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A STUDY ON THE ENDOCRINE DISRUPTING EFFECT OF ESTROGENIC COMPOUNDS ON THE ZEBRA MUSSEL, (*DREISSENA POLYMORPHA*): AN *IN VIVO* / *IN VITRO* APPROACH

MICHAEL BRIAN QUINN

This thesis is submitted in fulfilment of requirements for the degree of Doctor of Philosophy to the University of Dublin, Trinity College

Department of Zoology

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Trinity College

August 2002

DECLARATION

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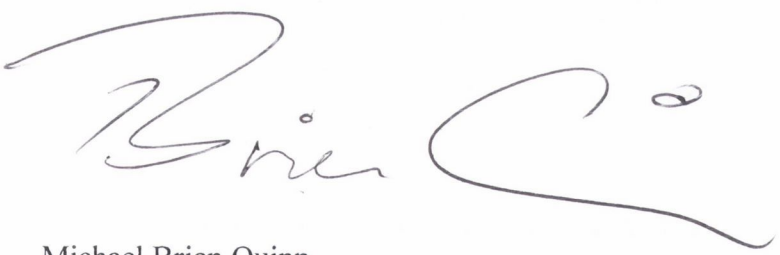

Michael Brian Quinn.

August, 2002

SUMMARY

Endocrine disruption (ED) by environmental estrogens has become a major research area in environmental toxicology after the discovery that chemicals entering the environment had a feminising effect on exposed male animals. This phenomenon has been well studied in vertebrates particularly in fish, where the production of vitellogenin (Vtg), an egg yolk precursor protein normally only produced by females, by males has been developed as a biomarker of exposure to environmental estrogens. Despite they're ecological importance, little work has been carried out in invertebrates. Through a series of experiments this ED effect was investigated on the zebra mussel in an attempt to develop a method of using the zebra mussel as a bioindicator of endocrine disruption by estrogenic compounds in the freshwater environment. Mussels were first exposed to the endocrine disrupting chemical nonylphenol (NP) at varying concentrations in the laboratory to establish the LC_{50} of this chemical for the zebra mussel. Mussels were then exposed to low concentrations (5 $\mu g\ l^{-1}$ and 500 $\mu g\ l^{-1}$) of NP during the gametogenic phase of their life cycle for 112 days in a controlled laboratory experiment to measure any ED effect. Mussels were also exposed (for 112 days) to tertiary treated effluent during an *In situ* experiment in a sewage treatment works (STWs). Using the indirect Alkali-label phosphate assay and gel electrophoresis a significant increase in the levels of Vitellin (Vn), the major protein found in oocytes of invertebrates synthesised from vitellogenin, was found in both male and female exposed mussels. Exposed mussels also showed much higher levels of cholesterol, the parent compound for sexual steroids in bivalves, both indicating that ED had occurred. Histological evidence of ED was also

found in the effluent exposed mussels with the discovery of a huge increase in the interstitial tissue in the male gonad, leading to a severe reduction in fecundity, that could possibly have an effect at the population level.

A cell culture method was developed for the *in vitro* culture of tissues from the zebra mussel. Once this technique was established cell suspensions were taken from the gill and digestive gland and were exposed to nonylphenol to calculate the *in vitro* LC_{50} concentration for comparison with the *in vivo* LC_{50}. In conclusion endocrine disruption did occur in the zebra mussel and this animal appears to be a suitable potential bioindicator for estrogenic compounds in the freshwater environment.

ACKNOWLEDGEMENTS

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And of course to my children, the zebra mussels without whom there would be no research!!!

p.s. a word of advice, don't even think about running a 112 day *in vivo* experiment!!!!

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GLOSSARY OF ABREVIATIONS

ALP	Alkali-labile phosphate
Bge	Cell line developed from the freshwater snail *Biomphalaria glabrata*
CHSE	Chinook salmon embryo cell line
DES	Diethylstilbestrol
DG	Digestive gland
E1	Estrone
E2	17β-Estradiol
ED	Endocrine disruption
EDC	Endocrine disrupting chemical
EE2	17α-ethinylestradiol
EM	Electron microscopy
ER	Estrogen receptor
FCS	Foetal calf serum
GC/MS	Gas chromatography / mass spectrometry
GE	Gel electrophoresis
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HPTLC	High-performance thin layer chromatography
HPLC	High pressure liquid chromatography
HSP	Heat shock protein
LC_{50}	Lethal concentration needed to kill 50% of the exposed population
LOEC	Lowest observable effect concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyl-tetra-zolium bromide
NOEC	No observable effect concentration
NP	Nonylphenol
NPEO	Nonylphenol ethoxylate
PBS	Phosphate buffer solution
PCNA	Proliferation cell nuclear antigen
SPE	Solid phase extraction

STW	Sewage treatment works
TIE	Toxicity identification and evaluation
TSK	Trimmed Spearman-Karber
TT	Toxicity threshold
Vn	Vitellin
Vtg	Vitellogenin
YES	Yeast estrogen screen

Chapter 1.

GENERAL INTRODUCTION

For a number of years there has been growing concern over changes in the health and fecundity of both humans and wildlife associated with the disruption of hormonal systems by endocrine disrupting chemicals (Colborn & Clement, 1992). Although many international committees have suggested various different definitions of endocrine disrupting chemicals (EDCs) the generally accepted definition is 'an endocrine disrupting chemical is an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations' (CSTEE, 1999). One of the reasons why so much research has been concentrated in this area is due to the possible effects of endocrine disruption on humans. A number of worrying trends in human reproductive health have been reported, such as declining sperm counts and increases in the incidence of testicular, prostate and breast cancer (Vines, 1995, Vos et al., 2000). One suggested explanation for these trends was increased exposure to certain environmental chemicals (Phillips & Harrison, 1999) although a direct link has yet to be established. However definite links have been established between exposure to EDCs and adverse affects on the health and reproductive ability of animals in the wild.

Endocrine disruption (ED) has been suspected of causing a large number of adverse effects on the health and reproductive ability of various animal species in the wild and has been linked to population decline. Numerous field and laboratory studies have revealed a number of ED effects at the population and individual level on a vast array of animals. The diversity of species affected includes mammals in which disturbed fertility of male Baltic grey and ringed seals (Bergman and Olsson, 1985; Haraguchi et al., 1992), Beluga whales (De Guise et al., 1994) and Florida panthers (Facemire et al., 1995) has been reported. In bird populations, feminisation and demasculinisation along with eggshell thinning resulted in reproductive failure (Fry, 1995) and abnormally developed male reproductive organs have been found in reptiles (Guillette et al., 1994). In male fish abnormal testicular development, intersex and the production of a female yolk protein (vitellogenin) have been reported (see below), while imposex (the imposition of the male sexual organs including a penis and vas deferens on the female reproductive organs) in marine molluscs has been proven to be a widespread problem (Oehlmann, et al., 1998). These are just some of the many examples of ED in wildlife available in the literature.

As research into this area has grown many new substances have been found that are thought to disrupt the endocrine system of animals, so that today thousands of compounds are either suspected or proven to have some form of endocrine disrupting effect. Due to the sheer number and chemical diversity of EDCs it would not be practical to categorise them based on their chemical properties or structure. Instead EDCs are categorised based on their biological effect. EDCs effect the endocrine and reproductive system using one of three mechanisms. They can either (i) mimic or alternatively antagonise (bind or block)

the effects of hormones by interacting with the hormone receptor (ii) alter the pattern of synthesis and metabolism of hormones or (iii) modify hormone receptor levels (Soto et al., 1995) (Figure 1.1). It can be seen from Figure 1.1 that endocrine disruption is a huge area of research with many different compounds having diverse biological effects on a potentially large number of species. However the group of EDCs that have been identified as being of greatest concern and that have subsequently been the focus of most research are the environmental estrogens that fall into the first category above. This study has focused on these chemicals, to assess the extent of their endocrine disrupting effect on the freshwater bivalve mollusc, *Dreissena polymorpha*.

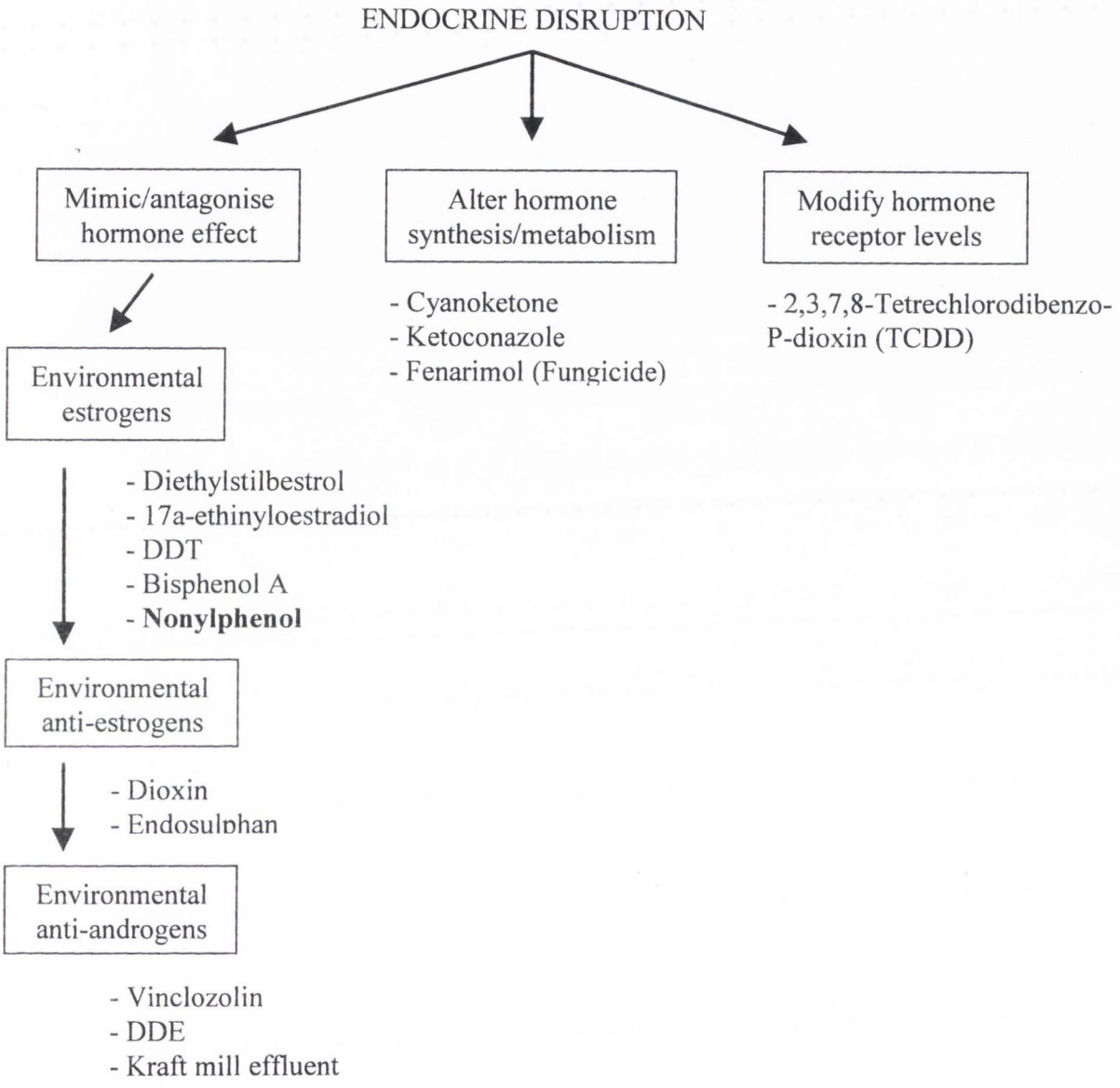


Figure 1.1. Diagram summarising the 3 mechanisms of action of endocrine disruption giving examples of endocrine disrupting chemicals.

Environmental estrogens

Although much research has been conducted on numerous endocrine disrupting substances, the group of compounds most studied are environmental estrogens otherwise known as estrogen mimicking chemicals, estrogen xenobiotics or xenoestrogens (Pinder & Pottinger, 1998). These are among the most effective EDCs, and are commonly found entering the environment via municipal and domestic effluent. They have been implicated in the disruption of reproductive and developmental processes in both wildlife and humans (Palmer & Selcer, 1996). As with other EDCs there is such a vast array of estrogen mimicking compounds that they are defined by their biological action rather than their chemical structure (Mclachlan et al., 1993). Estrogens are the hormones responsible for the primary and secondary sexual characteristics in females by promoting cell proliferation and hypertrophy of female secondary sex organs and inducing the synthesis of cell type-specific proteins (Hertz, 1985).

Environmental estrogens affect an animal's hormonal system by acting as either estrogen agonists that bind to the estrogen receptor in a cell and elicit a biological response or as estrogen antagonists (anti-estrogens/androgens), that bind to the receptor and block the response to the natural hormone (Dempsey & Costello, 1998). They usually contain a phenolic group in the molecule and as pollutants are characterised by being lipophilic, persistent and having the ability to bioaccumulate and bioconcentrate up the food chain, and are usually slow to breakdown and eliminate in the environment. They may also act cumulatively, with the effect of the total estrogen load being more relevant than the sum

of the effects of each individual contaminant. Table 1.1 lists some of the estrogenic compounds that are commonly found in the environment. As can be seen from this table most environmental estrogens were not designed to have an endocrine function and are inadvertently estrogenic and include pesticides and many common industrial chemicals. From this vast array of environmental estrogens only the estrogen agonists were studied in this report. More specifically the effect of the estrogen mimicking chemical nonylphenol, a by-product resulting from the breakdown of alkylphenol ethoxylates and natural and synthetic estrogens, both of which are commonly found in municipal sewage effluent.

Table 1.1. Environmental estrogenic compounds reported to have an endocrine disrupting effect and their sources.

Estrogenic compounds	**Source**	**Reference**
Natural estrogens: estrone & 17β-estradiol	Anthropogenic, domestic sewage, livestock	Turan 1995; Desbrow et al., 1998; Ternes et al., 1999.
Synthetic hormones: 17α-ethinlyestradiol, diethylstilbestrol	Domestic sewage, livestock	Purdom et al., 1994; Routledge et al., 1998.
Organochlorine pesticides & organotins	Used in pest control & anti-fouling	Soto et al., 1992; Colborn & Clements, 1992; Sharp et al., 1995.
Alkylphenol ethoxylates (APEs)	Industrial detergents, paints & pesticide formulations	Ahel, 1994; Jobling et al., 1996; Giesy et al., 2000.
Polychlorinated biphenols (PCBs), Bisphenol-A	Industrial chemicals	McKinney & Waller, 1994; Bergeron et al., 1994; Soto et al., 1995.
Dioxins & furans	Waste products from industry	Safe et al., 1991; Brinbaum, 1995.
Phytoestrogens & phytosterols	Naturally present in plants & trees	Institute of Environmental Health, 1995;
Some phthalates & plasticisers	Produced by industry for use in plastics	Jobling et al., 1995; Gaido et al., 1997.

Alkylphenol ethoxylates and Nonylphenol

Alkylphenol ethoxylates (APEs) are the second largest group of non-ionic surfactants in production and have been used for more than 40 years as surfactants in a variety of industrial processes and cleaning products (White et al., 1994). Annual world wide production of APE is estimated at over 300,000 tonnes, with nonylphenol ethoxylates (NPEs) taking approximately 80% of the market and octylpheol ehtoxylates (OPEs) accounting for the remaining 20% (White et al., 1994). It is estimated that annually between 200-500 tonnes of APE are used in Ireland, primarily for industrial detergent formulation and the manufacture of water based emulsion paints and pesticides (Dempsey & Costello, 1998).

APEs themselves are not actively estrogenic compounds, but their biodegradation products have estrogenic properties. As these surfactants are most commonly used in aqueous solutions, they are discharged into the marine and freshwater environment via industrial and municipal waste water treatments (Ahel et al., 1994). During conventional mechanical biological sewage treatment, APEs are microbially degraded to the mono- and diethoxylates (NP1EO and NP2EO) by the shortening of the hydrophilic ethoxylate chain. These products are further degraded in anaerobically stabilised sewage sludge to produce the increasingly lipophilic metabolites, 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP) (Figure 1.2). These compounds are resistant to further microbial degradation and tend to accumulate in sewage sludge and river sediments (Giger et al., 1987; Ahel et al., 1994). While parent APEs were efficiently eliminated during biological treatment in sewage

treatment works (STWs), the overall rate of degradation was limited due to the formation of these biorefactory metabolites. It has been estimated that about 60% of the APEs produced end up in the aquatic environment as NP, NP1EO and NP2EO (Kvestak et al., 1994; Jobling et al., 1996). It therefore appears that STWs generate compounds with a higher toxicity than the parent compounds.

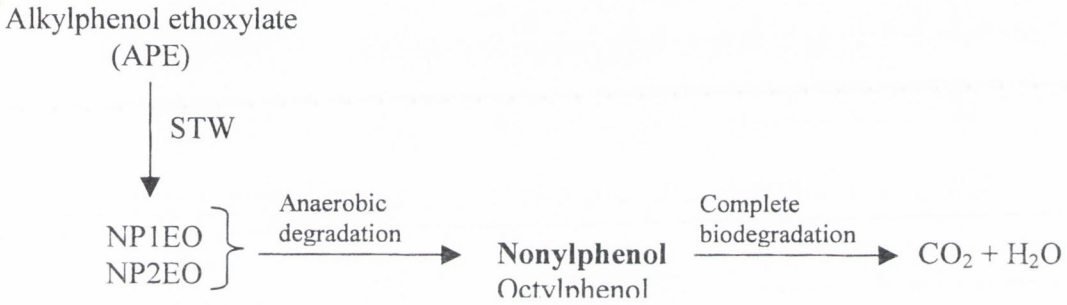


Figure 1.2. Biodegratation of APE to nonylphenol mono- and diethoxylate (NP1EO, NP2EO) in a sewage treatment Works (STW), which is anaerobically degraded into nonylphenol and octylphenol and is further completely biodegraded to carbon dioxide and water.

APE consist of a branched chain alkylphenol which has been reacted with ethylene oxide, producing an ethoxylate chain (Warhurst, 1994). When degraded to NP the ethoxylate chain is shortened increasing its hydrophobic nature (Figure 1.3). The phenol ring has lipophilic properties resulting in the compounds highly lipophilic character. It is this phenolic ring that binds to the estrogen receptor and exerts estrogenic actions *in vivo* and *in vitro* (Jobling & Sumpter, 1993; White et al., 1994).

4-Nonylphenol

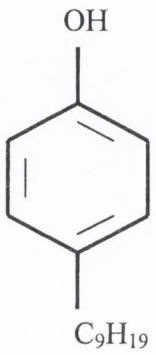


Figure 1.3. Chemical structure of 4-nonylphenol consisting of a phenol ring with an OH group attached at one end and an ethoxylate chain at the other.

The endocrine disrupting effects of these non-ionic surfactants in the aquatic environment is well documented in studies using numerous teleost fish species. Exposures to sewage discharges containing alkylphenols including NP resulted in the stimulation of the synthesis of the female egg yolk precursor protein vitellogenin (Vtg) in male fish and the inhibition of testicular growth (Jobling & Sumpter, 1993; Purdom et al., 1994; Lech et al., 1996; Jobling et al., 1996; Harries et al., 1997; Ashfield et al., 1998; Giesy et al., 2000). Vtg production is non-existent to low in males and immature females and is under hormonal regulation by estradiol in mature females (Cheek, et al., 2001). A significant elevation of Vtg levels in male fish is now a recognised highly specific biomarker of exposure to xenoestrogens. Direct exposure of fish to NP in laboratory tests has resulted in significant effects on the growth and ovosomatic index (Ashfield et al., 1998), fecundity (Giesy et al., 2000; Nichols and Pierens, 2000), Vtg induction (Lech et al., 1996; Nichols et al., 2000; Thorpe et al., 2000; Foran et al., 2000; Hemmer et al., 2001),

inhibition of testicular growth (Jobling et al., 1996; Kinneberg et al., 2000), intersex (Gray & Metcalfe, 1997) and a reduction in somatic growth and the gonadosomatic index (Thorpe et al., 2000; Kinneberg et al., 2000).

Although not as extensively studied, some effects of endocrine disruption resulting from NP exposure have been observed in invertebrates also. Baldwin et al., (1997) found an accumulation of exogenous testosterone in *Daphnia magna* exposed to NP. Reduced fecundity in adult female *daphnia* (Shurin & Dodson, 1997) and enhanced fertility, reduced growth and a significant increase in the length of antennae (secondary sexual characteristic) of exposed male *Corophium volutator* were also observed after NP exposure (Brown et al., 1999). Notable increases in the level of cyprid major protein occurred after exposure of barnacle larvae (*Balanus amphitrite*) to low concentrations of (1.0 $\mu g\ l^{-1}$) NP (Shimizu et al., 1996; Billinghurst et al., 2000). There is therefore abundant evidence that alkylphenolic compounds and NP in particular are capable of endocrine disruption.

Concentrations of hundreds of micrograms per litre have been recorded for nonylphenolic compounds found in both secondary treated effluents in STWs and in receiving waters in Europe and Canada (Ahel et al., 1994; Bennie, 1999). These concentrations are well above the threshold of response of fish (10-50 $\mu g\ l^{-1}$) (Gray & Metcalfe, 1997). However most studies of river waters generally report a concentration <10 $\mu g\ l^{-1}$ NP (Blackburn & Waldock, 1995; Lye et al., 1999; Korner et al., 2000). Significant bioconcentration of these lipophilic metabolites has been observed in various species of aquatic organisms

(Ekelund et al., 1990; Ahel et al., 1993). Bioconcentration factors (BCF) of 3400 and 230 were reported for mussels (*Mytilus edulis*) exposed directly to NP (Ekelund et al., 1990) and for caged mussels exposed to effluent (Granmo et al., 1989) respectively. Bioconcentation factors of 90-110, 2740-4120 and 1200-1300 were found in shrimp (*Crangon crangon*), mussel (*Mytilus edulis*) and fish (*Gasterosterus aculeatus*) respectively (Ekelund et al., 1990). Concentrations of around 400 $\mu g\ kg^{-1}$ fresh weight were found in caged *Mytilus edulis* at a wastewater effluent discharge site where the concentration in the ambient water was around 2 $\mu g\ l^{-1}$ (Granmo et al., 1989). In freshwater mussels (*Ellipitio complanata*) introduced to the St. Lawrence river non-ionic surfactants levels of up to 12.4 $\mu g\ g^{-1}$ were found using GC/MS analysis (Cathum & Sabik, 2001). Due to this highly lipophilic character NP has the ability to bioconcentrate up the food chain, possibly affecting animals not directly in contact with the chemical.

