nonylphenol exposure. The fact that nonylphenol treated oocytes retained a high fertilisation success suggest that nonylphenol did not strongly associate with the oocyte surface membrane where it may potentially have affected sperm/egg binding. This observation was further supported by exposure of high concentrations of nonylphenol to females of the nereided polychaete *Platynereis dumerili* with the result that they were normally maturing with fertilised eggs and normal offspring (García-Alonso et al., 2011). Compared with oocytes, sperm were exposed to nonylphenol without any protection, thus adverse effects of nonylphenol on fertilisation are suggested to be attributed to its effects on the sperm.

5.4.2 Effects of combining ocean acidification and nonylphenol.

A stepwise multiple regression was used in this study to determine which predictors were significantly fixed to the model and to predict the outcome of the response. In this study, I wanted to predict the effects on sperm motility and fertilisation success when the gametes were exposed to increasing time, reduced seawater pH and increasing nonylphenol concentration.

For motility and swimming speed, exposure of sperm in decreased seawater pH, increased nonylphenol concentration and for an increasing period of time, showed 'additive' models and all predictors were significant. Exposure time was the biggest

contributor followed by pH, then nonylphenol concentration. The reduction of sperm motility was predicted to be additive for each of these variables in combination.

For fertilisation success, exposure time was once again the main influence followed by pH and nonylphenol concentration. The results of pre-incubation of *A. rubens* oocytes was also influenced by exposure time, pH and nonylphenol concentration; however, for pre-incubation of *P. miliaris* oocytes, exposure time and pH were the main contributing factors.

Sperm swimming velocity and the number of motile sperm are important components in fertilisation dynamic models, and the resulting decrease in fertilisation success observed in these experiments confirmed the initial hypothesis that fertilisation success is affected by changes in seawater pH, exposure time and nonylphenol concentration. Exposure time was the main factor and can be explained, in part, by the lifespan of sperm for these species. Echinoderm sperm can live for up to 24 hours while sperm from the polychaete worm can live for up to 72 hours. Williams and Bentley (2002b) reported that fertilisation success was influenced by gamete 'age'. Older gametes had a reduced fertilisation success.

A second bigger contributor to predict sperm motility was seawater pH. Effects of reduced pH on marine invertebrates are increasingly well documented. A sea urchin study using *Heliocidaris erythrogrramma* reported that a significant reduction in sperm motility and sperm straight line swimming speed (VSL) resulted in a lowering of fertilisation success (Havenhand et al., 2008a). The coral *Acropora digitifera* also experienced a reduction of sperm motility of up to 30% when exposed to 1000ppm CO_2 (Nakamura and Morita, 2012). The sea urchins *Strongylocentrotus francisanus* (Reuter et al., 2011b), *Paracentrotus lividus* (Moulin et al., 2011) and *Strongylocentrotus nudus* (Sung et al., 2014), had fertilisation processes negatively affected at pH 7.81, 7.6 and 7.94 respectively. In the urchin *Sterechinus nuemayeri*, a reduction in fertilisation success in high CO_2 treatments (pH 7.3) was observed but only at low sperm concentrations (Ericson et al., 2010c). However, the effects of ocean acidification on reproductive success of marine invertebrates were shown to not always be negative. Exposure to reduced pH seawater was reported to increase sperm motility in *P. miliaris* (Caldwell et al. (2011a). The authors found an increase of motile sperm and sperm swimming speed in sperm exposed to 7.98-7.97 compared to their control pH 8.06 at

14°C. The effects of reduced seawater pH are varied. For example, Havenhand and Schlegel (2009) found no effects on sperm motility and fertilisation success in the oyster *Crassostrea gigas*. Bryne et al. (2009, 2010a, 2010b) found a number of echinoderms to be robust to near future acidification conditions (pH from 8.1 to 7.6). Even though in this chapter, one of the factors responsible for the decline in sperm motility was pH, it has been suggested that reduced sperm swimming under ocean acidification conditions could actually increase sperm longevity due to a lowered consumption of limited endogenous energy provisioning (Mita and Nakamura, 1998). An increase of sperm longevity may increase the chances of successful fertilisation with sperm/egg encounter rates remaining sufficient over prolonged periods of times (Levitan, 2000a, Marshall, 2002). However, the current study showed that this does not occur in the species tested here.