Natural and synthetic estrogens

Recently concern has shifted from the endocrine disrupting effect caused by the estrogen mimicking alkylphenolic compounds most notably NP, to the effect of natural and synthetic hormones. As with alkylphenolic compounds natural and synthetic estrogens enter the environment via treated effluent. Humans and all other animals produce estrogens. They are metabolised in the liver, transported in the blood and discharged with urine as conjugated metabolites. 17β-estradiol is the main product of these excretions. To gauge the quantities of these hormones being excreted, a woman's daily estrogen secretion is 24-100 μg/day depending on the phase of her menstrual cycle, which can rise

to 30 mg per day at the end of pregnancy. Livestock is an important source of these hormones as a pregnant mare secretes about 100 mg of estrogen per day (Turan, 1995). Synthetic hormones have been used in humans and animals for a number of years. 17α-ethynylestradiol, the principle estrogen in the contraceptive pill is believed to be the main estrogenic compound found in STW effluent (Purdom et al., 1994).

In STWs the microbial degradation of synthetic estrogens (17α-ethinylestradiol (EE2), diethylstilbestrol (DES)) releases the deconjugated biologically active form into the effluent (Routledge et al., 1998). This effluent is subsequently released into the marine and freshwater aquatic environments. Although these substances are found in lower quantities (ng l^{-1} range) than alkylphenolic compounds they are much more effective estrogens. Similar biological effects are found for exposure to these chemicals as to NP. Effects on male fish exposed to these hormones include increased Vtg levels (Anderson et al., 1996; Kinnberg et al., 2000) changes in testis morphology (Chang et al., 1995; Kinnberg et al., 2000) and intersex (Metcalfe et al., 2001). Effects on invertebrates include reduced moulting frequency in juvenile *Daphnia magna*, decreased fecundity of second generation and altered steroid hormone metabolic capabilities in adults exposed to DES (Baldwin et al., 1995). In another study female *Daphnia* exposed to DES stimulated the development of the abdominal process, a secondary sexual characteristic (Olmstead & LeBlanc, 2000).

The natural endogenous estrogens 17β-estradiol, and estrone and the synthetic estrogens EE2, DES and mestranol have all been detected in STW effluents at concentrations

between 1-70 ng l^{-1} (Desbrow et al., 1998; Ternes et al., 1999). In one study DES has been found in effluent at concentrations up to 40,007 μg l^{-1} (Hansen et al., 1998). These concentrations are within the thresholds of response for fish species as the LOEL for EE2 and estrone are 1 and 10 ng l^{-1} respectively (Metcalfe et al., 2001). In another study concentrations of EE2 as low as 0.1 ng l^{-1} were shown to cause a significant rise in fish plasma Vtg levels (Sheahan et al., 1992). Estrogenic hormones are much more potent estrogens than NP and NPEO mixtures by a factor of 10^3 to 10^6 (Metcalfe et al., 2001). In fact in a recent study levels of phenols found in effluent only accounted for 1.4% to 4.3% of the total estradiol equivalent concentrations found in the effluent (Korner et al., 2000). Therefore natural and synthetic hormones appear to be the most estrogenically active chemicals in STW effluent.

Effluent from both industrial and municipal STWs contains a mixture of xenoestrogens that when released into the environment were shown to produce a synergistic positive effect (Dempsey & Costello, 1998). Using the E-SCREEN test (MCF-7 human breast cancer cells) Soto et al., (1994) revealed that when mixed together xenoestrogens induced estrogenic responses at concentrations lower than those required when each compound was administered alone. This was also shown by Arnold et al., (1996) using the recombinant yeast assay or YES assay, involving the yeast cell *Saccharomyces cerevisiae* encoded with a human estrogen receptor. Therefore measuring the total estrogenic burden of the effluent may be more meaningful than assessing the exposure by measuring the levels of each known xenoestrogen (Soto et al., 1994). It is for this reason that whole

effluent toxicity exposure to treated effluent was established in this study as well as an *in vivo* laboratory exposure to NP.

Effects on invertebrates

As can be seen above the vast majority of work on endocrine disruption has concentrated on the effects on the health and reproductive status of vertebrates, with most work on xenoestrogens concentrating on fish. Although a few examples of the effects of ED on invertebrates have been given above, these mostly concentrated on one species (*Daphnia magna*). Very little work has been carried out to look at the effects of EDCs on invertebrates. Practically all the reported incidences of ED in invertebrates involve the induction of imposex in gastropod molluscs (*Nucella lapillus*) after exposure to organotin compounds (for review see Matthiessen & Gibbs, 1998). There has been quite a lot of controversy regarding what constitutes an endocrine disrupting effect in invertebrates. Although different effects have been observed after exposure to EDCs (see above) these could be normal toxicological responses, not necessarily linked to any endocrine effects.

In mammals and birds sex is defined by genes located on the sex chromosome. But in lower vertebrates and invertebrates the mechanisms of sex determination and differentiation are less defined and less rigid, resulting in invertebrates being potentially more susceptible to influence by environmental factors, such as xenogenous substances with hormonal activity (CSTEE, 1999). EDCs could therefore have a larger effect on invertebrates, affecting entire populations, that could in turn be detrimental to an entire

ecosystem. Due to their abundance and diversity and their importance in ecotoxicological testing and monitoring of environmental conditions, the need to investigate ED in invertebrates has been highlighted and has been the focus of several major international meetings (see DeFur et al., 1999).

Although relatively little is know about the endocrine system in invertebrates significant evidence exists to indicate that invertebrate protostomes (including molluscs) make use of vertebrate-type sex steroids for reproductive maturation and function, and possess an endocrine pathway that is theoretically susceptible to disruption (DeFur et al., 1999). This is described in detail in Chapter 3. The process of vitellogenin synthesis in bivalves appears to be susceptible to ED in a manner similar to that of fish, by increasing levels of vitellogenin (Vtg) or vitellin (Vn)-like proteins (Li et al., 1998; Blaise et al., 1999; Gagne et al., 2001b) possibly making them a suitable indicator species for ED.

Zebra mussels

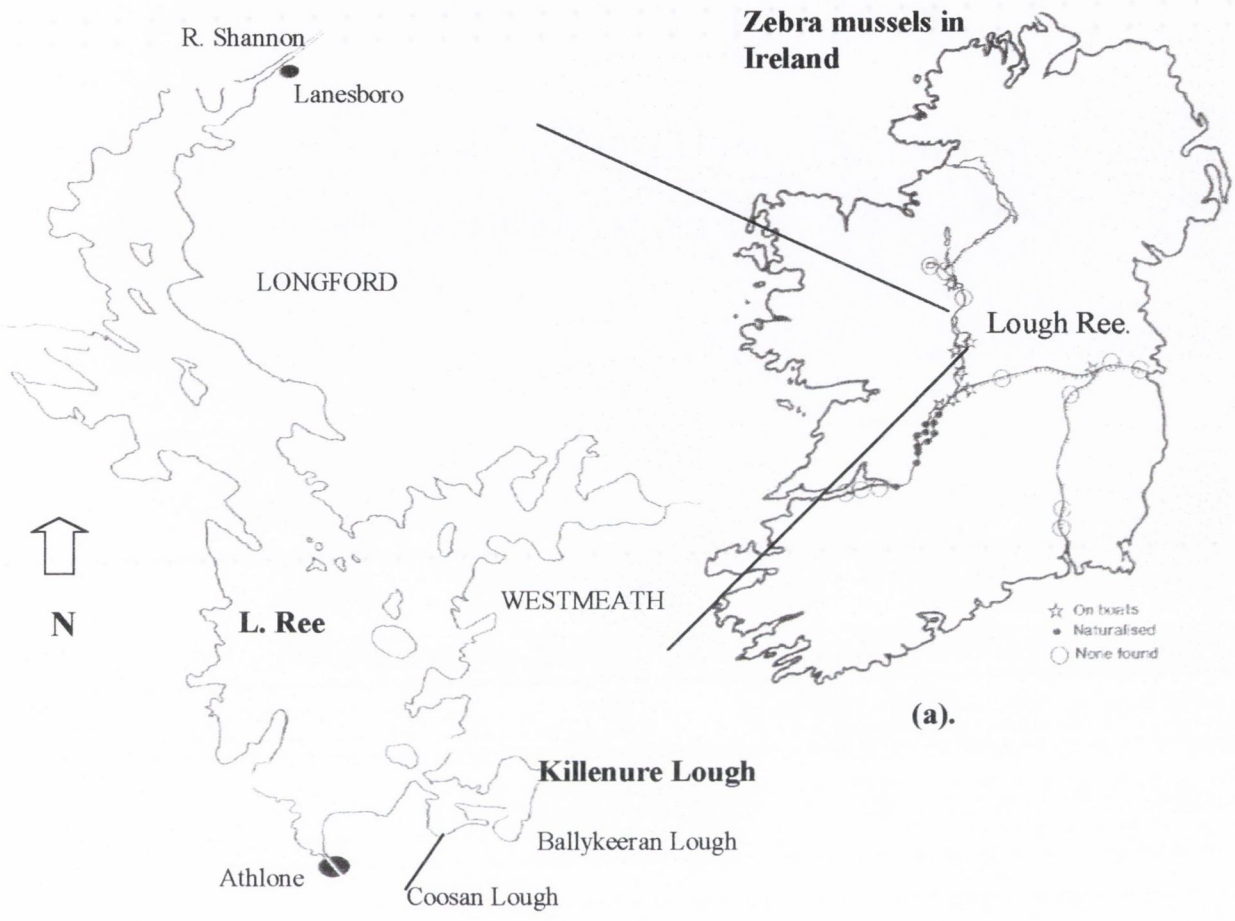
The zebra mussel *Dreissena polymorpha* is a small (< 5 cm), freshwater bivalve mollusc originating from the drainage system surrounding the Black, Caspian and Aral seas and is easily identified by its dark and light coloured patterning (Figure 1.4). Its dispersal from its native area was aided by the development of the European canal system in the late 1700's early 1800's and it has now become one of the most dominant species in many European rivers and lakes (Borcherding, 1991). More recently the zebra mussel has become established throughout North America in 1985/86 having entered the Great lakes

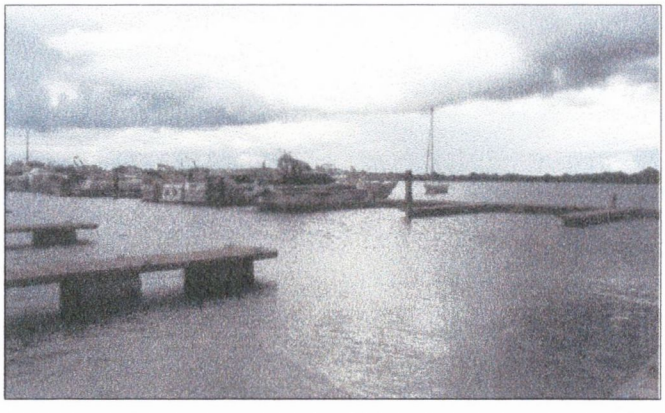
Derg in 1997. Since then they have spread throughout the Shannon waterway from Limerick docks up to Carrick-on-Shannon (Figure 1.5). This figure also shows the location of the zebra mussel population used throughout the study. All mussels used throughout the experiments were taken from the same population located at a depth of above 1 m on 2 floating bouys in Quigley's marina in Killenure Lough, one of 3 inner lakes in Lough Ree, county Westmeath, Ireland.

(a).

(b).

Figure 1.4. The zebra mussel *Dreissena polymorpha.* **(a).** Photograph showing the distinctive dark and light bands on the bivalve shell responsible for the mussel's common name 'zebra mussel'. **(B).** The two size classes of mussel used in the experiments, 5-15 mm and 15–30 mm.





(C).

Figure 1.5. (a). A map showing the distribution of zebra mussels in Ireland in 1997 (http://www.marine.ie/frc/exotic/Zebra/technic.html-ssi). **(b).** A map of Lough Ree with the inner lakes where the sampling site was located. **(c).** The sample site in Quigley's marine in Glassan where mussels were scraped from floating buoys.

Although generally regarded as a pest species biofouling hard substrates in huge densities zebra mussels have been used to some advantage as a bioindicator species for pollution. Their high filtration rate, ability to accumulate and bioconcentrate toxicants, widespread distribution and abundance and their primarily stationary, endobenthic life-cycle has made them a valuable bioindicator species (Neumann & Jenner, 1992). They have been proposed as the freshwater counterpart of the blue mussel *Mytilus edulis*, in mussel watch programmes for freshwater environments (Busch & Schuchardt, 1991; Mersch et al., 1992). Zebra mussels have been used in both passive and active biomonitoring (Bowmer et al., 1991) for heavy metals (Busch and Schuchardt, 1991; Kraak et al., 1991; Mersch et al., 1992; Camusso et al., 1994) and organic contaminants (Comba et al., 1996: Perret et al., 1996; Dauberschmidt et al., 1997) and are regarded as sentinel organisms for monitoring environmental quality.

Zebra mussels are also suitable as possible bioindicators for ED as they are oviparous, sexually dimorphic and their biology and reproductive cycles have been well studied and reported. Although cases of hermaphroditism have been reported in the literature not one hermaphroditic animal was observed from the present sample site (n= ~600), nor in other studies involving zebra mussels from the Shannon waterway (n= ~900) (O'Toole, 2002). Like most bivalves zebra mussels also produce sexual steroids to control their sexual differentiation and development (Reis-Henriques et al.,1990) and are large enough for the measurement of these steroids. Being stationary, endobenthic animals that filter very high volumes of water including suspended solids, zebra mussels are particularly at risk to chemicals found in the aquatic environment (Gagne et al., 2001a). Due to their biofouling

ability zebra mussels are also of economic and ecological importance. They have a very widespread distribution in Europe and North America and have the potential for use in both field and laboratory studies.

The primary criteria for the selection of an indicator species for endocrine disruption has been outlined (Weybridge, 1996) and included a widespread distribution, ease of field sampling, sensitivity to contaminants, location in an environmental compartment in which the EDC may concentrate, ability to maintain the species under laboratory conditions and possession of a well characterised endocrine system. Although not meeting all, the zebra mussel (*Dreissena polymorpha*) meets most of these criteria and its suitability as a bioindicator species for endocrine disruption using vitellogenin-like proteins as a biomarker of exposure to xenoestrogens was investigated.

Biomarkers of endocrine disruption

Biomarkers are defined as biological responses that can be measured in tissue samples, body fluids or at the level of the whole organism, which signal exposure to or adverse effects of anthropogenic chemicals and radiations (Depledge &Billinghurst, 1999) and have been identified as an extremely useful means of detecting endocrine disruption *in situ* (Depledge et al., 1999). The most useful biomarkers for ED are biochemical (e.g. vitellogenin) or histopathological (development of eggs by males) as are commonly used in fish. At present the only biomarker for ED in invertebrates is the development of imposex and there is no biomarker for exposure to xenoestrogens. Through a series of

this study investigated the use of vitellogenin-like proteins as a biomarker of ED in the zebra mussel *Dreissena polymorpha* and the possible use of the zebra mussels as a bioindicator for endocrine disruption.

Zebra mussels were exposed *in vivo* to the xenoestrogen NP in a series of controlled laboratory experiments. The first of these was to measure the toxicity of NP to the zebra mussel, and the next was to measure the possible ED effect on the mussel. This ED effect was also measured in mussels exposed to tertiary treated effluent in an *in situ* exposure at a sewage treatment works. ED in the zebra mussels was measured by looking at elevated levels of vitellin-like proteins and the sterol cholesterol in the exposed animals and by histological examination of the gonads. An *in vitro* method of culturing tissues of the zebra mussel was also established and the toxicity of NP to the cells of various tissues was investigated.

Objectives of study

The aim of the experiments described in this thesis were:

- Primarily to develop the zebra mussel (*Dreissena polymorpha*) as a bioindicator of endocrine disruption in the freshwater environment through a series of *in vivo* and *in situ* experiments and to establish the extent to which bivalves are susceptible to endocrine disruption.
- To investigate for the first time whether exposure to the xenoestrogen NP under laboratory conditions can induce an endocrine disrupting effect in the zebra mussel.
- To investigate for the first time whether exposure to tertiary treated effluent can induce endocrine disruption in the zebra mussel.
- To use vitellin-like proteins and the sterol cholesterol as biomarkers of ED in the zebra mussel.
- To investigate at a histological level the effects of exposure to the xenoestrogen NP and tertiary treated effluent on the zebra mussel.
- To establish a cell culture method for the maintenance of tissues from the zebra mussel *in vitro* for exposure to the endocrine disrupting chemical nonylphenol.
- To investigate the potential for the development of an *in vitro* technique for the quick identification of xenoestrogens using oestrogen receptors in cultured zebra mussel gill.
- To find and compare the LC_{50} of nonylphenol for the zebra mussel both *in vivo* and *in vitro*.
- To develop the zebra mussel as a sentinal species for ED in the environment.

Chapter 2.

ACUTE AND CHRONIC TOXICITY OF NONYLPHENOL TO THE ZEBRA MUSSEL (*DREISSENA POLYMORPHA*).

INTRODUCTION

4-n-Nonylphenol is one of a group of chemicals known as estrogen mimicking compounds or xenoestrogens. As previously mentioned (Chapter 1) nonylphenol (NP) and nonylphenol ethoxylates (NPEO) are formed by the breakdown of alkylphenol ethoxylates, non-ionic surfactants used in the formulation of detergents and emulsion paints and are commonly released into the environment in areas polluted by sewage effluent (Ahel et al., 1994; White et al., 1994). The endocrine disrupting effects of these chemicals has been well documented in numerous vertebrate species including fish, alligators and birds (for review see Vos et al., 2000). NP is an estrogen mimicking chemical, that works as an estrogen agonist, binding to the estrogen receptor and eliciting a biological response. Exposure to sewage discharges containing alkylphenols including NP has resulted in stimulation of synthesis of the female yolk protein vitellogenin (Vtg), in male rainbow trout and an inhibition of testicular growth (Purdom et al., 1994; Jobling et al., 1996; Harries et al., 1997). A significant elevation of Vtg levels in fish is now a recognised biomarker of exposure to xeno-oestrogens.

One of the methods of measuring the acute toxicity of a substance to an organism is to measure the lethal concentration needed to kill 50% of the exposed population, the LC_{50}. Most research regarding the toxicity of NP has focused on vertebrates, particularly fish. Generally LC_{50} values reported for fish vary between 0.13 and 0.9 mg l^{-1} (Table 2.1). Lethal dose rates of NP have been calculated for numerous invertebrate species, and work is also beginning to be carried out to measure other effects, such as possible endocrine disrupting effects on invertebrates. Fish seemed to be more susceptible to the lethal affects of NP than crustaceans, while bivalves were considerably more resistant with recorded LC_{50} values of between 0.5 to 5 mg l^{-1} NP (Table 2.1). Although the LC_{50} values (3.0 mg l^{-1}, 0.5 mg l^{-1} and 0.14 mg l^{-1} after 96h 15d and 35d exposure respectively) for exposure of *Mytilus edulis* to NP have been reported (Granmo et al., 1989), they have yet to be calculated for the zebra mussel.

In previous chronic studies the lowest observed effect concentration (LOEC) for NP was in the range of 20-60 μg l^{-1} for fish, bivalves and crustaceans (Bechmann, 1999) and estimated to be 76 μg l^{-1} for zooplankton communities (O'Halloran et al., 1999). The no observed effect concentration (NOEC) for bivalves was reported to be 23 μg l^{-1} NP (Schmude et al., 1999). These concentrations were calculated for the zebra mussel using chronic and acute parameters (mortality, attachment and siphon extension).

Proliferating Cell Nuclear Antigen (PCNA) is a highly conserved molecule, occurring throughout all eukaryotic cells including plants (Hall & Levison, 1990). PCNA, also termed polymerase δ auxiliary protein (Fairman, 1990) is essential for the synthesis of

DNA during the S-phase of cell division and is thus an absolute requirement for cellular proliferation (Gray et al., 1992). Its immunoreactivity is localised to sites of DNA synthesis (Bravo & McDonald-Bravo, 1987). Although the antibody employed (PC-10) and the immunohistochemical method used were developed for use with mammalian tissues, cross-reactivity with the zebra mussel was investigated using immunohistochemical techniques.

Induction of the heat shock protein, HSP 70 by exposure to NP was also investigated by this method. These proteins are induced to compensate for the physiological impact of an array of environmental stresses and appear to be part of a general stress response, universally conserved in organisms of all phyla (Morimoto et al., 1990). HSP 70 synthesis has been induced by exposure to heavy metals, anoxia, heat and many inorganic and organic chemicals (Nagao et al., 1990; Bierkens, 2000; Ait-Aissa et al., 2000).

This is the first in a series of experiments to expose the zebra mussel to the well-known endocrine disrupting chemical NP. This preliminary experiment was undertaken to find the LC_{50} of NP, in order to find a concentration for use in further experiments that would be high enough to possibly induce an ED effect on the mussels but low enough to allow a long term exposure with minimal mortality.

Table 2.1. LC_{50} values of various aquatic organisms exposed to NP.

Test organism	Common name	LC_{50} (mg l^{-1})	Exposure (h)	Reference
Fish				
Lepomis macrochirus	Bluegill sunfish	0.65	96	Kline et al., 1996
Pimephales promelas R.	Fathead minnow	0.77	96	Kline et al., 1996
Salmo salar (juvenile)	Salmon	0.9	96	McLeese et al., 1980
Gadus morhua L.	Cod	3.0	96	Swedmark et al., 1971
		0.1	369	Swedmark et al., 1971
Invertebrates				
Crangon septemspinosa	Shrimp	0.4	96	McLeese et al., 1980
Homarus americanus	Lobster	0.2	96	McLeese et al., 1980
Corophium volutator	Amphipod	1.67	96	Brown et al., 1999
		0.262	240	Brown et al., 1999
Daphnia magna	Freshwater flea	0.3	24	Comber et al., 1993
		0.19	48	Comber et al., 1993
Tisbe battagliai	Marine copepod	0.03-0.06	1272 (53 d)	Bechmann, 1999
Mytilus edulis	Blue mussel	3.0	96	Granmo, et al., 1989
		0.5	360	Granmo, et al., 1989
Anadonta cataractae	Freshwater mussel	5.0	144	McLeese et al., 1980
Mya arenaria	Clam	>1.0	360	McLeese et al., 1980

MATERIALS AND METHODS

Test compound

The compound under investigation was 4-n-nonylphenol (98^{+}% purity, Lancaster Synthesis Ltd., England) and was tested at the concentrations of 0.1, 1.0, 5.0, 10.0 and 100.0 mg l^{-1}. Stock solutions were prepared by dissolving the relevant quantity of NP into a known volume of solvent and were stored in the dark at room temperature. Having a very low solubility in water (0.007 g l^{-1} at 25°C), NP needs to be dissolved in a suitable solvent. Acetone was the solvent of choice in a number of experiments (Comber et al., 1993; Billinghurst et al., 1998; Bechmann, 1999) and was used here. Ethanol has also been used (Giesy et al., 2000; Foran et al., 2000) but was found to cause extensive bacterial growth in exposure tanks (Granmo et al., 1989; Brown et al., 1999). An acetone concentration of 1 ml l^{-1} was decided upon, keeping within the no observable effect limits found in other studies (Granmo et al., 1989; Bechmann, 1999: Ekelund et al., 1990). A solvent control tank was included in the experiment to measure any solvent effects.

NP is photodegraded by light and being hydrophobic is absorbed out of solution. For these reasons attempts were made to analyse the actual concentration of the NP in water samples taken from the exposure tanks using the GC/MS method described by Billinghurst et al. (1998). However these attempts were unsuccessful and nominal concentrations are quoted hereafter. Previous work showed that the definitive

of the nominal concentrations after 24 h and 60-70% after 48 h (Comber et al., 1993; Billinghurst et al., 1998; Bechmann, 1999).

Test animal

Zebra mussels were collected from the sampling site (Chapter 1) on June 4th 2000. The water temperature at time of collection was 15°C. The mussels were transported back to the laboratory, graded by size (5-15 mm and 15-30 mm) and placed onto the underside of ceramic tiles held in large 40 litre storage tanks filled with de-chlorinated, aerated tap water at a temperature of 15°C and a 12 hour light/dark cycle. They were fed a commercial phytoplankton feed 'Phytoplex' and were allowed to acclimatise for 7 days and attach onto the tiles (Figure 2.1). The water in these tanks was changed every 3 days.

Figure 2.1. Zebra mussels graded by size and attached onto a tile ready to be placed into the glass tank for exposure to NP.

Experimental design

The experiment took place in a laboratory with a controlled light source giving a photoperiod of 12 h light:dark and a relatively constant temperature (15-18°C) (Figure 2.2). 5 l of de-chlorinated tap water was added to 10 litre glass aquarium tanks (acid washed) and aerated using a Rena Air 300 pump. The water was de-chlorinated by passing air through it for 24 h at room temperature (16 ± 1°C). The NP stock solution (1 ml) was slowly pipetted into each tank (Table 2.2) while continuously stirring with a glass rod for 2 minutes, with the controls received 1m of solvent (acetone) or water. Contamination was prevented by working from the lowest NP concentration up to the highest and by washing all equipment with acetone after use. Once the solvent had been mixed into the water the tiles with the zebra mussels attached were added. The number of mussels attached to each tile was recorded and tiles were paired up to give a total of around 100 mussels of varying sizes (5 - 30 mm) per tank (Table 2.2). In the tanks containing 10 mg l^{-1} and 100 mg l^{-1} the NP appeared to have precipitated out of solution and coagulated into white particles in the water, resulting in the water turning cloudy. Increasing the amount of stirring in these tanks did not succeed in further dissolving the solution. This was a semi-static exposure with the water in the tanks changed 3 times a week and fresh chemical added after each water change. Waste water was poured through a filter of fine particulate carbon to remove the nonylphenol, which was later disposed of by incineration. Mussels were fed 1-2 ml of phytoplankton food (Phytoplex, Kent Marine, USA) after each water change.

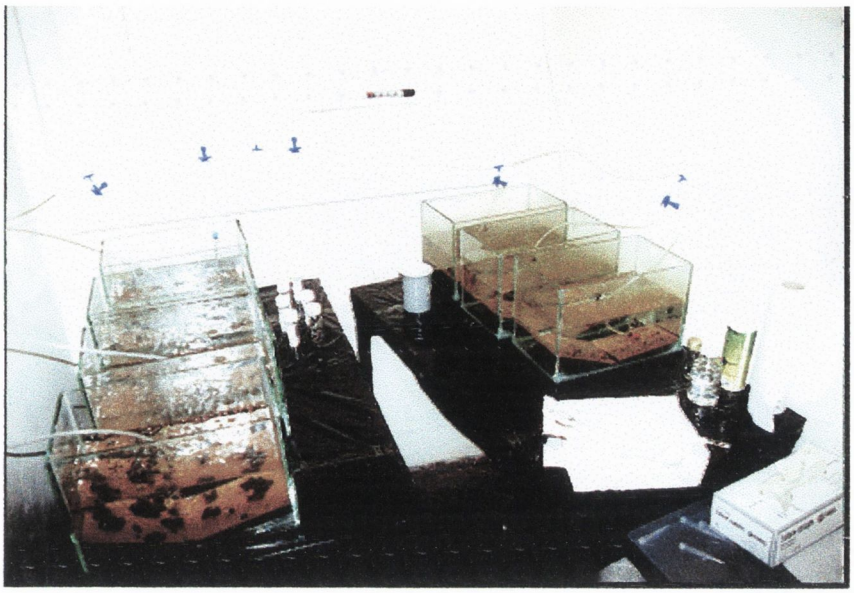


Figure 2.2. Experimental design for *in vivo* toxicity exposure of zebra mussels to NP. 7 glass tanks were used in a room with a controlled temperature and light regime.

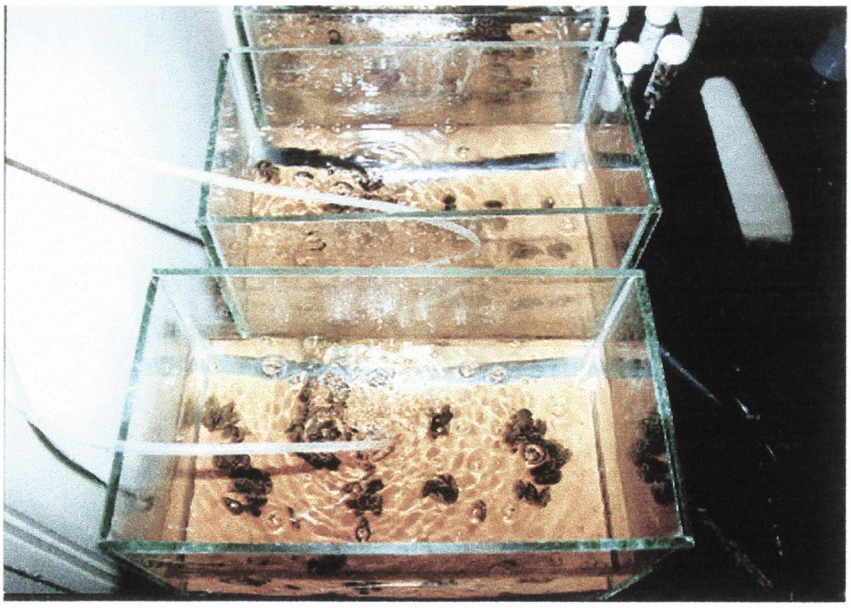


Figure 2.3. Tanks used for the *in vivo* toxicity test showing zebra mussels attached to tiles placed in glass aquarium tanks and exposed to NP dissolved in solution.

Table 2.2. Number of zebra mussels in each tank exposed to varying concentrations of NP.

Tank No.	NP Concentration	Tile No.	Mussels per tile	Total mussels
1	Control	7	34	
		10	63	97
2	Acetone control	4	55	
		6	45	100
3	0.1 mg l^{-1}	3	47	
		13	47	94
4	1 mg l^{-1}	9	57	
		12	43	100
5	5 mg l^{-1}	8	41	
		11	57	98
6	10 mg l^{-1}	1	63	
		5	41	104
7	100 mg l^{-1}	2	49	
		14	48	97

A fine film of algae/bacteria coated all the surfaces in the tank, including the mussels themselves, resulting in reduced filtration. To prevent this the walls of the tanks were wiped with a paper towel and rinsed with clean water every 9/10 days. A small toothbrush was used to clean the tiles around the mussels. The mussels themselves were gently brushed to remove the build up of algae around their siphons. Some mussels were dislodged during this process but their reattachment was observed.

The experiment lasted 50 days, from the 12th of June until the 1st of August 2000. Samples of live mussels were taken from each tank at regular intervals (Table 2.3). Mussel length and weight were measured before dissection. The soft body part was dissected and placed intact in 10% formalin (Appendix 2.1) for 24-35 hours, processed through graded alcohol and xylene and embedded in paraffin wax. The larger size class of mussels (>15 mm) were dissected posterior-anteriorly along the mid-region, to expose the visceral sac containing the gonad and digestive gland. Tissue sections 5 μm thick were cut from these blocks and used in immunohistochemical studies and for histological analysis.

Table 2.3. The sample number of zebra mussels removed from each tank after each exposure period and preserved in formalin for analysis.

Date	Exposure (days)	Mussels removed & preserved in formalin (from each tank)
13 June	1 d (24h)	4
14 June	2 d (48h)	4
16 June	4 d (96h)	4
20 June	8 d (192h)	2
28 June	16 d (384h)	2
6 July	24 d (576h)	2
13 July	31 d (755h)	2
1 August	50 d (1200h)	3

Biological functions studied

The tanks were inspected daily and the dead mussels were removed. Mortality was based on complete inactivation of mantle-edge muscles, cessation of function of the adductor muscles (no response to prodding) and cessation of siphon retraction (Swedmark et al., 1971). Gaping of valves (not fully closed after prodding) was also measured and was often observed. These dead mussels were sexed using the squash technique by looking at the gonads under x 200 and x 400 magnification using a light microscope.

Sub-lethal chronic effects were also measured. Byssal activity offers a good indicator of the mussel health, measured by the amount of mussels attached to any substrate within the tank (Swedmark, et al., 1971). Re-attachment after dislodgement also offers an easily observable indicator as to the animal's state of health (Swedmark et al., 1971; Granmo et al., 1989). Four mussels of varying size from each tank were marked with a small amount of Tippex on their shell. These mussels were dislodged from the tiles, their byssal threads were removed and their re-attachment measured after 24 hours. The filtration activity was measured by counting the number of mussels per tank with their siphons extended beyond the bivalve shells. This was done with the minimum of disturbance to ensure the mussels were filtering as normally as possible.

Immunohistochemistry

PCNA

The effect of NP exposure on cell proliferation was investigated using immunohistochemical analysis, by looking at the expression of the monoclonal mouse Anti-Proliferating Cell Nuclear Antigen antibody (PCNA) (Clone PC-10, DAKO, Cambridge, UK). The immunogen used was rat PCNA made in the protein A expression vector pR1T2T. This antibody reacts with all vertebrates and invertebrate species investigated so far.

Immunoreactivity was investigated using the avidin-biotin complex (ABC) method of immunoperoxidase staining, using a mouse monoclonal vectastain Elite ABC kit (Vectastain Corporation Burlingame, USA). This method is based on the standard indirect immunoperoxidase technique. Sections 5 μm thick were cut from the paraffin wax preserved animals, mounted on silinized slides and dried in the oven at 60°C for 1 hour. Sections were deparaffinized through xylene and graded alcohol and rehydrated. In order to retrieve the anitgens masked by formaldehyde fixation slides were placed in citrate buffer (1.2 g citric acid in 1000 ml adjusted to pH 6 with 2M NaOH) and underwent the following treatments to enhance immunostaining:

20 minutes in microwave (800 Watt) followed by 20 minutes in heated buffer.

15 minutes in microwave (800 Watt) followed by 15 minutes in heated buffer.

10 minutes in microwave (800 Watt) followed by 10 minutes in heated buffer.

Overnight at 80°C in buffer.

60 seconds in pressure cooker at high pressure followed 10 minutes in heated buffer.

The protocol was as follows:

All staining procedures took place in a moisture chamber. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (Sigma, St. Louis, USA) in methanol (BDH, Poole, England) for 3-5 minutes. Slides were rinsed with PBS (Appendix 2.2) and horse blocking serum (Vectastain ABC kit) was added for 5-10 minutes. Excess serum was removed and the primary antibody (PC-10) was applied to the slides for 60 minutes. Concentrations of the antibody used were 1:100; 1:200; 1:400; 1:800; 1:1600; 1:3200. Following a PBS rinse the biotinylated secondary antibody solution (Vectastain ABC kit) was added for 10 minutes. Slides were rinsed with PBS and the avidin DH: biotinylated horseradish peroxidase H complex (Vectastain ABC reagent) was added for 10 minutes. Following a PBS rinse 3,3'-diaminobenzidine tetrahydrochloride (DAB), the peroxidase substrate (0.625 mg ml^{-1} diaminobenzaldehyde (Sigma, St. Louis, USA) in PBS with 10 µl of 30% H_2O_2) used as a chromagen was added to the slides for 10 minutes and washed off with tap water. The slides were counterstained with filtered Mayers haematoxylin (Sigma, St. Louis, USA) (30 seconds) and blued in running hot water. Slides were dehydrated through graded alcohols, cleared in xylene and coverslipped with Distrene Plasticiser Xylene (DPX, BDH, Poole, England). Brown staining was indicative of a positive reaction. Negative controls were sections in which all the reagents were added except the primary antibody. Positive controls used were normal human tonsil sections. Both were included in all immunostaining runs.

HSP 70

The polyclonal Rabbit Anti-Heat Shock Protein 70 antibody (HSP 70) (DAKO, Cambridge, U.K.) was used to detect and quantify the expression of the 70 kDa heat shock protein in the paraffin preserved zebra mussels exposed to NP. The iummunogen used was purified heat shock protein (DnaK) from *E. coli*, found to cross react with epithelial cells from many mammalian tissues (DAKO, Cambridge, U.K.).

To visualise the heat shock protein the avidin-biotin complex method of immunoperoxidase staining was used, employing the rabbit polyclonal vectastain ABC kit (Vectastain Corporation Burlingame, USA). The method and protocol used were the same as the PCNA protocol, substituting goat blocking serum for the horse blocking serum used for the monoclonal kit and the primary antibody for HSP 70. The slides were also subjected to the same unmasking techniques as described above.

Expression of HSP 70 was found in the control mussels. It was therefore necessary to carry out a range of antibody dilutions in order to find the optimum concentration that would not give a positive reaction in the control mussel but would show increased expression in the NP exposed mussels. The recommended dilution for the Anti-HSP 70 in mammalian tissues is 1:250-500 for immunohistochemical studies (DAKO, Cambridge, U.K.). A dilution range of 1:100, 1:200, 1:400, 1:800, 1:1200, 1:1600, 1:1800, 1:2000, 1:2400, 1:2800, 1:3000, 1:3200 was tested, with PBS used to dilute the antibody. An optimal dilution of 1:3000 was found to give little positive staining in the control slides and was used in all subsequent exposures.

Quantification of HSP 70 expression was based on quantitative image analysis, using the Leica Image Analyser (Leica Microsystems Holdings, Ernst-Leitz-Strasse, Germany). A macro program was set up to measure all areas of the field of view with the same colour intensity. 12 intensities were measured ranging from weak, medium to strong staining by the antibody. HSP 70 expression was quantified in the digestive gland and it was ensured that this was the only tissue in the field of view. In order to overcome intra-sample variation, staining was quantified in 6 fields of view from 5 slides taken from each sample. Total sample size was 5 slides from 2 mussels from each of the 7 exposure tanks after 96 hour exposure.

Statistics

The LC_{50} value for NP on the zebra mussel was calculated with 95% confidence limits by using the Trimmed Spearman-Karber (TSK) program version 1.5 from the US Environmental Protection Agency ToxTest computer program, designed for the analysis of mortality data from acute and chronic toxicity tests. Calculations were verified using the Spearman-Karber equation. The long term LC_{50} value was calculated using a second order quadratic equation to the curve LC_{50} : exposure time (days) using Cricket Graph 3. A toxicity threshold (TT) was calculated as follows: $TT = (NOEC \times LOEC)^{1/2}$ (Horning & Weber, 1985). Tests for significance were done using the paired students T-test on the SigmaPlot 7 computer program and correlation analysis was undertaken using Pearson-moment correlation (Microsoft Excel). Significance was set at $p=<0.05$.

RESULTS

Acute toxicity

LC_{50}

The LC_{50} values obtained for zebra mussel exposed to NP with upper and lower 95% confidence limits are expressed in Figure 2.4 with the data presented in Table 2.4. The long term LC_{50} for zebra mussels exposed to NP was calculated as 1.61 mg l^{-1}.

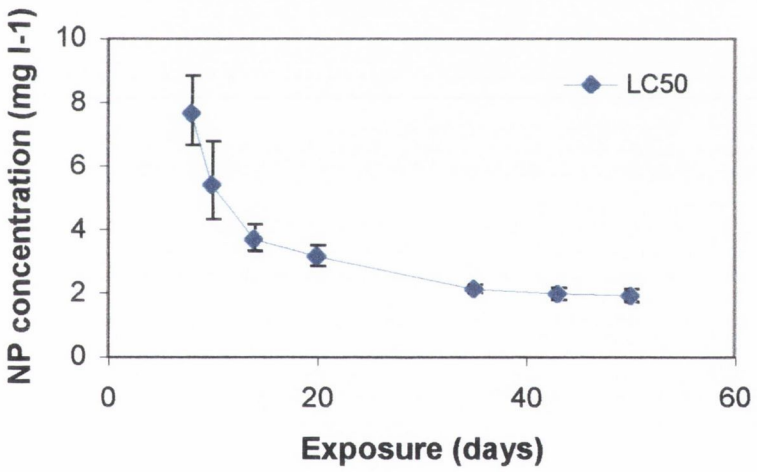


Figure 2.4. LC_{50} values for the zebra mussel (*Dreissena polymorpha*) exposed to NP with 95% upper and lower confidence limits.

Table 2.4. LC_{50} values for the zebra mussel exposed to NP calculated using the Trimmed Spearman-Karber method with 95% confidence limits (CL). The lowest observable effect concentration (LOEC) represents the lowest NP concentration showing a significant difference (p=0.05) in mortality to the control. The no observable effect concentration (NOEC) is the highest concentration at which no significant (p=0.05) difference in mortality occurred when compared to the control. Toxicity threshold (TT) = (NOEC x LOEC)$^{1/2}$.

	LC_{50} mg l^{-1}	95% CL mg l^{-1}	LOEC mg l^{-1}	NOEC mg l^{-1}	TT mg l^{-1}
8 day	7.67	6.67-8.83			
15 day	3.71	3.31-4.16			
35 day	2.13	2.0-2.26			
50 day	1.76	1.54-2.0	1.0	0.1	0.32

Mortality

The rate of mortality for the zebra mussels exposed to NP is expressed as a cumulative percentage of dead mussels in each tank per exposure day (Figure 2.5). When compared with the control, the solvent control showed no significant increase in mortality (p=0.65). From Figure 2.5 it is evident that the higher concentrations of NP (100, 10 and 5 mg l^{-1})

were lethal to the mussels, with 100% mortality after an exposure period of 14, 14 and 24 days respectively. When compared with the solvent control these exposures showed a very highly significant increase in mortality ($p<0.001$). The effect of exposure to these high concentrations of NP on morphology can be seen in Figure 2.6 showing the deterioration of the digestive gland after 14 days exposure to 100 mg l^{-1} NP compared to that of a normal mussel. Exposure to the lower concentrations of NP (1 and 0.1 mg l^{-1}) did not result in mass mortalities although at 1 mg l^{-1} NP there was a significant rise in mortality towards the end of the exposure ($p=0.03$). The lowest concentration of 0.1mg l^{-1} NP did not significantly effect mortality ($p=0.24$). All mortality results are presented in Appendix 2.3.

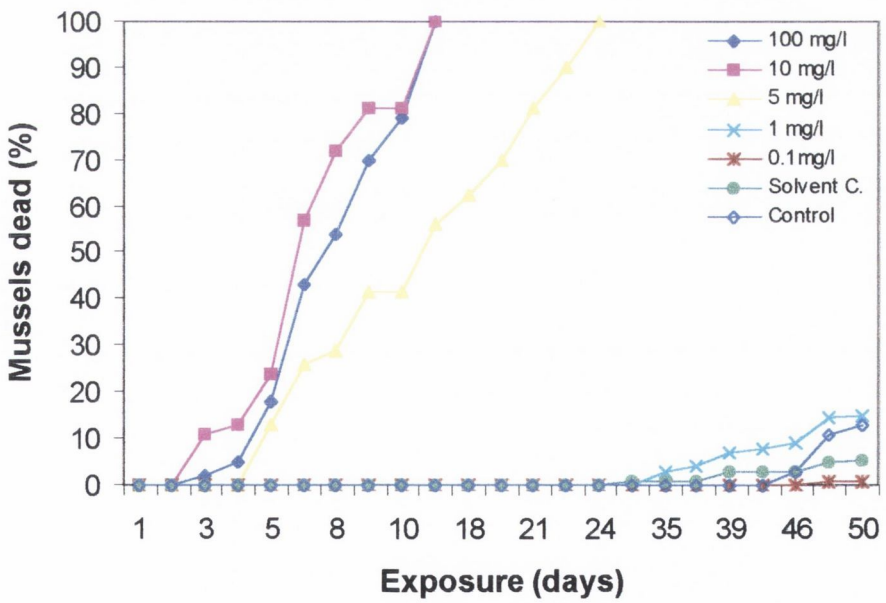
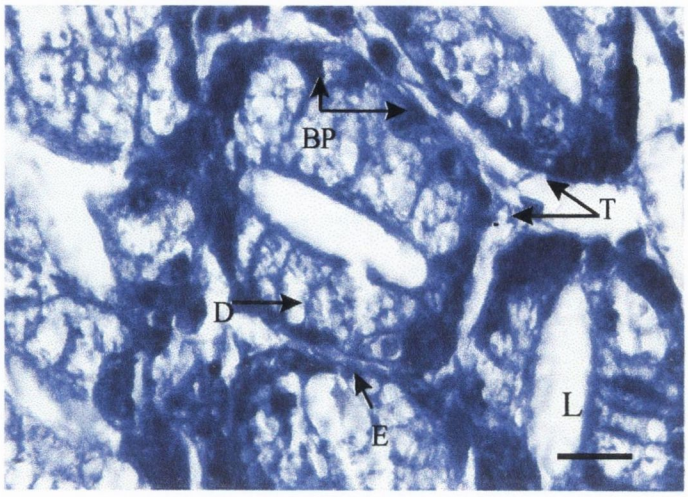
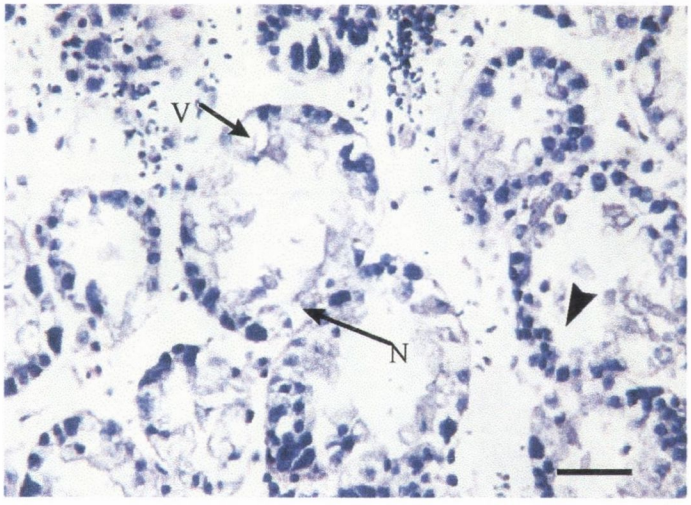


Figure 2.5. Cumulative percentage mortality of zebra mussels after exposure to various concentrations of NP.