The least significant influence on sperm motility and fertilisation success was nonylphenol. Ocean acidification can hinder the degradation of organic contaminants by modifying nutrient bioavailability and metal speciation, and may thus promote the release and toxicity of organic pollutants (Jurado et al., 2005, Erses et al., 2008. In this study, the results showed the additive effect of nonylphenol can contribute to a reduction of sperm motility and fertilisation success. Nonylphenol toxicity to gametes during fertilisation has been reported at a range of environmentally relevant concentration for a number of aquatic species. Exposure to gametes of the Japanese medaka, *Oryzias latipes* showed a reduction in percentage sperm motility (Kawana et al., 2003a). Further study using the same species, reported a similar result where a decrease in motile sperm and swimming speed was observed after 60 seconds in 100μmol nonylphenol (Hara et al., 2007). Nice (2005) indicated that the oyster *Crassostrea gigas* is affected by nonylphenol with decreasing sperm motility and fertilisation success. An in vitro study of *Bufo raddei* done by Feng et al. (2011) found that sperm motility was affected by 400μg/l after nine minutes of exposure. Niu et al. (2013) demonstrated that nonylphenol at ≥1.0μg/l significantly reduced sperm motility of barbell chub, *Squaliobarbus curriculus*. As gametes are released in the seawater, the ability to locate oocytes by sperm in potentially challenging hydrodynamic condition is of great importance (Mead and Denny, 1995a). Any external stressors, including exposure to toxicants that may affect sperm fitness and could potentially compromised fertilisation success (Lewis et al., 2008, Lewis and Galloway, 2010).

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Even though studies that combine the effects of environmental contaminants and ocean acidification on sperm motility are rare, my results for *P. miliaris* and *A. rubens* add useful evidence that gametes will respond negatively to deceasing seawater pH and increasing nonylphenol concentration with increasing exposure time. However, in the *P. miliaris* oocyte pre-incubation test, only exposure time and pH were significant influences. This shows that oocytes of this species were robust to nonylphenol at the concentrations tested.

5.5 Conclusions

This series of nonylphenol exposure experiments have confirm the hypothesis that toxicity of nonylphenol at $\geq 0.1\mu g/l$ significantly reduced sperm motility and swimming speed. Pre-incubated sperm and sperm plus oocytes in nonylphenol $\geq 0.1\mu g/l$ experienced reduced fertilisation success; however, pre-incubating oocytes in nonylphenol had no effect on fertilisation success. Therefore, it is concluded that nonylphenol is a potential threat to reproductive success of marine invertebrates at the environmentally relevant concentrations tested in this thesis. Nonylphenol concentration, exposure time and seawater pH affected sperm motility and fertilisation success of *A. rubens* and *P. miliaris*. Only oocytes of both species were unaffected by nonylphenol. Exposure time was always the biggest contributor followed by reduced pH and nonylphenol concentration having the least effect.

Nonylphenol is known to be responsible for feminization of aquatic species. We may in fact be underestimating to impact of ocean acidification for marine invertebrates from these contaminants. There is a huge gap in knowledge regarding how previous exposure to environmental contaminants or adaptation to live in contaminated areas might affect future responses to climate change. Further experiments regarding the mixture of organic contaminants on reproductive success of marine invertebrates are required. A better mechanistic understanding of the potential interactions between contaminants with different modes of toxicity and ocean acidification is therefore important for our understanding of the broader implications of ocean acidification for marine invertebrates. As our Earth has entered a new phase in its history, experiencing a pace of climate change far greater than the planet has experienced over evolutionary timescales, predicting the outcomes of interactive effects of these stressors for marine biota remains a significant challenge.

Chapter 6: Conclusions and future directions

6.1   Conclusions

Sperm spawned from broadcast spawning animals such as echinoderms and polychaetes are single-celled free swimming gametes. Sperm motility is defined as the ability of a sperm to move/ swim towards an egg. The capacity to swim is therefore a critically important mechanism if fertilisation is to be successful. Fertilisation is a fusion of sperm and egg to produce an offspring. Most marine invertebrates are broadcast spawners meaning that fertilisation happens externally i.e. directly in contact with seawater. Clearly, there are costs and barriers for species that have evolved strategies for spawning and fertilising in this manner – even in a pristine ocean. For example, the timing of spawning must be simultaneous (within a given temporal window), and for many species spawning has been tuned to a lunar cycle or cued by pheromone production from individuals (Fox, 1924). Even after simultaneous spawning, fertilisation success can be low if the distance over which the sperm must travel is great or if water turbulence is too strong. Turbulence dilutes the concentration of sperm and eggs in the water column, dispersing them in all directions thereby making encounters less likely (Mead and Denny, 1995a). Aside from physical challenges, the gametes will also face biological challenges such as encountering the gametes of other species (risk of species incompatibility) or perhaps more commonly the risk of predation on the gametes. Predation is particularly relevant for eggs but sperm may also be lost to filter feeders such as mussels. If we then overlay these challenges to successful fertilisation with additional threats carried by pollution and global climate change, then the likelihood of successful fertilisation should, in theory, reduce even further.