(a).



(b).

Figure 2.6. (a). Normal digestive gland of control zebra mussel. The epithelium of the digestive tubules (T) is composed of columnar digestive cells (D), with a basal nucleus and various vacuoles, and large columnar basophilic cells (BP). Note uniform epithelia (E) and lumen (L). Haematoxylin and eosin x 1000. Scale bar = 20 μm **(b).** Highly vacuolated (V) digestive cells showing evidence of necrosis (N) in mussel exposed to 100 mg l^{-1} for 14 days. Note atrophic tubules with enlarged lumen. There is a loss of part of the digestive cells (arrow) in the digestive tubule, reduction in digestive epithelium thickness and general breakdown of epithelial integrity. Haematoxylin and eosin x 400. Scale bar = 40 μm.

Chronic toxicity

Attachment

The percentage of zebra mussels not attached to any substrate in each tank was recorded and presented in Figure 2.7. This graph follows a similar trend as Figure 2.5 with the highest percentage of mussels not attached closely correlated (r=0.969 to 0.986) with exposure to the highest NP concentrations (100, 10 and 5 mg l^{-1}). Mussels exposed to 1 mg l^{-1} NP showed a highly significant (p=0.004) reduction in attachment when compared with the solvent control mussels. However those exposed to 0.1 mg l^{-1} did not show a significant reduction in attachment (p=0.58). The mussels ability for re-attachment after removal of the byssal threads was also investigated (Table 2.5). After 48h exposure to NP only the control, 0.1 and 1 mg l^{-1} exposed mussels re-attached after 24 h. After 24 d exposure re-attachment was only observed in the control tanks (Table 2.5).

Table 2.5. Re-attachment of zebra mussels 24 h after removal of byssal threads after exposure to NP. C = control; SC = solvent control.

	Re-attachment after 24 h						
Exposure	C	SC	0.1mg l^{-1}	1mg l^{-1}	5mg l^{-1}	10mg l^{-1}	100mg l^{-1}
48 h	Yes	Yes	Yes	Yes	No	No	No
24 d	Yes	Yes	No	No	No	No	No

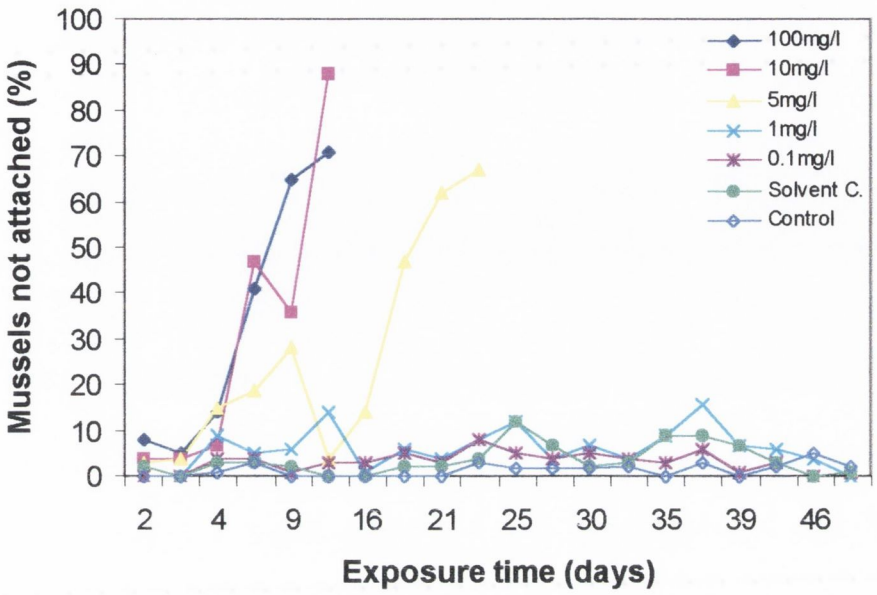


Figure 2.7. Percentage of mussels not attached to any surface within the exposure tank after exposure to NP.

Filtration

The percentage of zebra mussels with their siphons extended indicating filtration is shown in Figure 2.8. Although there was considerable day to day variation in siphon extension rate it is clear that the highest concentrations of NP (100, 10 and 5 mg l^{-1}) reduced very significantly ($p<0.00004$) the rate of siphon extension. Mussels exposed to 1 mg l^{-1} NP showed a very erratic pattern with rates dropping from over 60% siphon extension one sample day to below 20% the next (day 36) and a highly significant reduction ($p=0.01$) when compared with the solvent control. Those mussels exposed to 0.1 mg l^{-1} NP had the most constant rate of extension, with around 60% of the mussels consistently with their siphons extended and a non-significant difference when compared with the solvent control

with the solvent control (p=0.66). A similar trend can be seen in Table 2.6, which shows the average percentage siphon extension rate for each exposure.

Table 2.6. Average percentage siphon extension rates of zebra mussels exposed to NP.

Exposure	Average siphon extension (%)
Control	24.7
Solvent control	32.8
0.1 mg l^{-1} NP	31.3
1 mg l^{-1} NP	25.5
5 mg l^{-1} NP	5.3
10 mg l^{-1} NP	6
100 mg l^{-1} NP	4.4

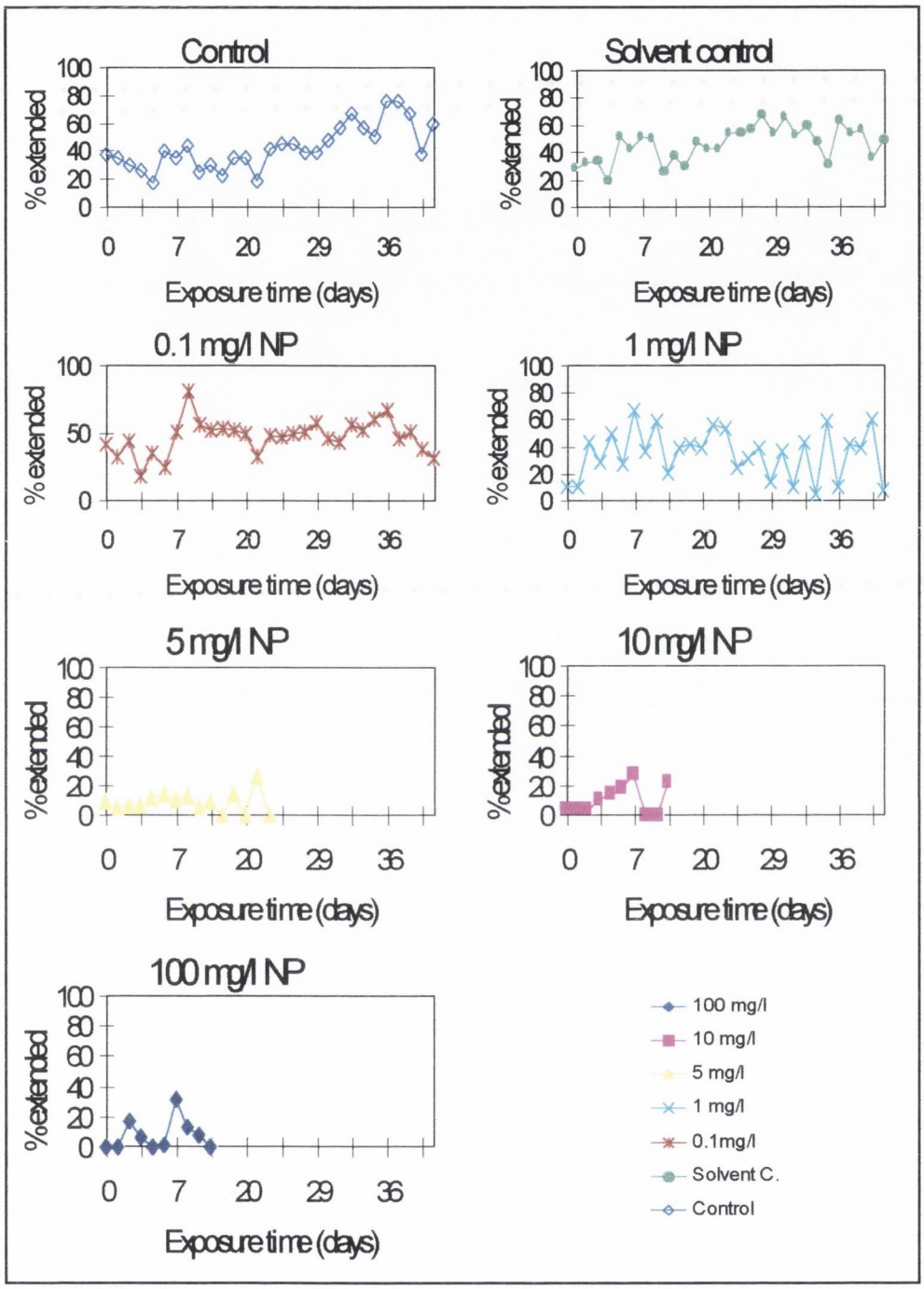


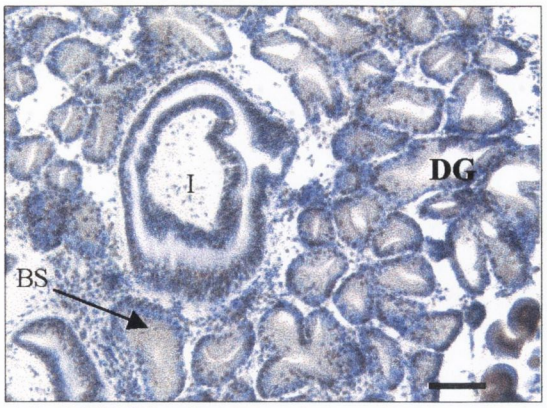
Figure 2.8. Series of graphs showing the effect of exposure to various concentrations of NP on zebra mussel siphon extension. As NP concentration increases fewer mussels have their siphons extended indicating a reduction in filtration.

PCNA

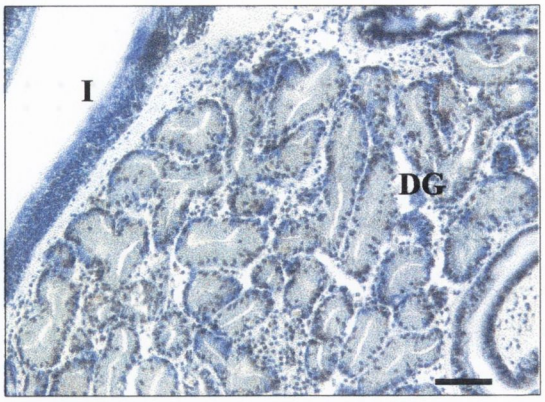
Zebra mussel tissue sections underwent various treatments to enhance immunostaining by unmasking the anitgen masked by formaldehyde fixation. The effectiveness of these different unmasking techniques is summarised in Table 2.7 and can be seen in Figure 2.9 showing the differences in staining in the zebra mussel tissue subjected to different unmasking techniques. The unmasking protocol best suited to zebra mussel tissue involved microwaving the sections for 15 minutes in citric buffer and leaving sit for 15 minutes. This technique offered the best compromise between good unmasking of the anitgen with the least deterioration of the tissue. Treatment in the pressure cooker gave the best unmasking results but the tissues were seriously deteriorated.

Table 2.7. Effectiveness of various unmasking treatments on slides taken from zebra mussels exposed to 1 mg l^{-1} NP for 50 days, a control mussel and tonsil control. PCNA dilution 1:1600. MW = microwave; P.C. = pressure cooker; O/N = overnight. *** staining too strong/bad, ** staining good, * staining too weak/bad.

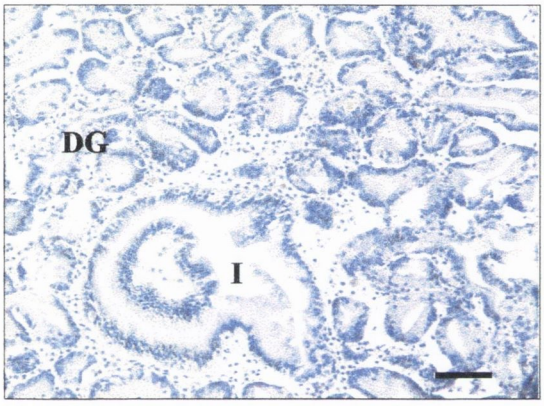
Treatment	Unmasked	Staining	BG staining	Tissue state
MW 20 min	***	***	***	***
MW 15 min	**	**	**	**
MW 10 min	*	*	**	**
P.C.	**	**	**	***
O/N @ 80°C	***	***	***	**



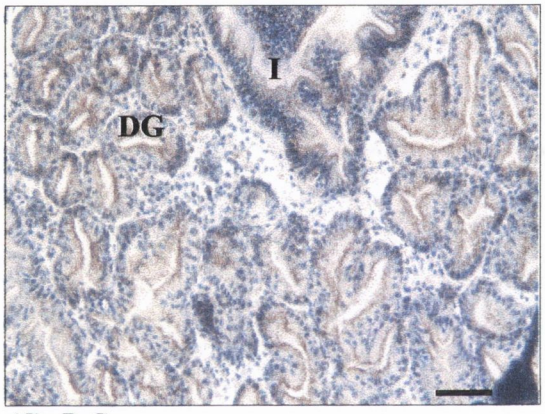
(a). MW 20 min



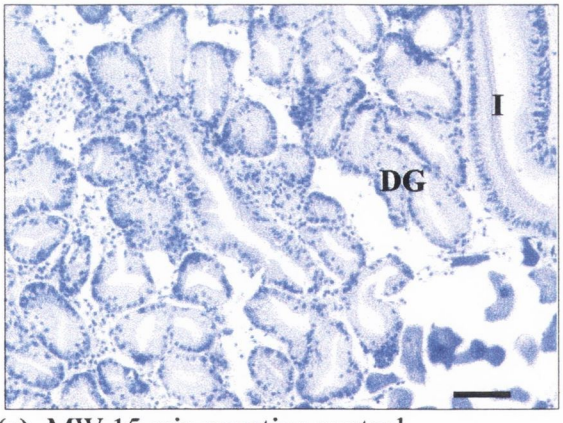
(b). MW 15 min



(c). MW 10 min



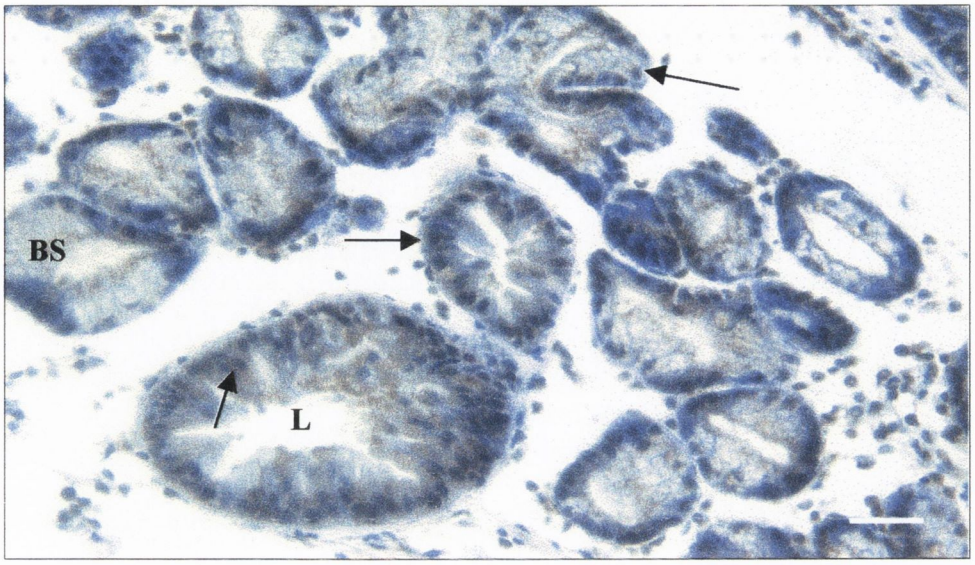
(d). P.C.



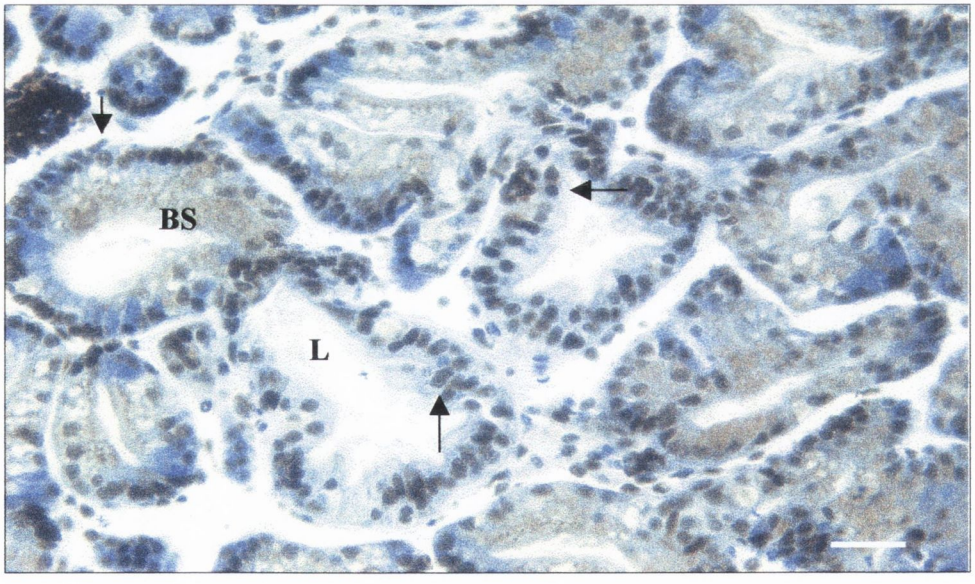
(e). MW 15 min negative control.

Figure 2.9. Series of photographs showing the effects of the various unmasking techniques used on zebra mussel tissue samples. All samples show the digestive gland (DG) and intestine (I) of the same control mussel. **(a).** Section microwaved (MW) for 20 min. Notice dark background staining (BS) around lumen in digestive gland (DG). **(b).** Section microwaved for 15 min. Less BS. **(c).** Section microwaved for 10 minutes, little BS. **(d).** Section treated in pressure cooker (PC), a lot of BS. **(e).** Negative control microwaved for 15 min, no brown staining. All photographs X 200. Scale bar = 100 μm.

From the dilution range tested a dilution of 1:1600 was found to give the best results, with the most dark stained cells showing positivity and least background staining in both the tonsil control and control mussels tested. This concentration was used to test the PCNA reactivity in mussels exposed to NP. Surprisingly mussels exposed to 10 mg l^{-1} NP for 8 days showed a similar if not higher degree of positivity when compared to the control mussel (Figure 2.10). This was also found for the mussels exposed to 1 mg l^{-1} NP after 50 days (Figure 2.11). These mussels seemed to show a higher degree of PCNA reactivity in the digestive gland and intestine, although this was extremely difficult to quantify as the colour intensity changed for each mussel tested. Immunohistochemical staining using the PCNA antibody was therefore not considered to be a reliable marker of cell proliferation in the zebra mussel.

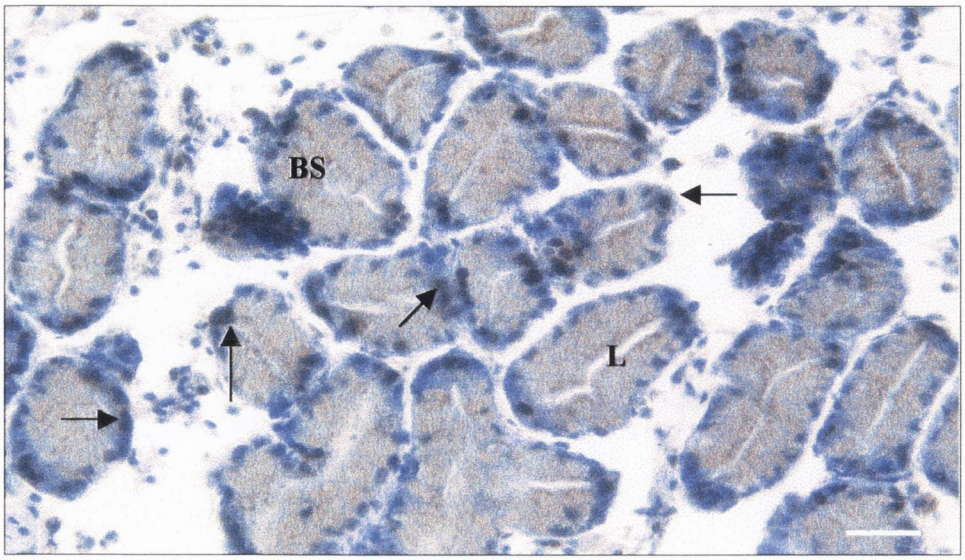


(a).

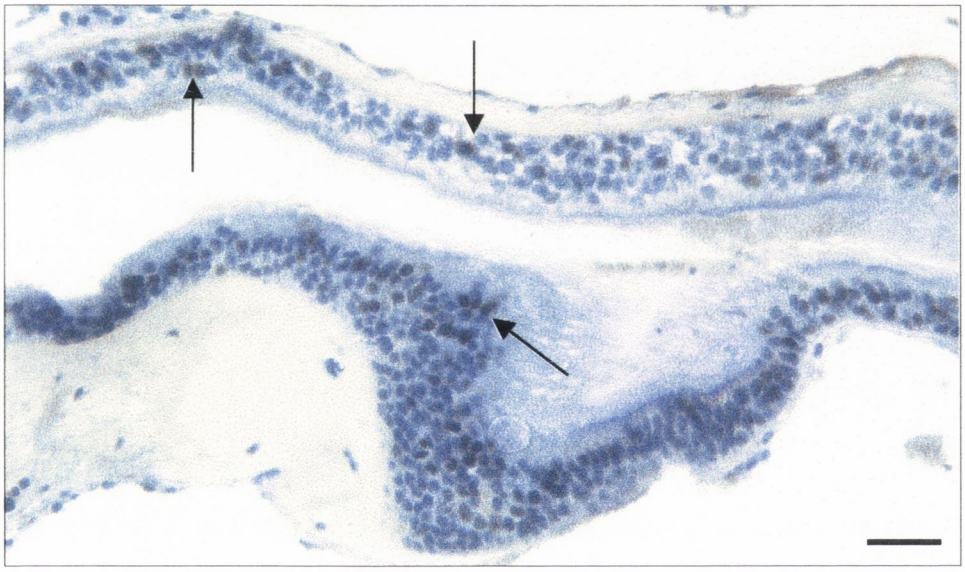


(b).

Figure 2.10. Slides of a cross section of the digestive gland tubules from zebra mussels showing positive staining with PCNA (1:1600) after demasking in microwave for 20 min. **(a).** Section of control mussel after 8 days exposure. **(b).** Section of mussel exposed to 10 mg l^{-1} for 8 days. Arrows indicate basophilic cells showing PCNA positivity (brown). BS = background staining; L = lumen. Magnification X 400. Scale bar = 40 μm



(a).



(b).

Figure 2.11. Slides of zebra mussel tissue 50 days after exposure stained with PCNA (1:1600) with anitgens unmasked in microwave for 20 min. **(a).** Cross section of digestive gland showing PCNA positivity in basopilic cells from control mussel. **(b).** Cross section of intestine showing PCNA positive cells in 50 day 1 mg l^{-1} NP exposed mussel. Arrows indicate cells showing PCNA positivity. BS = background staining; L = lumen. X 400. Scale bar = 40 μm.

HSP 70

The polyclonal rabbit HSP 70 antibody gave a positive reaction when tested on formalin fixed paraffin embedded zebra mussel samples. The dilution range tested indicated that the 1:3000 dilution was suitable for use with these samples (Figure 2.12). The unmasking techniques used to enhance immunostaining were not effective in unmasking the antigen for the HSP 70 antibody. Increased expression of the antibody was found in both the tonsil controls and in the control and exposed mussels where no unmasking had taken place. The unmasking technique was therefore dropped from the protocol.

The HSP 70 protein was located in the nucleus and in the cytoplasm of the tissue observed with a higher labelling intensity in the cytoplasm. Initial tests showed that the staining intensity was not related to NP exposure, with no increase in protein expression (darker staining) in mussels exposed to higher concentrations of NP as would be expected (Figure 2.12). Although some intense staining did occur in samples from mussels exposed to high NP concentrations (Figure 2.13), a large degree of variation was found in the staining intensity of different slides taken from the same animal (intra-sample variation) (Figure 2.14). Even with a high number of replicates the results showed a very high degree of inconsistency and were very difficult to quantify. Problems of patchy staining within the tissue on the same slide were also observed. These problems persisted after repeated testing. For these reasons immunohistochemical staining using HSP 70 was not considered to be a reliable method for measuring stress in tissues of the zebra mussel.

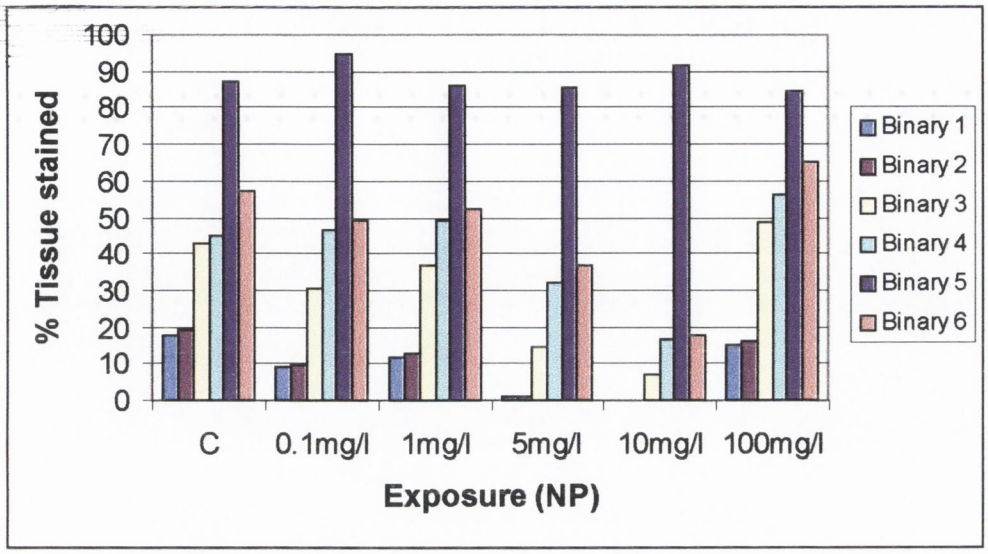


Figure 2.12. Graph showing the intensity of HSP 70 antibody staining in sections cut from zebra mussels exposed to increasing concentrations of NP. Each binary number represents a staining colour intensity with intensity increasing with number. Each bar represents the average of 5 counts. Mussels exposed to higher NP concentrations do not show any significant increase in staining intensity compared with the controls.

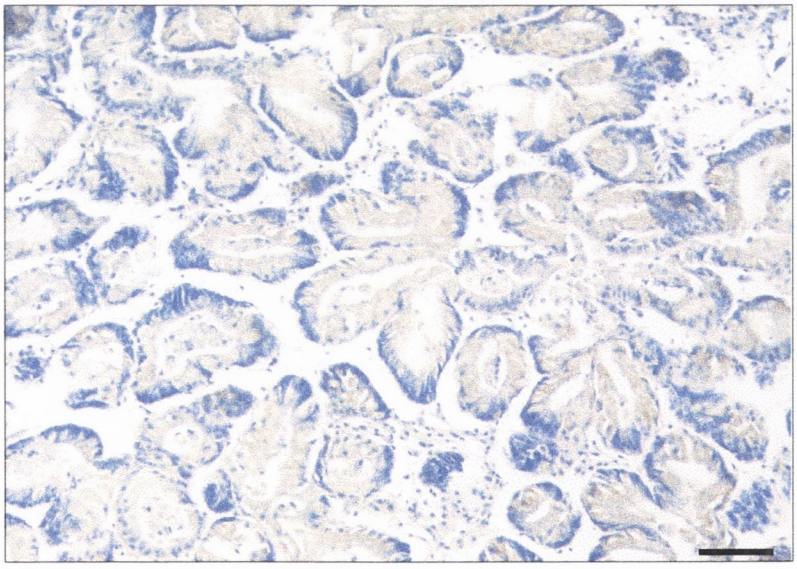
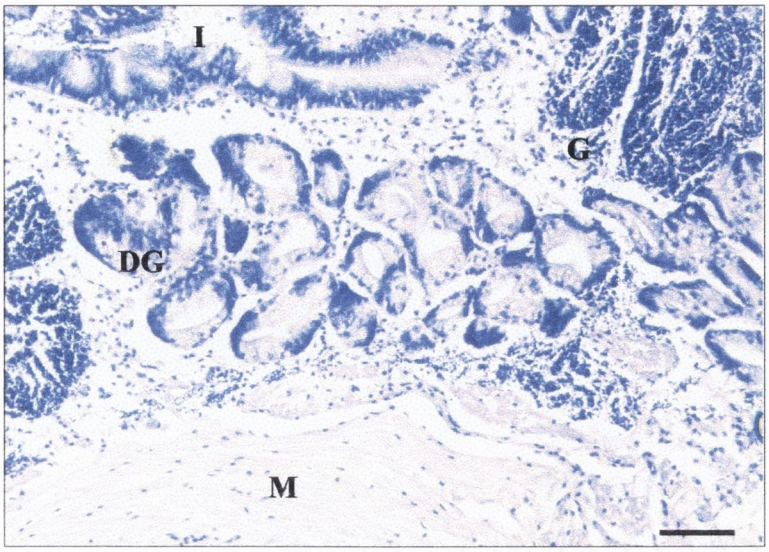
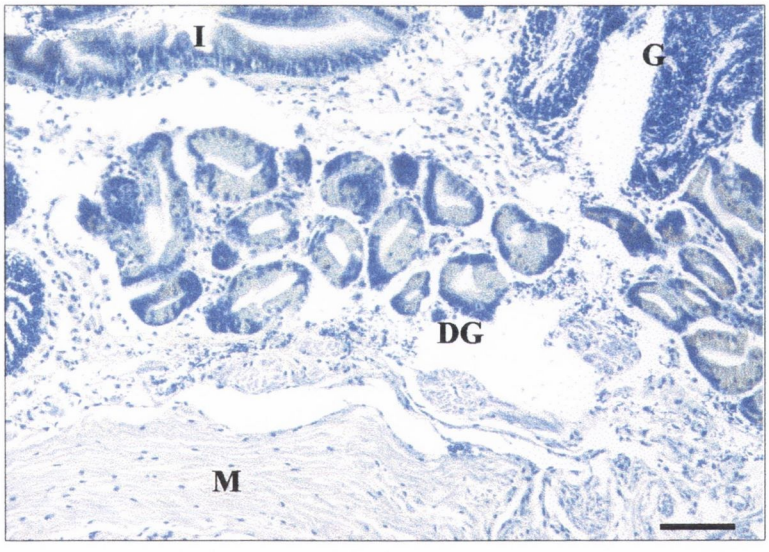


Figure 2.13. Section showing HSP 70 positivity in the digestive gland of a zebra mussel after exposure to 100 mg l^{-1} NP for 7 days. This slide does show increased staining in the cytoplasm of the tubules of the digestive gland. However such staining was difficult to replicate, providing unreliable results. Magnification X 200. Scale bar = 100 μm.



(a).



(b).

Figure 2.14. Evidence of the variation in the staining of the HSP 70 antibody in zebra mussel tissues during immunohistochemical analysis. **(a)** and **(b)** are 2 slides of the same tissue cut from the same zebra mussel, stained using the same antibody during the same immunostaining run. These 2 slides should be identical but have stained differently with slide **(b)** showing a lot more immunoreactivity. DG = digestive gland; M = muscle; I = intestine; G = male gonad. Magnification X 200. Scale bar = 100 μm.

DISCUSSION

The recommended time scale for a lethality experiment is 96 h (APHA, 1971). However as observed by Eisler (1971) this may not be long enough for acute effects to be determined accurately. For this reason the present experiment was allowed to run for 50 days. The LC_{50} of NP for the zebra mussel *Dreissena polymorpha* was unexpectedly high with values of 3.71, 2.13 and 1.76 mg l^{-1} (nominal concentration) for 15, 35 and 50 days exposure respectively. The long term LC_{50}, the concentration that would result in 50% mortality after an indefinite period, was calculated at 1.61 mg l^{-1}. The LC_{50} values for other invertebrate species is shown in Table 2.1. *Mytilus edulis* which is often used in comparative studies with the zebra mussel had much lower LC_{50} values measuring 3.0, 0.5 and 0.14 mg l^{-1} (nominal concentration) after 4, 15 and 35 days respectively (Granmo et al., 1989). In another experiment 100% mortality was recorded for *Mytilus edulis* after 8 day exposure to 20 mg l^{-1} NPEO (Swedmark, et al., 1971).

Based on previous experiments it would appear that NP is less toxic to the zebra mussel than to other bivalve species. This may be the case as methods of detoxification are well developed in the zebra mussel. Hemocytes rich in lysosomal hydrolases (Giamberini et al., 1993) a very efficient lysosomal-vascular system in the podocytes of the pericardial glands (Giamberini & Pihan, 1996; Giamberini & Pihan, 1997), and increased production of esteratic enzymes and acid mucopolysaccharides after exposure to toxins (Janssen et al., 1992) increase the zebra mussels ability for detoxification. These factors could explain the high LC_{50} value for zebra mussels exposed to NP. However care should be

taken when comparing freshwater and seawater toxicity to include any possible matrix effect resulting from the different chemical properties of the two media.

The hydrophobic property of surfactants and their ability to accumulate on surfaces and interfaces makes it probable that in aquatic animals they effect the gill epithelia where gas and ion exchange occur. The swelling of gill epithelia and an increase in mucus secretion of gill tissues in fish affecting respiratory function have been observed (Swedmark et al., 1971). *Mya arenaria* demonstrated a decrease in oxygen uptake after exposure to NPEO and surfactants are thought to also have an influence on ion balance and osmoregulation in bivalves (Swedmark, et al., 1971). In this exposure high concentrations of NP (5, 10 and 100 mg l^{-1}) had a severe effect on the morphology of the zebra mussel tissues, resulting in the complete breakdown of the tubules of the digestive gland.

This hydrophobic nature may have caused the actual concentration of NP available to the mussels to differ from the nominal concentration added to the tanks due to absorption of the compound onto the tank and test animals. At the highest concentrations (10 and 100 mg l^{-1}) the NP appeared to precipitate out of solution, reducing the amount of NP available to the mussels. However it is possible that absorption of NP onto the algae on which the mussels fed could increase exposure to the toxin. When exposed to a toxin bivalves have the ability to close their shell, potentially reducing their exposure. This was observed in the zebra mussels along with a reduction in siphon extension with increased exposure to NP and has previously been observed in other bivalves (Swedmark et al., 1971). This ability may result in delaying the toxic effects of NP on the animal.

Exposure of the zebra mussels to high concentrations of NP (5, 10 and 100 mg l^{-1}) had a negative effect on the sub-lethal chronic parameters also, with reduced attachment and siphon extension (indicating a reduction in filtration). Significant sub-lethal effects of decreased attachment and siphon extension were also found at exposure to 1 mg l^{-1} NP, the LOEC for the experiment. After 23 days mussels exposed to 1 mg l^{-1} were gaping and were much slower in closing their bivalve shells in response to prodding, indicating poor health (Swedmark et al, 1971). No such effect was observed at 0.1 mg l^{-1}, although re-attachment was not observed after 24 d exposure to 0.1 mg l^{-1}. A similar response of reduced byssal thread formation at 0.1 mg l^{-1} NP was also observed in *Mytilus edulis*, with a reduction in byssal thread strength at 0.056 mg l^{-1} (Granmo et al., 1989). These results indicate that with regard to sub-lethal parameters zebra mussels have a similar sensitivity to NP as *Mytilus*. These sub-lethal effects observed in the lab may actually be lethal in the natural habitat. The development of byssal thread is related to survival as it is used in attachment, orientation and locomotion in the wild (Granmo et al., 1989). A toxicity threshold of around 0.32 mg l^{-1} NP was calculated for the zebra mussel. As this is the nominal concentration the actual concentration could be considerably lower nearing the range of NP found in rivers of Europe (<0.1->500 μg l^{-1}) (Ahel et al., 1994; Blackburn & Waldock, 1995; Bennie, 1999; Korner et al., 2000). Due to its ability to bioaccumulate and its hydrophobic nature bivalves could be particularly vulnerable to exposure to NP as they live in intimate contact with and feed on particulate matter where this chemical accumulates (Blackburn & Waldock, 1995).

Although successfully used in immunocytochemistry to indicate a reduction in cell proliferation in fish exposed to various toxins (Suzuka, et al., 1989; Kilemade & Mothersill, 2001), PCNA did not indicate reduced cell proliferation in the zebra mussel after exposure to NP. It was expected that mussels exposed to increasing concentrations of NP would show a reduction in PCNA expression, indicating a reduction in cell proliferation. This could be quantified and used to indicate the aquatic toxicity of NP to bivalves. Although most tissues (digestive gland, gill, intestine, gonads) of the zebra mussel did show reactivity to the antibody and were positively stained, exposure to concentrations of NP clearly toxic to the animal did not reduce PCNA expression when compared to the control. A limitation to using cell proliferation as an indicator of aquatic toxicity is that PCNA protein has a long half-life (Bravo & MacDonald-Bravo, 1985) and will remain detectable for an extended period in cells that have left the cell cycle, resulting in an overestimation of cell proliferation. PCNA expression may also be influenced by the compound the animal was treated with, again possibly resulting in an overestimation of the actual proliferation rate (Deitrich, 1993). For these reasons it was felt that the measurement of PCNA using immunohistochemistry was not a good indicator of cell proliferation in zebra mussels exposed to NP.

Expression of heat shock proteins are commonly measured in fish exposed to numerous pollutants (Williams et al., 1996; Iwama et al., 1999) and has been observed in *Mytilus edulis* both *in vivo* and *in vitro* after exposure to TBT (Sanders, 1990; Steinert & Pickwell, 1993). The heat shock protein HSP 70 was also expressed in zebra mussels exposed to NP as observed using immunohistochemistry. An appropriate dilution range

(1:3000) was found that gave little or no background or natural positivity while increased expression after exposure of the mussels to NP, indicating a stress response. However it was not possible to quantify this response due to a large amount of intra and inter-sample variation. One explanation for this variation between slides of the same animal was the differing thickness of the tissue cut. In order to reduce this variation a large sample number was employed, antibody dilutions were checked, care was taken when cutting samples and the protocol was rigorously adhered to. Despite this it was still not possible to quantify an increase in HSP expression in NP exposed zebra mussels using immunohistochemistry.

CONCLUSION

The object of this exposure was to determine the LC_{50} value of 4-nonylphenol for the zebra mussel *Dreissena polymorpha* in order to find a concentration that could induce an endocrine disrupting effect in the mussel without being overtly toxic to the animal. Despite having a relatively high LC_{50} value when compared to other bivalve species, results indicate levels of around 0.1 mg l^{-1} NP may have observable effects on sub-lethal parameters on zebra mussels in the field. Gaps in mussel distribution could thus be compared with pollution sources. Although not a particularly sensitive species to 4-NP, zebra mussels may be useful indicators where moderate levels of pollution occur.

Appendix 2.1.

10% Neutral buffered formalin

28.6 mM sodium dihydrogen phosphate monohydrate

46 mM disodium hydrogen phosphate anhydrous

100 ml 40% formaldehyde

Add to 900 ml with distilled water.

Appendix 2.2.

Phosphate buffer saline (PBS):

12 mM sodium dihydrogen orthophosphate dihydrate ($Na_2H_2PO_4.2H_2O$)

37.7 mM disodium hydrogen orthophosphate anhydrous (Na_2HPO_4)

72.7 mM sodium chloride (NaCl)

Add to 5 litres of distilled water.

APPENDIX 2.3. Results of the lethality exposure of *Dreissena polymorpha* to NP. M = Male; F = female; ? = sex not determined. Expo = exposure

Date	Expo. (days)	Tank	Mussels removed	Mussels dead/gaping	No. Preserved (Formalin)	Male / Female
13/06/00	1	All	6		4	
14/06/00	2	All	4		4	
15/06/00	3	5	2		2	
		6	10	10	7	
		7	4	2	4	
16/06/00	4	1 to 5	4		4	
		6+7	6	2	4	
17/06/00	5	1+2	2			
		5	11	11		
		6	10	10		
		7	11	11		
19/06/00	7	5	11	11		6M: 4F
		6	29	29		16M: 12F
		7	21	21		12M: 8F
20/06/00	8	1 to 5	2		2	
		5	4	2	2	2F
		6	14	12	2	6M: 5F
		7	10	8	2	4M: 4F
21/06/00	9	5	10	10		5M: 3F
		6	8	8		3M: 4F
		7	13	13		7M: 6F
22/06/00	10	7	7	7		
26/06/00	14	5	12	12		6M: 3F
		6 empty	15	15	2	4M: 2F: 4?
		7 empty	17	17	2	5M: 6F: 1?
28/06/00	16	1 to 5	2		2	
30/06/00	18	5	4	4		1M: 1F: 2?
02/07/00	20	5	6	6		4M: 2F
03/07/00	21	5	9	9		5M: 2F: 2?
05/07/00	23	5	7	7		3M: 3F: 1?

Date	Expo. (days)	Tank	Mussels removed	Mussels dead/gaping	Preservation	Male / Female
06/07/00	24	1 to 4	2		2	
		5 empty	7	6		3M: 2F
10/07/00	28	2	1	1		
13/07/00	31	1 to 4	4		2	
17/07/00	35	4	2	2		1M
19/07/00	37	4	1	1		
21/07/00	39	2	1	1		
		4	2	2		
25/07/00	43	4	1	1		
28/07/00	46	1	2	2		
		4	1	1		
31/07/00	49	1	5	5		5?
		2	2	2		2?
		3	1	1		1M
		4	4	4		1M:1F:1?
01/08/00	50	1	4	1	3	1F
		2 to 4	3		3	

Chapter 3.

THE ENDOCRINE DISRUPTING EFFECT OF EXPOSURE TO TERTIARY TREATED EFFLUENT AND NONYLPHENOL ON THE ZEBRA MUSSEL (*DREISSENA POLYMORPHA*).

INTRODUCTION

In Ireland municipal effluents have been responsible for the majority of moderate and serious pollution incidences in the freshwater environment (EPA, 2000a). In addition municipal effluents contain substances such as pesticides, polyaromatic hydrocarbons and heavy metals known to be toxic to aquatic animals (Secombes et al., 1991; Costello & Read, 1994). But the relatively recent discovery of compounds capable of interfering with an animals hormonal system known as endocrine disrupting chemicals or EDCs, triggered a major new area of research in ecotoxicology. Municipal effluents have been shown to contain a cocktail of EDCs including oestrogen mimicking chemicals or xenoestogens and synthetic and natural hormones (e.g. 17α-ethinylestradiol and 17β-estradiol) (see Table 1.1). Xenoestrogens mimic or interfere with an animals endogenous hormones by acting as an estrogen agonist, binding to the estrogen receptor and eliciting a biological response (Jobling et al., 1996; Routledge and Sumpter, 1996). An example of such a compound is nonylphenol (NP) formed during sewage treatment by the breakdown of

alkylphenol ethoxylates, non-ionic surfactants used in the formulation of detergents, pesticides and emulsion paints. These compounds are generally found at concentrations $<0.1\ \mu g\ l^{-1}$ in municipal effluent and in environments polluted by this effluent, (Kvestak et al., 1994; Blackburn & Waldock, 1995), but have been found at concentrations up to hundreds of $\mu g\ l^{-1}$, high enough to elicit a biological endocrine disrupting response (Ahel et al., 1994; Ternes et al., 1999; Bennie, 1999).