This study was set out to explore the reproductive success of three ecologically important marine invertebrates, *Psammechinus miliaris, Asterias rubens* and *Arenicola marina* under conditions of environmental pollution and ocean acidification. Sperm motility and fertilisation success were used as key measures of fertility and likely reproductive success. The main findings of the thesis are chapter specific and were summarised within the chapters.

6.1.1 The effects of pharmaceuticals on sperm motility and fertilisation success of selected marine invertebrates.

This chapter addressed the effects on sperm motility and fertilisation success of exposure to pharmaceuticals or organic contaminants; diclofenac, ibuprofen, and sildenafil citrate. Pharmaceutical contamination of the oceans is reported worldwide. Pharmaceuticals were invented to improve the quality of human health; however, recent data indicates the possibility of negative impacts on different ecosystems. This study contributes further evidence that pharmaceuticals may adversely affect the reproductive success of marine invertebrates. Diclofenac is known to induce high mortality in vulture species, *Gyps bengalensis*, *G. indicus* and *G. tenuirostris* (Prakash et al., 2003, Gilbert et al., 2006). My data confirmed diclofenac reduced sperm motility and fertilisation success so indicating that diclofenac is not only toxic to birds but also marine invertebrate gametes. In an environmental context, diclofenac has been classified as very toxic according to EU Directive 93/67/EEC, and therefore under the EU Water Framework Directive (2013) (WFD 2013/39/EU), diclofenac is listed on the 'watch list' of emerging aquatic pollutants. My data also indicated that ibuprofen negatively affected the reproductive success of *P. miliaris* when exposed to sperm; however, oocytes were robust. Thus, ibuprofen would be classified as very toxic to sea urchin gametes. Exposure to sildenafil citrate showed opposite results, where increases in sperm motility and fertilisation success of *A. rubens* and *P. miliaris* were observed. Therefore, the effects of pharmaceuticals appear to have an aspect of species specificity. Pharmaceuticals are designed to have a specific mode of action, hence they have different pharmacokinetic and pharmacodynamic properties. These species specific results may be due to the factors of the drugs themselves rather than marked variation in the biology of the individual species (Fent et al., 2006). Worldwide action to enforce legislative approaches (e.g. the WFD) to control the concentration and occurance of pharmaceuticals and their metabolites in the environment are vital for future environmental management. My findings highlight the first toxicity data for diclofenac, ibuprofen and sildenafil citrate on marine invertebrate gametes. These data give us the opportunity to make a valuable contribution to the risk assessment of these pharmaceuticals. This will help to take proper action to reduce or prevent the occurrence of specific pharmaceuticals in the aquatic environment.

6.1.2 The effect of metals on sperm motility and fertilisation success of marine invertebrates.

In this chapter I examined the effects of copper and cadmium on reproductive success, concluding that both copper and cadmium reduced sperm motility and fertilisation success in a dose and time dependent manner. Copper proved more toxic than cadmium; this agrees with the scientific literature based on other bioassays. My finding was similar to Campbell et al. (2014), where it has been proven that copper negatively affected fertilisation success of *A. marina*. In terms of an environmental context, the species used in this study provides an effective and sensitive tool for detecting metal toxicity by detecting the reduction of sperm motility and fertilisation success. The present study also adds to the significant weight of evidence for use in regulatory frameworks and environmental quality standards by setting individual concentration limit in aquatic environments. However, in the natural environment metals are not present alone but in mixture. Therefore experimental data based on single compound exposure should be interpreted with an element of caution.

6.1.3 The effects of ocean acidification on sperm motility and fertilisation success of marine invertebrates.

For this chapter, the main objective was to examine the effects of an acidifying ocean on reproductive success. Sperm motility and fertilisation success was affected by elevated seawater pCO$_2$, in other words a reduction in the pH of seawater reduced sperm motility and fertilisation success. However, this chapter only focused on ocean acidification, this is a limitation as marine life is exposed to multiple climate change stressors; for example, global warming, UV radiation and salinity changes. Thus, experiments involving multiple stressors are advantageous for exploring the survivability of marine life. Moreover, it help to predict how marine populations and ecosystems may adapt and fare in a changing ocean.