As mentioned in Chapter 1 the presence of EDCs in municipal effluents was confirmed by the feminisation of male fish exposed to the effluent itself, downstream from effluent sources and to NP in controlled laboratory conditions (Purdom et al., 1994; Harries et al., 1997; Jobling et al., 1996; Giesy et al, 2000; Hemmer et al., 2001). Biological responses have been well studied in aquatic vertebrates where effects on male fecundity, testicular growth and the gonadosomatic index have been investigated in many fish species (for summary see Vos et al., 2000). Increased levels of the egg yolk precursor protein vitellogenin (Vtg) in the plasma of male fish has been broadly accepted as a biomarker for estrogen or xenoestrogen exposure in aquatic systems (Jobling & Sumpter, 1995).

Despite their obvious ecological importance, relatively little work on endocrine disruption (ED) has been carried out on invertebrates, with the exception of imposex in gastropod molluscs exposed to organotin compounds (Gibbs et al., 1988; Oehlmann et al., 1998). However there is growing evidence that invertebrates may be susceptible to this form of ED as exposure to sewage effluent resulted in abnormal sex ratios and intersexuality in harpacticoid copepods (Moore & Stevenson, 1994) and ovotestes in male lobster

(Sangalang & Jones, 1997). Exposure to the synthetic estrogen diethylstilbesterol and the xenoestrogen nonylphenol both commonly found in effluent, resulted in the stimulation of secondary sexual characteristics and the accumulation of exogenous testosterone in daphnids (*Daphnia magna*) (Baldwin & LeBlanc, 1994; Brown et al., 1999; Olmstead & LeBlanc, 2000). Other possible ED effects include altered steroid metabolism in the gonads and pyloric caeca of the sea star *Asterias rubens* exposed to cadmium and zinc (Den Besten et al., 1991).

Although relatively little is known about the endocrine system in invertebrates, the use of hormones to control and co-ordinate biochemical, physiological and behavioural processes is common to all invertebrate taxa (DeFur et al., 1999). Most invertebrate species studied possess a complex mixture of vertebrate-type hormones (testosterone and estrogen) in measurable quantities (De Longcamp et al., 1974; Jarzebski, 1985; Reis-Henriques et al., 1990). Cholesterol has been identified as the main component of this steroid mixture (Reis-Henriques et al., 1990). In bivalves vitellogenesis occurs in the gonad where the oocytes are believed to produce yolk proteins called vitellin (Vn) autosynthetically from vitellogenin (Vtg) (Jong-brink et al., 1983; Pipe 1987) through a process induced and regulated by estrogen (Eckelbarger and Davis, 1996; Li et al., 1998). This process appears to be susceptible to ED in a manner similar to that of fish, by increasing levels of Vtg or Vn-like proteins. This response has been reported in male and female clams (Blaise et al., 1999), mussels (Gagné et al., 2001b) and oysters (Li et al., 1998) exposed to estradiol, municipal effluent and NP respectively. A recent insight into the mechanism behind this action has been provided by Gagné et al. (2001a) with the

identification and characterisation of the estrogen receptor in *Elliptio complanata*, through which interaction with municipal effluent and NP resulted in increased Vtg levels in exposed animals.

The freshwater bivalve *Dreissena polymorpha* commonly known as the zebra mussel has been widely used as a sentinal species for the monitoring of various environmental pollutants (see Chapter 1). Due to their high filtration rate, an ability to accumulate and bioconcentrate toxicants and a primarily stationary, endobenthic life-cycle freshwater mussels may be at particular risk to EDCs found in the aquatic environment.

This chapter deals with the ED effect on the zebra mussel of exposure to both tertiary treated effluent in an *in situ* experiment at a sewage treatment works (STW) and exposure to an environmentally relevant (5 $\mu g\ l^{-1}$) and an elevated (500 $\mu g\ l^{-1}$) concentration of NP in a controlled laboratory experiment. The reproductive cycle in bivalves is thought to be the stage in the life cycle that could be particularly sensitive to endocrine disturbance (Mori et al., 1969). For this reason prolonged exposures (112 day) were undertaken between mid December to mid May, to coincide with the onset of early gametogenesis in the mussels. Zebra mussels in Irish rivers and lakes begin gametogenesis in mid December when the gonads begin the process of maturation and development of spermatocytes or ovocytes in pre-vitellogenesis begins, stage B in the gonad maturation index (Wang & Denson, 1995). This stage is predominant until the end of May when spermatozoids develop and become mobile and developed ovocytes appear (stage C1) (O'Toole, 2002). It was felt that this stage of gonad development represented the period of

the life cycle that was potentially the most susceptible to endocrine disruption. Due to practical reasons both experiments could not be started together, therefore the *in vivo* laboratory experiment was initiated in mid January 2001 while the *in situ* exposure began before Christmas in mid December 2000.

The endocrine disrupting effect of both exposures was examined by measuring the impact on levels of Vn-like proteins in both male and female mussels using an indirect alkali-labile phosphate (ALP) assay and gel electrophoresis (GE). This data was primarily normalised against the mussels total protein, but data from normalisation against mussel volume was also included. Attempts were also made to characterise the estrogen receptors in the zebra mussel. The effect on cholesterol levels and the bioaccumulation of NP by the mussels was measured by HPTLC. Condition indices were used to measure the overall impact of these exposures on the mussels. Effects at a histological and cellular level were investigated using both light and electron microscopy. The identification and quantification of EDCs in both effluent and lake water samples was undertaken. The potential use of the zebra mussel as a possible bioindicator of endocrine disruption in freshwater environments was investigated.

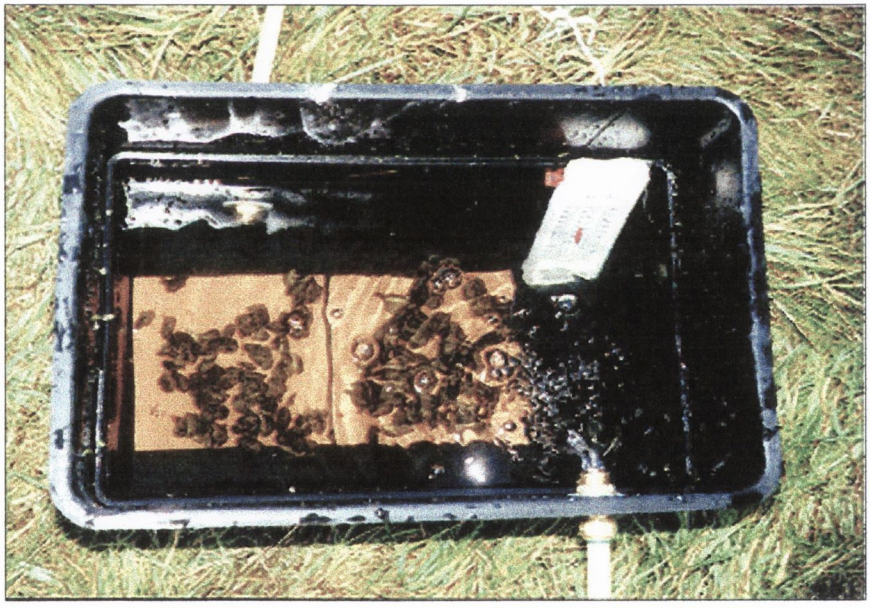
MATERIALS AND METHODS

In situ exposure

Mussel collection and experimental set up

Freshwater zebra mussels were collected in late November (water temperature 5-6°C) from Quigley's marina, Lough Ree (see Chapter 1.). Mussels were transported back to the laboratory, cleaned and graded by size (14-29 mm). They were acclimated for 7 days, fed a commercial bivalve food 'Phytoplex' (Kent Marine, USA) and allowed to attach onto tiles in 60 l tanks containing de-chlorinated, aerated tap water at 8°C. The tiles with the mussels attached were then transported damp to the sewage treatment works (STW) in Athlone, County Westmeath, where they were placed into 18 l plastic tanks and exposed to tertiary treated effluent (Figure 3.1(a)). Effluent was pumped into a large 135 l header tank where it was fed by gravity at a constant flow (2 l min^{-1}) into 3 exposure tanks, each containing 250 mussels. The exposure lasted 112 days from December 6th 2000 until April 2nd 2001. The tanks were checked roughly every two weeks when the mussels were cleaned of particulate matter, the clogged pipes un-blocked, and the temperature recorded using a max/min thermometer (Figure 3.1(b)). At the end of the exposure samples of exposed and control mussels (taken from the wild mussel population in Lough Ree) were taken back to the laboratory for analysis.

(a).



(b).

Figure 3.1. Experimental set up of *in situ* exposure of zebra mussel to tertiary treated effluent in Athlone STWs. **(a).** Effluent pumped from an effluent well into a large 135 l header tank and fed by gravity into 3 smaller 18 l plastic tanks containing the zebra mussels. Continuous flow experiment with effluent released onto the grassy area. **(b).** Mussels attached to tiles exposed to a continuous flow of tertiary treated effluent. Note max/min thermometer to record temperature in the tank.

In vivo laboratory exposure to nonylphenol

Test Solution

Working solutions were prepared by dissolving NP (4-n-nonylphenol, 98+% Lancaster Synthesis Ltd., U.K.) in the carrier solvent ethyl acetate (99.9%, Romia Chemicals). For exposures to 5 $\mu g\ l^{-1}$ and 500 $\mu g\ l^{-1}$ NP, concentrations of 1 mg and 100 mg NP respectively were dissolved in 10 ml of solvent. 250 µl of this working solution was added to tanks containing 5 l of de-chlorinated tap water for a final solvent concentration of 50 $\mu g\ l^{-1}$, keeping within the no observable effect limits for ethyl acetate (Bluzat et al., 1979; Rayburn & Fisher, 1997). Stock solutions were stored in glass containers in the dark at 5-8°C. Nominal concentrations are presented hereafter. As mentioned in Chapter 2. the actual concentrations have been previously shown to be 70-90% and 60-70% of the nominal concentrations after 24 h and 48 h respectively (Comber et al., 1993; Billinghurst et al., 1998).

Mussel collection and exposure to nonylphenol

Zebra mussels were collected at the beginning of January (water temperature 5-6°C) from Quigley's marina, Lough Ree (see Chapter 1). Mussels were cleaned, graded by size (9-14 mm and 14-29 mm) and acclimated in 60 l tanks containing de-chlorinated, aerated tap water at 8°C for 14 days while fed a commercial bivalve food 'Phytoplex' (Kent Marine, USA). The mussels attached onto tiles which were placed into 15 l glass tanks (n=185 mussels per tank) containing 5 l of de-chlorinated, aerated tap water in a climate controlled room with a constant temperature of 5-8°C and a 12 h light:dark cycle (Figure

3.2). The mussels were exposed to NP (5 μg l^{-1} and 500 μg l^{-1}) in triplicate, in a semi-static exposure scheme. The solvent control tank received 250 μg l^{-1} of solvent and the control tank was left untouched. Both the water and chemical were changed 3 times a week, with the contaminated water being filtered through activated charcoal for the removal of NP and disposed by incineration. Mussels were fed 2 ml of a commercial bivalve food 'Phytoplex' (Kent Marine, USA) after each water change. As the exposure progressed a build up of material was noted at the inhalant and exhalent siphons of the mussels and all surfaces within the tank were covered with what appeared to be algae. To prevent this build up tiles with the mussels attached were cleaned every 10 days. This involved the cleaning of each mussel individually using a toothbrush. The exposure lasted 112 days (January 20th to May 12th) after which time samples from each tank were collected for analysis.

Figure 3.2. Experimental design of the *in vivo* laboratory exposure of zebra mussels to NP. Mussels were maintained in 12 glass tanks (control, solvent control, 5 μg l^{-1} and 500 μg l^{-1} NP in triplicate) in a climate controlled room for 112 d.

Analysis of *in situ* and *in vivo* exposed mussel samples

In the laboratory sub-samples of both the *in situ* and *in vivo* exposed mussels were taken for biometric measurements (length, width, total weight, shell weight and dry and wet tissue weight) to estimate the animals condition. Samples were also measured for length and weight and their visceral mass dissected, fixed in 10% formalin (Appendix 2.1) for 24-35 hours, processed through graded alcohol and xylene and embedded in paraffin wax for histological examination. Samples were also prepared for examination by electron microscopy. Mussels were weighed and measured for length and width. The soft body part was dissected out, weighed and placed in a solution of 2.5% glutaraldehyde made by diluting 25% glutaraldehyde $^{1}/_{10}$ in 0.1 M Sorensen's phosphate buffer (Appendix 3.1). Samples were left in fixative for 3-4 weeks when they were removed and stored in PBS (Appendix 2.2) until examination. All remaining mussels were frozen at -70°C for protein and steroid analysis.

Condition index

Two different condition indices (CI) were used to indicate whether prolonged exposure to tertiary treated effluent and NP affected the general physiological health of the zebra mussels. The ratio of tissue dry weight : wet weight, measures the body water content of the animal. The tissue dry weight : shell weight ratio measures the relative tissue to shell weight. Tissue dry weight and shell weight were determined after drying in an oven at 60°C.

Vitellin-like protein determination

Whole mussel homogenate preparation

The homogenate sample for each mussel was prepared individually. The shell length of each mussel was measured and mussels of similar size, 22-24 mm (n=8 mussels per exposure and control, 4 male and 4 female) were gradually thawed on ice for approximately 10-15 min until their valves were easily opened. Mussels were sexed after microscopic examination (X 400 magnification) of a gonad smear, with large oocytes indicating females and spermatozoa indicating males. After removal of the byssal thread and gills, the visceral mass was dissected and homogenised in an ice bath with a Teflon pestle tissue grinder in 1 ml of 10 mM Hepes-NaOH buffer, pH 7.5, containing 50 mM NaCl, 1 mM sodium molybdate, 1 mM dithiothreitol and 1 mM EDTA. The homogenate was maintained on ice until centrifugation at 10,000 x g for 15 min at 4°C. The supernatant was pipetted off, aliquoted and stored at -20°C for later analysis. The dissected gills were stored in buffer at –20°C. The total protein level in the supernatant was determined using the method of Bradford (1976) with bovine serum albumin as the standard.

Alkali-labile phosphate assay

Levels of Vn-like proteins were determined in the mussel homogenate using the alkali-labile phosphate (ALP) measurement procedure. This indirect assay exploits the fact that lipophosphoproteins such as Vn and Vtg contain alkali labile phosphates that can be ether extracted and subjected to an alkali treatment in order to release labile phosphates that

can be easily quantified (Copeland et al., 1986; Pereira et al., 1992). The assay has been previously used to determine Vtg and Vn levels in fish (Nagler et al., 1987; Pereira et al., 1992) and mussel haemolymph (Blaise et al., 1999; Lafontaine et al., 2000; Gagne et al., 2001a) and has been significantly correlated with other Vtg assays (Campbell & Idler, 1980; Gagne & Blaise, 1998). A 500 µl sample of visceral mass homogenate was defrosted over ice, mixed with 350 µl acetone and centrifuged at 10,000 x g for 5 min. The supernatant was discarded and the pellet dissolved in 200 µl of 1 M NaOH in a 70°C water bath for 30 min, and vortexed intermittently to increase solubility. The level of free phosphate in the NaOH phase was determined using a phosphorous inorganic kit (Sigma) based on the colorimetric phosphomolybdenum method developed by Stanton (1968), with standards of phosphate and trout vitellogenin. Total ALP was normalised to the total protein content of the visceral mass (Bradford,1976) and expressed as µg of phosphate / mg of protein. Data was also normalised against mussel volume and expressed as ng phosphate / mm^3.

Gel Electrophoresis analysis

This generic assay for the direct measurement of Vn-like proteins based on molecular weight used NuPAGE Bis-Tris Bis-acrylamide matrix gels (Invitrogen, USA). A 25 µl sample of visceral mass homogenate was added to a 96 well plate containing 25 µl of methelyne blue in PBS (350 µg ml^{-1}), with 50% glycerol and 0.1% SDS, pH 7.4. Samples were left overnight at 4°C to absorb the dye. A 20 µl sample was pipetted into each well in the gel and the gels enclosed in a close circuit cell. The running buffer (125 mM glycine, 25 mM Tris HCH, 0.1% SDS made up to 500 ml with distilled water, pH 8) was

added and gels were run for 3-4 hour at 100 volt. Gels were rinsed in distilled water and the proteins visualised by staining with Invitrogen Simply Blue Safestain for 1 hour. Gels were re-rinsed and left overnight in distilled water at 4°C. Band intensity was analysed by densitometric scanning using UN-SCAN-IT computer software. Calibration was carried out using trout Vtg as the standard. Data was normalised against total protein and expressed as μg of phosphate / mg of protein. As with the ALP data, data was also normalised against mussel volume and expressed as ng phosphate / mm^3.

Determination of estrogen-binding sites in the gills.

The binding of estrogens to gill cytosolic proteins was determined by an ultrafiltration method adapted from Gagné et al. (2001b). The gills that had previously been dissected from the female mussels exposed to both sewage effluent and NP and frozen in buffer solution were defrosted. These samples were homogenised at 4°C in 10 mM Hepes-NaOH buffer, pH 7.4, containing 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA and 10 mM ammonium molybdate. The homogenate was centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was collected and used in the binding assay. The binding assay was based on the separation of protein complexes with estradiol-17β (E2) linked to fluorescein-albumin (Sigma) from unbound or free albumin-E2 by ultrafiltration using a 100-kDa molecular weight cut off membrane. The albumin-E2 was labelled with fluorescein isothiocyanate (Sigma) and detected by fluorescence. To determine the dissociation constant (K_d) and the number of unoccupied binding sites ($\in_0$), the cytosol fraction is diluted 1:5 in buffer containing 125 mM NaCl, 1 mM EDTA, 1 mM

dithiothreitol, 0.5% albumin, 0.2% Tween 20 and 10 mM Hepes, pH 7.5. The diluted cytosol is mixed with increasing concentrations of E2-albumin-fluorescein (1/5, 1/10, 1/20, 1/100, 1/500) and allowed to incubate for 1 hour at room temperature. The mixture is centrifuged with an ultrafiltration membrane (400 µl polysulfone membranes) for 1 hour (until cytosol has passed through the membrane) at 12,000 x g to separate the protein-E2-albumin-fluorescein complexes from the unbound E2-albumin-florescein. Controls have 2.5 µl and 5 µl E2 (1.3 mM) and NP (13 mg/ml). Levels of free (eluate) and bound (retained on filter) E2 were determined by microfluorometry (excitation at 485 nm and 520 nm emission) (Dynatech, FL-1000). The dissociation constant (K_d) and the determination of the number of unoccupied sites ($\in_0$) were performed by Scatchard analysis (Bagshaw & Harris, 1987). The specificity of the binding E2-albumin was also examined by pre-incubating the diluted cytosols for 15 min with NP and processed as above.

Measuring cholesterol levels and nonylphenol uptake

Levels of the predominant steroid cholesterol and of the EDC nonylphenol absorbed by the mussels were measured using High-Performance Thin Layer Chromatography (HPTLC). A sample (n=5 per exposure and control) of mussels of similar size (22-24 mm) were dissected on ice and made up to 7 ml with 50 mM HCl. After homogenisation with a Teflon pestle tissue grinder 5 ml of ethyl acetate was added (to extract lipophilic compounds) and the sample was mixed on a rotator for 1 hour. The mixture was then centrifuged at 10,000 x g for 5 min to separate the phases. The ethyl acetate layer was

mixture was again centrifuged at 10,000 x g for 5 min and 10 x 7.5 µl of the ethyl layer were spotted onto a silica-based, HPTLC plate (10x10 cm, Sigma) and resolved for 25 min with the solvent: 75% hexane and 25% tert-butylmethyl ether. The bands were visualised by spraying with phosphomolybdate reagent (Sigma), followed by heating at 95°C for 10-15 min. The bands were then analysed by densitometric scanning using UN-SCAN-IT software (Figure 3.3). Standards of nonylphenol and cholesterol were used for retention factor (R_f) identification and quantification by densitometric analysis.

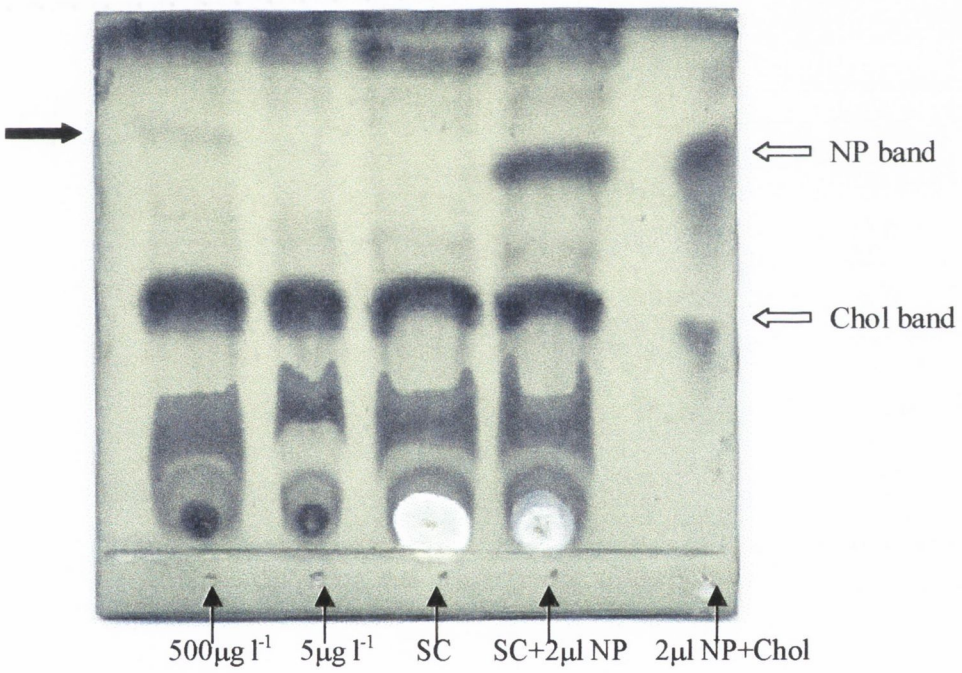


Figure 3.3. HPTLC plate showing the nonylphenol (NP) and cholesterol (Chol) bands migrating from samples of zebra mussels exposed to NP. In lane 1 (far left) from a 500 µg l^{-1} NP exposed mussel a faint band (full arrow) can be seen eluting with the same retention time as the NP control (lane 5). Lane 4 shows a solvent control (SC) mussel sample spiked with 2 µl NP. Lane 5 contains 2µl nonylphenol and cholesterol standards.