6.1.4 The effect of nonylphenol and ocean acidification on sperm motility and fertilisation success of marine invertebrates.

This chapter examined whether nonylphenol affected sperm motility and fertilisation success both with and without the additional effects of ocean acidification. Nonylphenol reduced sperm motility and fertilisation success even at the lower concentration range e.g. $\geq 0.1\mu g/l$. The literature addressing the impacts of nonylphenol on marine invertebrate sperm is very limited; therefore, this study has made a noteworthy contribution to this field. However, what was curious was that the oocytes were substantially more robust compared with sperm. To all intents and purposes the oocytes were unaffected by nonylphenol at the concentrations and times used in this study. When nonylphenol and ocean acidification were combined my study verified that time of exposure, reduction of seawater pH and nonylphenol concentration has additive effects on the marine invertebrate gametes. This adds more evidence to the 'library of reproductive success research' that pollutants have additive effects in an acidic ocean and will pose significant threats to marine invertebrates.

6.2   Future recommendations

Nonylphenol is a known xeno-estrogen that tends to feminize organisms. To date, echinoderm and polychaete gametes are not known to have any estrogen receptors that would allow estrogenic compounds to bind to the cells of the species. However, in the present study, nonylphenol affected sperm motility and fertilisation success. Because of the substantial knowledge gap in this field, further investigation is needed on the combination of the endocrinology mechanism(s) and ecological relevance required to understand and examine these questions. Experiments should not stop at only looking at fertilisation success but should include life history and development stages. The results will allow us to understand how this chemical reacts and affects the organisms.

Current prospective risk assessments are based on exposure to a single pollutant or single environmental stressor − as commonly done in ecotoxicology investigations; however, as we live in a 'mixed' environment, there is no single isolated exposure to a pollutant or stressor. Further study is required to identify the interaction mechanisms; synergistically, additively, and antagonistically of metals or organics with ocean

acidification or other environmental stressors. There will always be combinations of many factor effects, but these can prove difficult to study. Hence, research that examines the combination between pollutants and other environmental factors on the reproductive success, early life and development of marine organisms should be carried out. Not to stop at that level, we also need to study the habitats, physiology, morphology and adaptation potential in order to understand the consequences for ecosystems. Furthermore, understanding the level of species specificity and capacity for adaptation or acclimatization, and the degree of response to changes in seawater conditions in multi-stressor studies is important for predicting the future composition and dynamics of marine communities under a 'winner-loser' scenario (Schlegel et al., 2012a).

My experimental design doesn't show the current situation of the natural environment. Almost all marine invertebrates reproduce using broadcast spawning. Once the gametes are release into the water column, they will eventually be diluted and face predators. As in study, only the optimum sperm concentration (2.5×10^6 sperm ml^{-1} and oocytes 250 oocytes) was applied to get the highest chances of motile sperm and percentage of fertilisation success. It would be enlightening to amend the experimental design by using different concentrations of sperm and oocytes or setting up 'alle effect design' as it will show the real situation of natural aquatic environment (Hallows et al., 2007).

The populations of many aquatic species are declining for reasons that often cannot be well explained. Therefore, approaches from diverse parties are crucial. Academic researchers, industry risk assessors and regulators need to be better integrated to assess current and future risks from pollutants. The scarce data on what happens to organisms when various environmental variables are altered makes reliable predictions of global change challenging to perform. In future, consideration of all variables affecting marine systems must be taken into account when legislating.

The monitoring of prioritised pharmaceuticals and their metabolites in coastal environment should be considered as complementary to prospective risk assessments. The Water Framework Directive (WFD; Directive 2000/60/EC) which involved 17 countries, has placed two hormones (17α-ethinyloestradiol and 17β-oestradiol) and diclofenac on the watch list for emerging pollutants. Thus, in a global context, it is

crucial to develop a monitoring suite of priority pharmaceuticals and transformation products that can be used in conjunction with biological assays to identify risk.

Pollutants enter the aquatic system via different routes, and one of the main routes is the release of treated effluent from sewage treatment plants. The need to increase knowledge about the fate of pollutants during sewage treatment is imperative for implementation of better removal techniques. It is also important to assess the presence and actual state of pollutants in several environments in different countries with a view to gaining empirical knowledge of contamination levels. Only with further available information will it be easier to improve existing legislation in order to protect our ecosystems from the threat posed by pollutants.

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