Histological examination

Light Microscopy

Samples of control and exposed mussels from both exposures were preserved in paraffin wax and used for tissue histological examination. Sections 5 μm thick were cut using a microtome (Leica, Germany) and attached onto silinized slides and dried in an oven at 60°C. Slides were deparaffinized through xylene and graded alcohol and rehydrated. After rinsing in distilled water slides were placed in filtered Mayers haematoxylin for 30 seconds and were allowed to 'blue' in hot water. Slides were checked microscopically to ensure that the nuclei stained blue and the background remained clear. These slides were then placed in 1% Eosin for 1 min and were rinsed in tap water. Slides were then dehydrated through graded alcohol and xylene and mounted using Distrene Plasticiser Xylene (DPX). Samples were examined using a light microscope (Nikon) under X 200, X 400 and oil immersion X 1000 magnification.

Transmission Electron Microscopy

Mussel samples were also examined using transmission electron microscopy. After fixation in 2.5% glutaraldehyde and storage in PBS samples were dehydrated through ascending grades of alcohol to 100% ethanol. As most resins are immiscible with ethanol an intermediate solution, propylene oxide, which is miscible with both ethanol and the resin was used to ensure good infiltration of the resin into the tissue. After infiltration with the 1:1 epoxy resin/propylene oxide mixture, the tissue was incubated in fresh resin at 37°C to evaporate any remaining propylene oxide. The tissue pieces were subsequently

embedded in epoxy resin for 24 hours at 60°C. Thick sections (2 μm thick) were cut with a glass knife, stained with 0.1% toluidene blue and examined under the light microscope. Thin sections (60-80 μm) were then cut from the same block face with a diamond knife (Drukker), mounted on 100 mesh unsupported copper grids, stained with uranyl acetate and lead citrate and examined using a JOEL 2000 transmission electron microscope at X 2500, 5000, 10,000 and 40,000 magnification.

In situ effluent characteristics

The physical and chemical characteristics of the effluent were continuously measured throughout the *in situ* experiment by the sewage treatment works. All parameters were measured using Hach products and methods based on US EPA approved Standard Methods (Clesceri et al., 1998). Biological oxygen demand (BOD) and chemical oxygen demand (COD) were determined on homogenised, unfiltered, undecanted samples using the 5 day BOD test and the potassium dichromate COD method. Suspended solids were weighed from samples filtered through a 0.45 μm filter membrane and dried at 105°C. Ammonia was measured using the Nessler method and nitrogen and phosphate measurements were found by molecular absorption spectrophotometry using the Kjeldahl and Vanadomolybdophosphoric acid methods respectively.

Measurement of EDCs in effluent samples

The methodology used in this study for the identification and quantitation of estrogenic compounds is based upon the toxicity identification and evaluation method (TIE) described by Desbrow and co-workers (1998) with modifications. This work was undertaken by Dr. Craig McKenzie in Cork Institute of Technology. A brief overview of the methodology is described.

Sampling - A 20 l sample of effluent and lake water was collected from Athlone STW and Quigley's Marina, Lough Ree respectively. Methanol (2% of sample volume) was added to inhibit microbial activity. The samples were transported to the laboratory stored overnight at 4-6°C and filtered within 24 hours of receipt for the removal and calculation of suspended solids. A 5 l tap water blank and 5 l tap water samples spiked with a range of estrogenic chemicals were also filtered.

Extraction - The filtered sample was split into 4 x 5 l aliquots. Each 5 l aliquot was passed through a 5 g C18 solid phase extraction (SPE) cartridge (International Sorbent Technology, Hengoed, Wales, UK) at a flow rate of 10-20 ml min^{-1}. Following extraction the cartridges were dried for 2 hours to remove residual water. The chemicals adsorbed by the cartridges were sequentially eluted with solvents of decreasing polarity to produce 8 coarse fractions: A - H_20:methanol (75:25); B - H_20:methanol (50:50); C - H_20:methanol (20:80); D - H_20:methanol (10:90); E - methanol; F - diethyl ether; G - diethyl ether:hexane (50:50); H – hexane. These coarse fractions were then tested for estrogenic activity using the yeast estrogen screen (YES) bioassay which uses the yeast cell

Saccharomyces cerevisiae encoded with a human estrogen receptor to identify the presence of xenoestrogens.

Yeast Estrogen Screen (YES) – This bioassay detects both endogenous estrogens (17β-estradiol, estrone and estriol) as well as xenoestrogens (e.g. alkylphenolic compounds, phthalates, phytoestrogens, synthetic hormones and certain pesticides commonly present in environmental samples). The bioassay has been described in great detail elsewhere (Routledge and Sumpter, 1996; Jobling, 1998; Beresford et al., 2000). Yeast cells were obtained from Prof. J. Sumpter of Brunel University, London and the bioassay validated with a range of estrogenic and anti-estrogenic substances. The presence of estrogenic chemicals in sample extracts was confirmed via a colour change, the intensity of which formed a sigmoidal relationship with estrogenic chemical concentrations reported as ng l^{-1} 17β-estradiol equivalents. Steroidal hormones were the most potent and their detection limits are considerably lower than for other less potent estrogen mimics. Coarse SPE fractions showing estrogenic activity were retained for further fractionation.

Fine Fractionation – Estrogenically active coarse fractions were recombined, diluted in 2.5 l ultrapure water and re-extracted onto a single 5 g C18 SPE cartridge. The cartidges were dried and eluted with 2 x 15 ml of hot methanol (60°C) that were combined and evaporated to 1.2 ml. 600 µl was injected onto a semi-preparative HPLC system for fine fractionation. The HPLC system comprised of a Waters 2690 Separations Module fitted with a quaternary pump, large volume syringe and large volume sample loop. The injected sample components were separated using a 7.8 mm x 300 mm x 7 mm

SymmetryPrep C18 reverse phase column (Waters Corp, Massachusetts, U.S.A) with a flow rate of 3 ml min^{-1}. Gradient elution was used, starting with 40% methanol in water, holding for 3 min then increasing the methanol content to 100% after 30 min. This mobile phase was maintained for 10 min (total running time of 40 min). Separated sample components were detected using a Waters 996 photodiode array detector. Fractions eluting from the column were collected in 2 minute fractions (6 ml) using a Waters Fraction Collector 2. The retention times of 7 estrogenic chemicals were checked in duplicate prior to injection of sample extracts to qualitatively check column performance. These fine fractions were then tested for estrogenic activity using the YES bioassay (see above).

GC-MS analysis of active fine fractions – The active fractions were transferred to a separating funnel containing 300 ml of ultrapure water. The sample was liquid/liquid extracted twice with 30 ml dichloromethane. The extracts were dried with sodium sulphate and the volume reduced to 100 ml using both rotary and turbovap LV evaporation systems (Zymark Corp, Boston, U.S.A.). 400 ml of a deuterated polyaromatic hydrocarbon (PAH) internal standard mixture was added (0.3 mg l^{-1}) and the sample vialled and refrigerated until GC-MS analysis. GC-MS separations and compound identification were carried out using a Thermofinnigan GC8000 series gas chromatograph with AS800 autosampler, coupled to a Thermofinnigan MD1000 mass spectrometer. Sample components were separated using a 30 m x 0.25 mm x 0.25 mm DB5-MS column (J & W Scientific). The carrier gas (GC grade Helium, Irish Oxygen, Cork, Ireland) velocity was 1 ml min^{-1} and the sample injection volume was 2 µl. The GC program was as follows:

Injection mode: splitless. Injection port temp: 270°C. Transfer line temp: 280°C. Initial column temperature was 50°C, increasing to 170°C at 20°C min^{-1} then to 240°C at 3°C min^{-1} and to 280°C at 20°C min^{-1}. The column was held at the maximum temperature for 5 minutes. The GC-MS system was calibrated with 5 levels of calibration standards. The limit of detection for 17β-estradiol, estrone and 17α-ethinylestradiol was 4.5 ng l^{-1}.

Statistical analysis

The exposure of zebra mussels to effluent and to each NP concentration was performed in triplicate. Samples of 4 exposed and control mussels of either sex were analysed for ALP (n=8). For GE samples of 2 exposed and control mussels of either sex were analysed (n=4). Variability between the exposure tanks and between the control and exposed mussels was assessed for vitellin-like proteins for both GE and ALP data by one-way analysis of variance (ANOVA) using Microsoft Excel. These intra and inter-exposure differences in the condition index were also performed using ANOVA. Correlation analysis between ALP and GE methodologies was undertaken using Pearson-moment correlation. The level of significance was set at p=0.05.

RESULTS

General Physiological Health

In situ exposure

The mussels exposed to tertiary treated effluent appeared to be in good health throughout the experiment. Mortality was very low with only 7 mussels dying out of the 750 exposed (0.9% of exposed population). The sub-lethal parameter of gaping of the bivalve shells due to inactivity of the adductor muscle (Swedmark et al., 1971) was infrequently observed. Mussel attachment, another sub-lethal parameter, was very good with fewer than 3 mussels per tank not attached on each sample day (less than 1.2% of the exposed population). No significant ($p>0.4$) intra exposure tank difference in the mussels condition was measured between the 3 exposure tanks by either condition index. (Appendix 3.2). When the condition of the exposed mussels was compared with that of the control mussels using the dry : wet weight ratio condition index, no significant ($p>0.6$) difference in the condition was observed. However using the dry : shell weight ratio a highly significant ($P<0.0001$) decrease in condition was observed for the effluent exposed mussels. This was the more sensitive of the two indices and indicated that exposure of zebra mussels to tertiary treated effluent caused a significant decline in the mussels condition owing to a decline in flesh weight.

***In vivo* NP exposure**

During the *in vivo* experiment the mussels did not appear to be in such good health as they were in the *in situ* exposure. As the exposure progressed increased numbers of mussels became unattached from their tiles indicating poor byssal thread activity, another sub-lethal parameter of mussel health (Swedmark et al., 1971). However gaping of bivalve shells was not often observed. Mortality rates of 8% and 18% for the 5 $\mu g\ l^{-1}$ and 500 $\mu g\ l^{-1}$ exposed mussels (respectively) were not significant ($p>0.1$) when compared with the controls (10% and 12% mortality for control and solvent control respectively). However there was a significant ($p=0.02$) difference in mortality between the 5 $\mu g\ l^{-1}$ and 500 $\mu g\ l^{-1}$ exposed mussels.

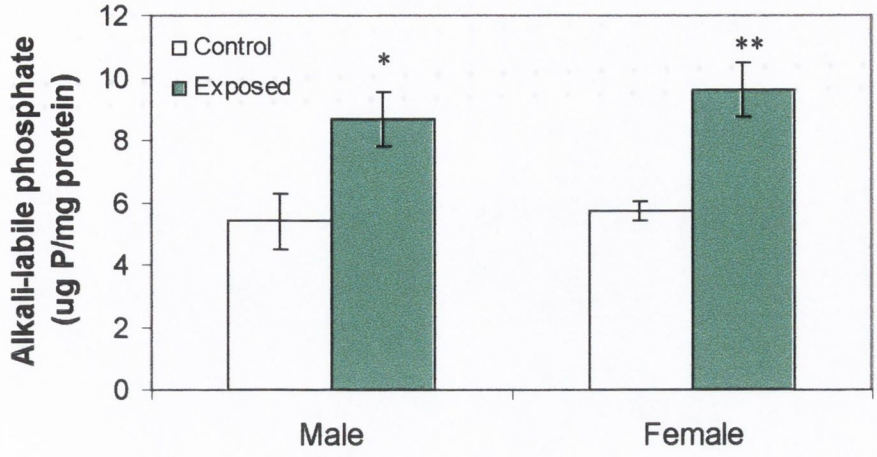
There was no significant ($p>0.05$) intra-exposure variation in the condition of the organisms as neither index showed a significant difference between the 3 tanks used for each exposure concentration (Appendix 3.3). As was seen with the *in situ* exposure the dry weight : wet weight ratio showed no significant ($p>0.1$) difference between the condition of the control mussels and those exposed to NP (Appendix 3.4(a)). This indicates that there was no difference in the water body content of the exposed mussels. However using the dry weight : shell weight ratio a significant decrease ($P<0.05$) in condition was found for the 500 $\mu g\ l^{-1}$ NP exposed mussels when compared with the control and the 5 $\mu g\ l^{-1}$ NP exposed mussels (Appendix 3.4(b)). This indicated a decrease in the flesh weight for mussels exposed to 500 $\mu g\ l^{-1}$ NP. As with the *in situ* exposure, this was the more sensitive of the two indices and showed an extremely significant ($p=0.00002$) decrease in condition for the control mussels used in the experiment when

compared with a sample of wild mussels taken from the original population living in their natural environment in Lough Ree (Appendix 3.5). However the dry : wet ratio index also showed a highly significant (p=0.009) decease in condition (Appendix 3.5). This indicates that the experimental design was not ideal for the animals and that even in the control mussels condition significantly declined.

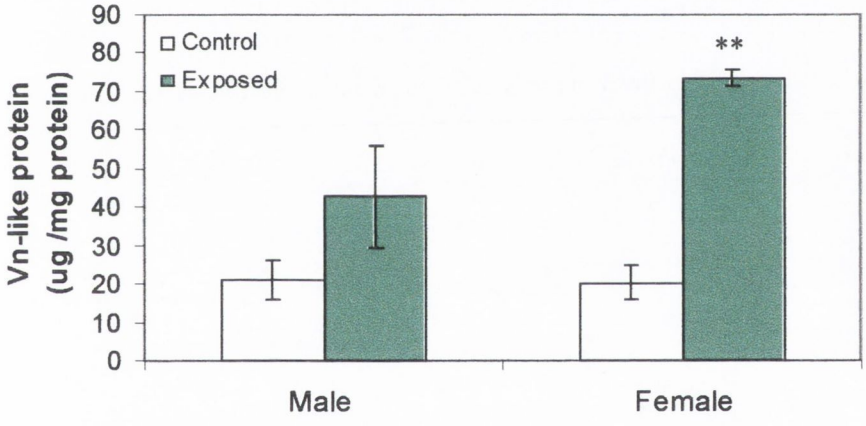
Effect on Vn-like protein

In situ exposure

Vn-like proteins measured indirectly by the ALP assay and directly using GE were standardised against mussel total protein. Both techniques showed an increase in the level of Vn-like proteins in male and female mussels exposed to tertiary treated effluent (Figure 3.4). For the ALP data this increase was statistically significant for both male and female exposed mussels (p=0.05 and 0.01 respectively), with the exposed females having slightly higher ALP levels than the exposed males (Figure 3.4(a)). These findings were confirmed by GE as similar results were found in both sets of data. Although an increase in Vn-like proteins measured by GE was found in both male and female exposed mussels, it was only significant in the females (p=0.008) (Figure 3.4(b)). From the GE data effluent exposure had a greater effect on the protein levels in female mussels as larger increases in Vn-like protein levels were recorded in the females. However the increase in exposed female Vn-like protein is not significant (p=0.1) compared with the male exposed level. Both ALP and GE data show relatively similar levels of Vn for both male and female control mussels.



(a).



(b).

Figure 3.4. Effect of 112d *in situ* exposure to tertiary treated effluent on Vn-like levels of male and female zebra mussels standardised against total protein. **(a).** Increase in Vn-like proteins measured by the alkali-labile phosphate assay. **(b).** Increase in Vn-like proteins measured using gel electrophoresis. P values calculated using one way ANOVA. Level of significance * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

Similar results were found when the data was normalised against mussel volume (mm^3) (Figure 3.5). For the *in situ* ALP data standardised against volume a significant increase in Vn-like proteins occurred in both male and female exposed mussels ($p= 0.04$ and 0.0009 respectively) (Figure 3.5(a)). While a marginally significant ($p = 0.08$ and 0.09 respectively) increase in Vn-like proteins was measured in both male and female exposed mussels using gel electrophoresis (Figure 3.5(b)).

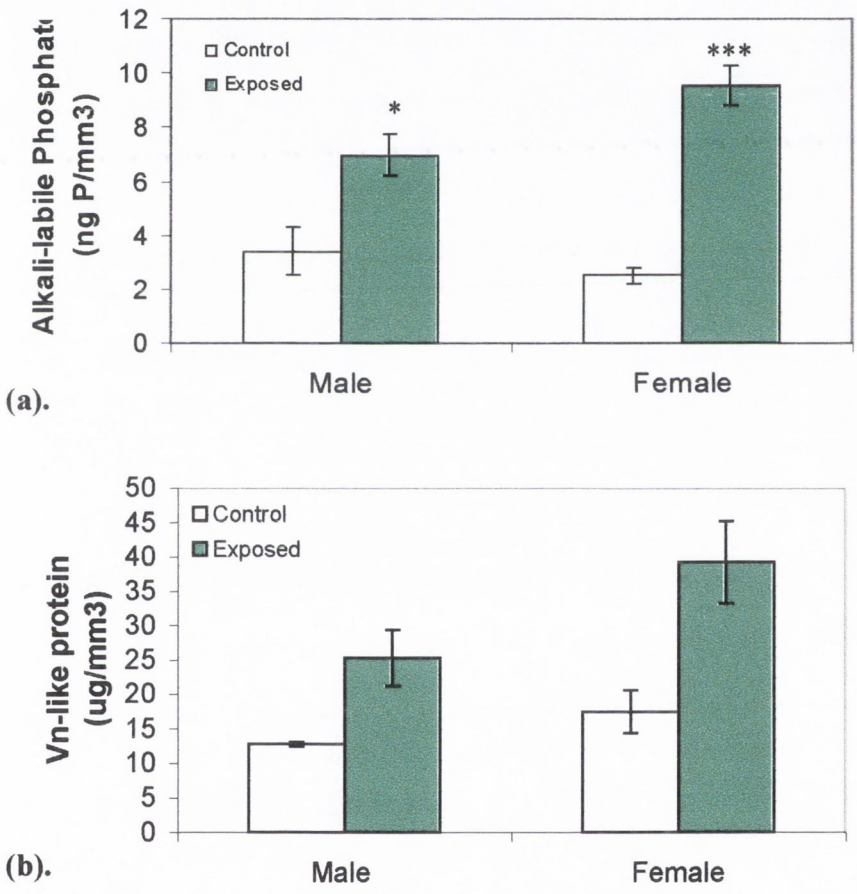
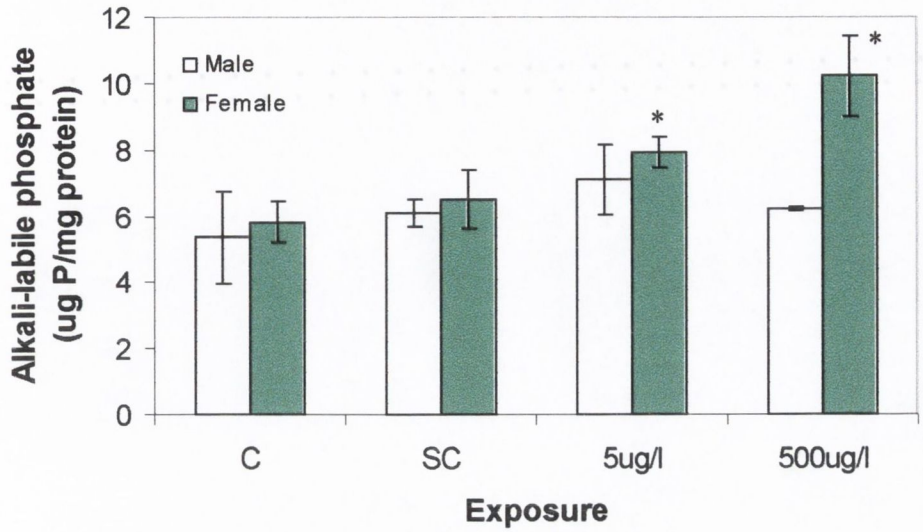


Figure 3.5 Effect of 112d *in situ* exposure to tertiary treated effluent on Vn-like levels of male and female zebra mussels standardised against mussel volume. **(a).** Increase in Vn-like proteins measured by ALP. **(b).** Increase in Vn-like proteins measured using GE. Level of significance * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

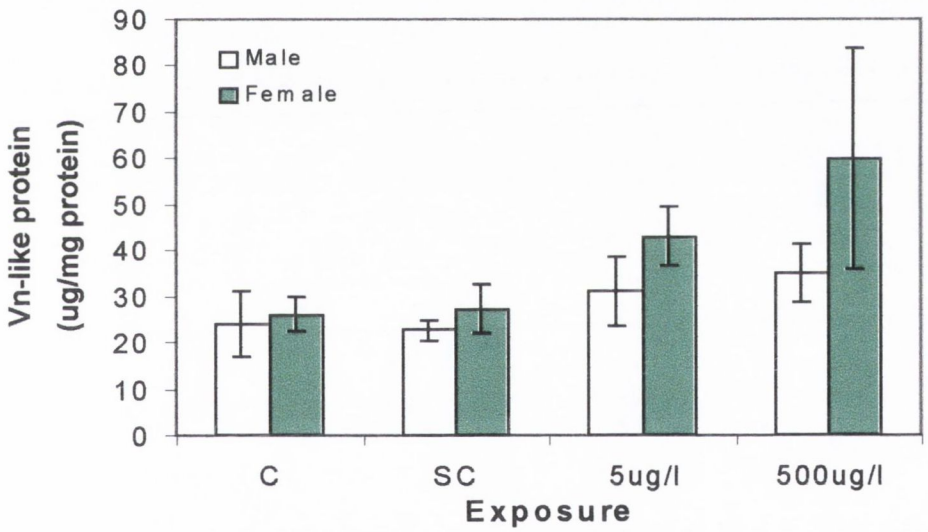
In vivo NP exposure

For the NP exposed mussels Vn-like proteins were also measured by ALP and GE and were standardised against total protein (Figure 3.6). For the ALP data a dose response increase in lipophosphoproteins with exposure to NP was evident, with a significant increase in ALP in female mussels exposed to 5 $\mu g\ l^{-1}$ and 500 $\mu g\ l^{-1}$ NP ($p=0.03$ and 0.02 respectively). Although an increase in ALP levels in male mussels exposed to 5 $\mu g\ l^{-1}$ NP was also evident (Figure 3.6(a)), this increase is not significant ($p=0.4$). A surprisingly low level of ALP (6.2 μg P/mg protein) is found in male mussels exposed to 500 $\mu g\ l^{-1}$ NP, significantly lower ($p=0.03$) than the ALP levels recorded for females exposed to the same dose (Figure 3.6(a)). This indicates that male zebra mussels do not respond to xenoestrogen exposure by ALP stimulation to the same extent as female mussels.

As in the ALP data the largest increase in Vn-like proteins measured by GE was seen in the female exposed mussels, although owing to large error bars these increases were not statistically significant from the control ($p>0.1$) (Figure 3.6(b)). GE measures only a slight increase in Vn-like proteins for male mussels after NP exposure. As with the *in situ* data above female mussels have responded to xenoestrogen exposure with a larger increase in Vn-like proteins. Figure 3.6(b) shows very little difference in Vn-like protein levels between male and female control mussels ($p=0.8$). However after exposure to 500 $\mu g\ l^{-1}$ NP the difference becomes much greater ($p=0.4$) due to increased Vn-like protein levels in the females.



(a)



(b).

Figure 3.6. Effect of 112d *in vivo* exposure to 5 μg l^{-1} and 500 μg l^{-1} NP on Vn-like levels in male and female zebra mussels standardised against total protein. **(a).** Increase in Vn-like proteins measured by the alkali-labile phosphate assay. **(b).** Increase in Vn-like proteins measured using gel electrophoresis. Level of significance * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$. C = control; SC = solvent control.

When normalised against mussel volume (mm^3) similar trends were observed in both the ALP and the GE *in vivo* exposure data (Figure 3.7). For the ALP data females exposed to 500 $\mu g\ l^{-1}$ NP showed a marginally significant ($p=0.07$) increase in ALP (Figure 3.7(a)). While the GE data showed a significant increase in Vn-like proteins in male mussels exposed to 500 $\mu g\ l^{-1}$. This data also showed significantly ($p=0.01$) higher levels of Vn-like proteins in female control mussels compared to the male controls (Figure 3.7(b)).

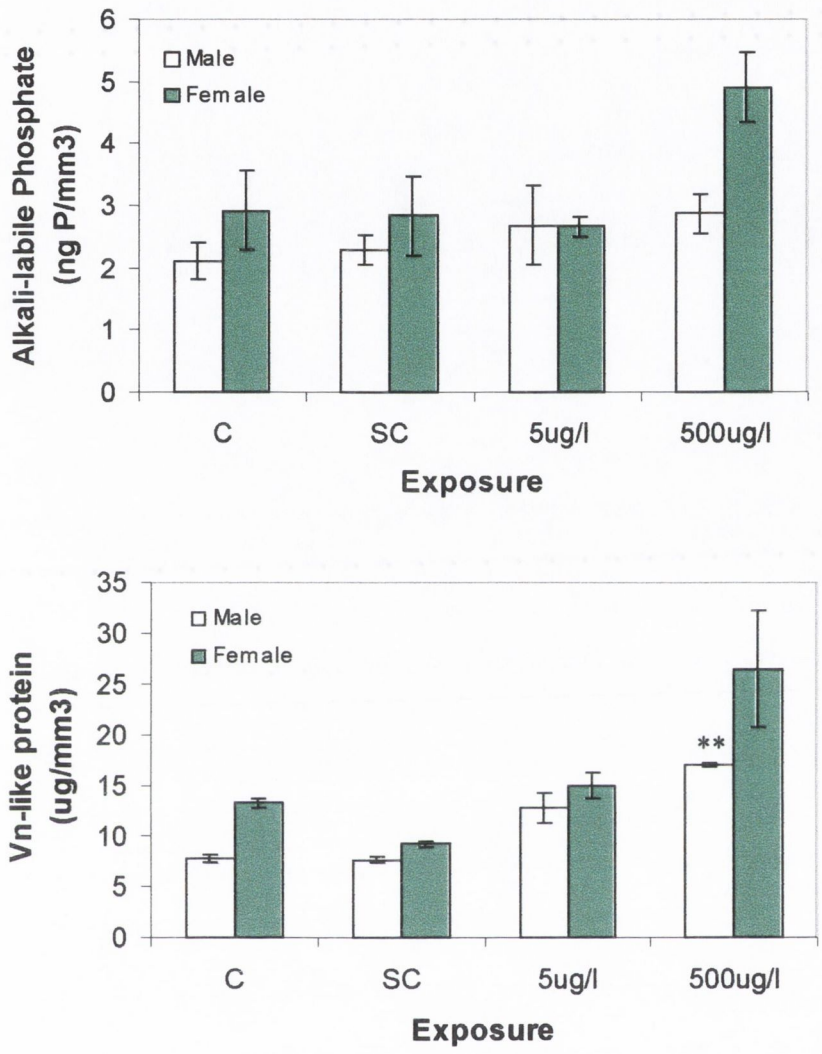
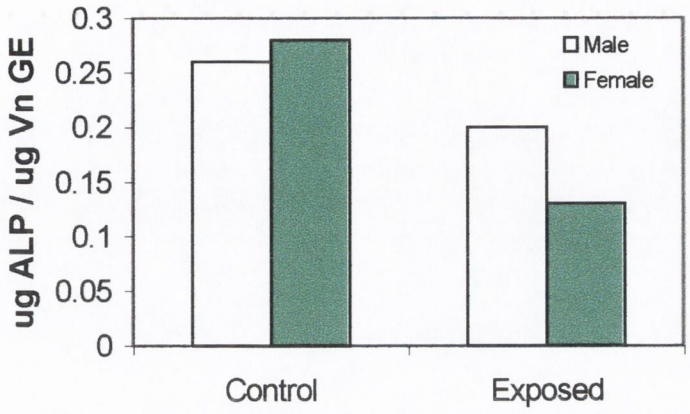
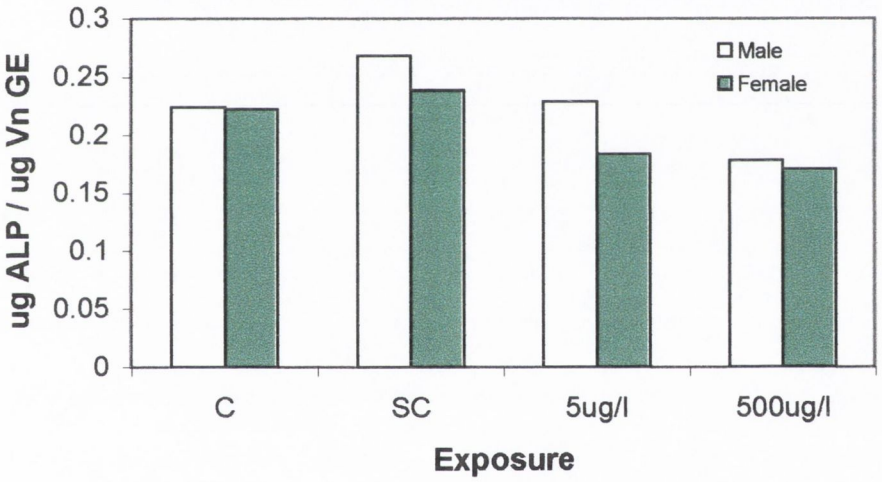


Figure 3.7. Effect of 112d *in vivo* exposure to 5 μg l^{-1} and 500 μg l^{-1} NP on Vn-like levels in male and female zebra mussels standardised against mussel volume. **(a).** Increase in Vn-like proteins measured by ALP. **(b).** Increase in Vn-like proteins measured using GE. Level of significance * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$. C = control; SC = solvent control.

Correlation analysis between these two methods of measuring xenoestrogenic response (ALP and GE) in zebra mussels for both exposure to NP and effluent *in situ* revealed that a highly significant correlation ($r=0.9$, $p=0.002$) existed between ALP and GE standardised against total protein. This suggests that both methods are related and that the state of phosphorylation of proteins is fairly constant. When the concentration of ALP is expressed per unit of Vn-like proteins measured by GE normalised against total protein, a decrease in the state of phosphorylation seems to occur after exposure to sewage effluent (Figure 3.8(a)) and after exposure to 500 $\mu g\ l^{-1}$ NP (Figure 3.8(b)). This response may indicate a toxic effect of these exposures.



(a).



(b).

Figure 3.8. State of phosphorylation in male and female zebra mussels calculated as ALP / VnGE normalised for total protein. **(a).** *In situ* exposed mussels **(b).** Mussels exposed to NP *in vivo*. C = control; SC = solvent control.

Estradiol binding sites and competitive binding study

Estradiol-17β was added to the samples in order to find a saturation curve of E2 binding to the gill cytosol. After linear transformation the slope of this response curve was used to find the dissociation constant (K_d) used to characterise the estrogen receptors (ER). However no saturation occurred with the conditions of the experiment so that the K_d value calculated is likely to be an overestimation. Preliminary results show a K_d of about 1.26 nM (pmole ml^{-1}) for ER in the gill of the zebra mussel.

E2 binding did occur in the gills of the zebra mussel and this was partially reversed by estradiol and NP. Preliminary results show cytosols pre-incubated with NP prior to addition of E2-albumin-fluorescein reduced the quantity of estradiol bound to the estrogen receptor binding sites. This suggests that NP is capable of competing with E2 for estrogen-binding sites in zebra mussel gill cytosols.

Cholesterol levels measured by HPTLC

Cholesterol levels were measured in the homogenate of 5 exposed and control mussels from both experiments using HPTLC. On the HPTLC plate a large band of cholesterol was obvious (Figure 3.3), eluting with a retention factor (R_f) of 0.45. From this band a clear response to sewage effluent exposure was evident with cholesterol levels more than doubling from 3.99 μM g^{-1} in control mussels to 9 μM g^{-1} protein in the exposed mussels (Figure 3.9(a)).

In the *in vivo* exposure to NP comparatively low levels of cholesterol (8.8 μM g^{-1} protein) were found in the controls when compared to the NP exposed mussels. This level doubled to 16 μM g^{-1} protein after exposure to 5 μg l^{-1} NP and increased 2.3 times to 20.5 μM g^{-1} protein after exposure to 500 μg l^{-1} NP (Figure 3.9(b)). The cholesterol levels in the control mussels used in the NP exposure were considerably higher than those for the *in situ* exposure control mussels. This could be due to the mussels used in the NP exposure being a month older and further into their reproductive cycle than the *in situ* exposed control mussels.

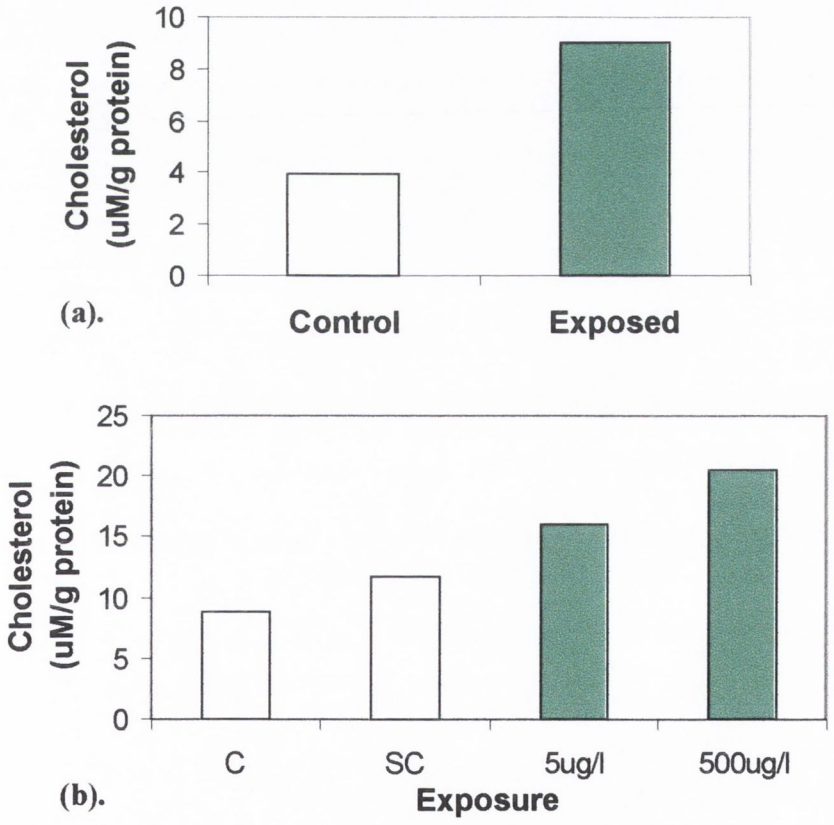
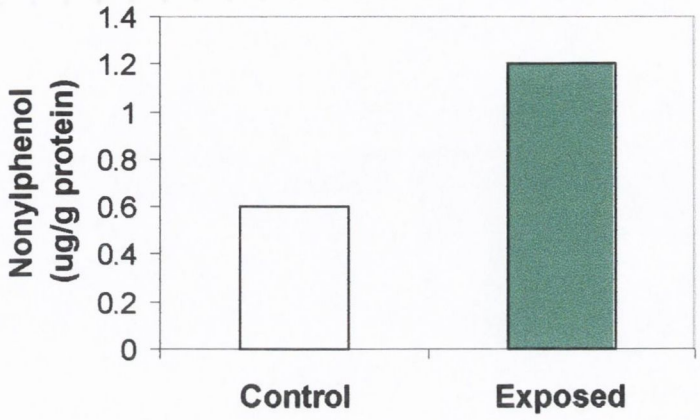


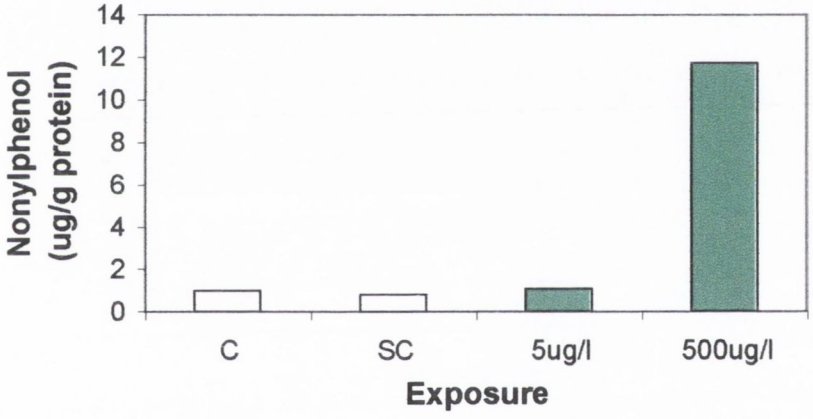
Figure 3.9. Cholesterol levels measured from mussels using HPTLC. **(a).** Cholesterol levels from *in situ* control and mussels exposed to tertiary treated effluent. **(b).** Cholesterol levels for the *in vivo* control and mussels exposed to 5 μg l^{-1} and 500 μg l^{-1} NP. C = control; SC = solvent control.

Nonylphenol levels measured by HPTLC

HPTLC was also used to measure the amount of NP in the mussel homogenate. NP produced a second more polar band than cholesterol, which eluted further up the plate with an R_f value of 0.73 (Figure 3.3). For the *in situ* exposed mussels a relatively low concentration (0.6 μg NP g^{-1} protein) of NP was found in the control mussels taken from Lough Ree. This concentration doubled with 1.2 μg NP g^{-1} protein found in the soft body homogenate of mussels exposed to effluent (Figure 3.10(a)), indicating that both the *in situ* control and exposed mussels bioaccumulated NP. In the NP exposed mussels the control, solvent control and mussels exposed to 5 μg l^{-1} NP had a relatively constant level of 0.8 - 1.1 μg NP g^{-1} protein (Figure 3.10(b)). This concentration was considerably higher (11.7 μg NP g^{-1} protein) for the soft body homogenate of mussels exposed to 500 μg l^{-1} NP.



(a).



(b).

Figure 3.10. Levels of NP found in zebra mussel homogenate, measured using HPTLC. **(a).** Level of NP found in the *in situ* control and mussels exposed to tertiary treated effluent. **(b).** NP level found in the *in vivo* control and mussels exposed to 5 μg l^{-1} and 500 μg l^{-1} NP. C = control; SC = solvent control.

Sewage effluent characteristics

Biological, physical and chemical parameters of the effluent were continuously measured throughout the experiment and are summarised in Table 3.1. Using the biological quality assessment index the effluent has a biotic index rating of Q1 (Class D) indicating a serious pollution status (EPA, 2000b). However the effluent readily meets the present legislative requirements, with BOD_5 values ranging from <5 mg l^{-1} to 21 mg l^{-1} (limit value: 25 mg l^{-1}), COD values ranging from 10 mg l^{-1} to 46 mg l^{-1} (limit value 125 mg l^{-1}) and total nitrogen values of between 0.89 mg l^{-1} and 1.32 mg l^{-1} (limit value 15 mg l^{-1}) (EPA, 2000b). But total phosphate which measured between 0.96 mg l^{-1} to 3.39 mg l^{-1} did exceed the 2 mg l^{-1} limit value.

Table 3.1. Biological, physical and chemical parameters of the tertiary treated effluent that zebra mussels were exposed to during the *in situ* exposure. COD = chemical oxygen demand; BOD = biological oxygen demand; SS = suspended solids; TKN = total nitrogen (sum of kjeldahl nitrogen); TP = total phosphate. * = data from EPA (2000b).

	Temp. °C	pH	COD mg l^{-1}	BOD mg l^{-1}	SS mg l^{-1}	Nitrate mg l^{-1}	Phosphate mg l^{-1}	Ammonia mg l^{-1}	TKN mg l^{-1}	TP mg l^{-1}
Sample range	- 8 to 20	6.7 to 7.5	10 to 45	<5 to 21	<5 to 15	11 to 43	0.5 to 1.5	0.39 to 4.32	0.89 to 1.32	0.96 to 3.39
Limit value *			125	25	35				15	2

ED properties of effluent

The concentration of suspended solids in the tertiary treated effluent sample was relatively low (1.45 mg l^{-1}) for effluent from a STW. During extraction the C18 cartridges turned bright green, indicating the presence of green dyes added to household cleaning products (bleaches, toilet blocks etc), a good indicator of pollution from domestic wastewater effluent. Of the 8 coarse fractions (A-H) eluted from the cartridges with solvent mixtures ranging from polar to non-polar, all except the non polar hexane showed some degree of estrogenic activity using the YES assay (Figure 3.11). Fractions A-G were therefore recombined, re-extracted and fine fractionated using semi-preparative HPLC.

Following system suitability checks with a standard containing 7 estrogenic compounds (Figure 3.12(a)), 600 ml of the sample was injected onto the semi-prep HPLC system. The HPLC chromatograms of the system suitability solution and the tertiary effluent extract are shown in Figures 3.12(a) and (b) respectively. The chromatogram revealed that the sample contains a complex mixture of compounds any one of which may elicit estrogenic activity. The fractions eluted from the HPLC were therefore tested using the YES assay and the results are shown in Figure 3.12(c). The vast majority of the estrogenic activity occurred in a single fraction eluting between 20 and 22 minutes (fraction 10). The retention time of this estrogenic fraction coincides with the retention time for 3 compounds in the calibration standard (Figure 3.12(a)): 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and Bisphenol A, indicating that these 3 compounds accounted for the majority of the estrogenic activity of the sample.

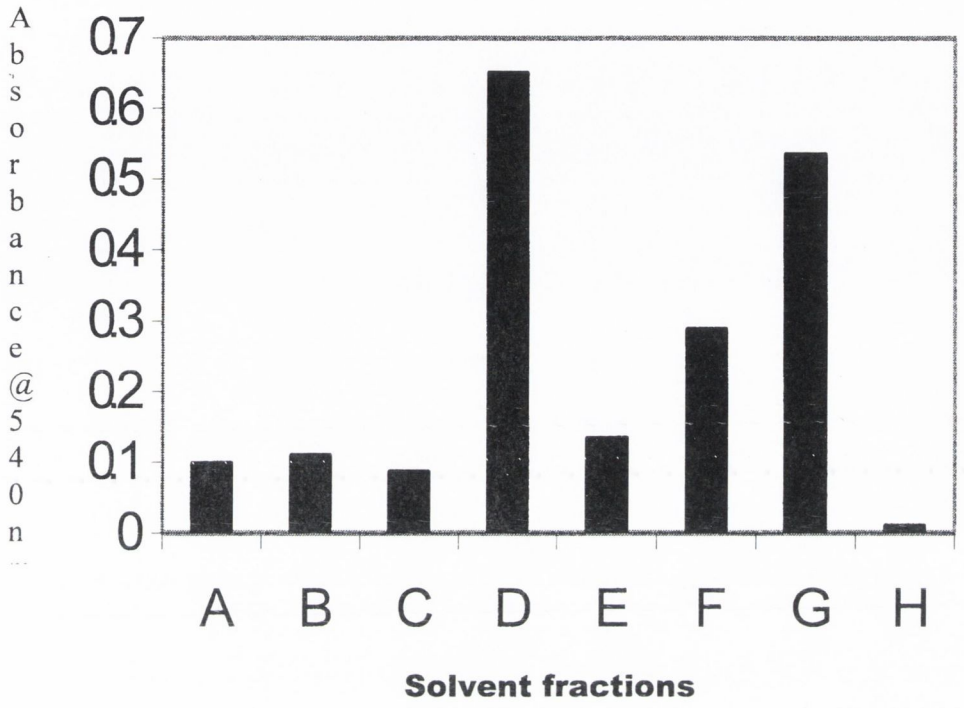


Figure 3.11. Results of qualitative YES bioassay analysis, estrogenic activity in the yeast screen following incubation of a 20 µl sample aliquot of each collected coarse SPE fraction. The fractions are: A - H_20:methanol (75:25); B – H_20:methanol (50:50); C - H_20:methanol (20:80); D - H_20:methanol (10:90); E - methanol; F - diethyl ether; G - diethyl ether:hexane (50:50); H – hexane.

No activity was observed in the method blank while the majority of the activity in the spiked water was observed in the fraction containing the steroidal hormones. Attempts to identify these compounds in the sample using GC/MS were unsuccessful and further work needs to be undertaken to confirm they are present in the effluent.

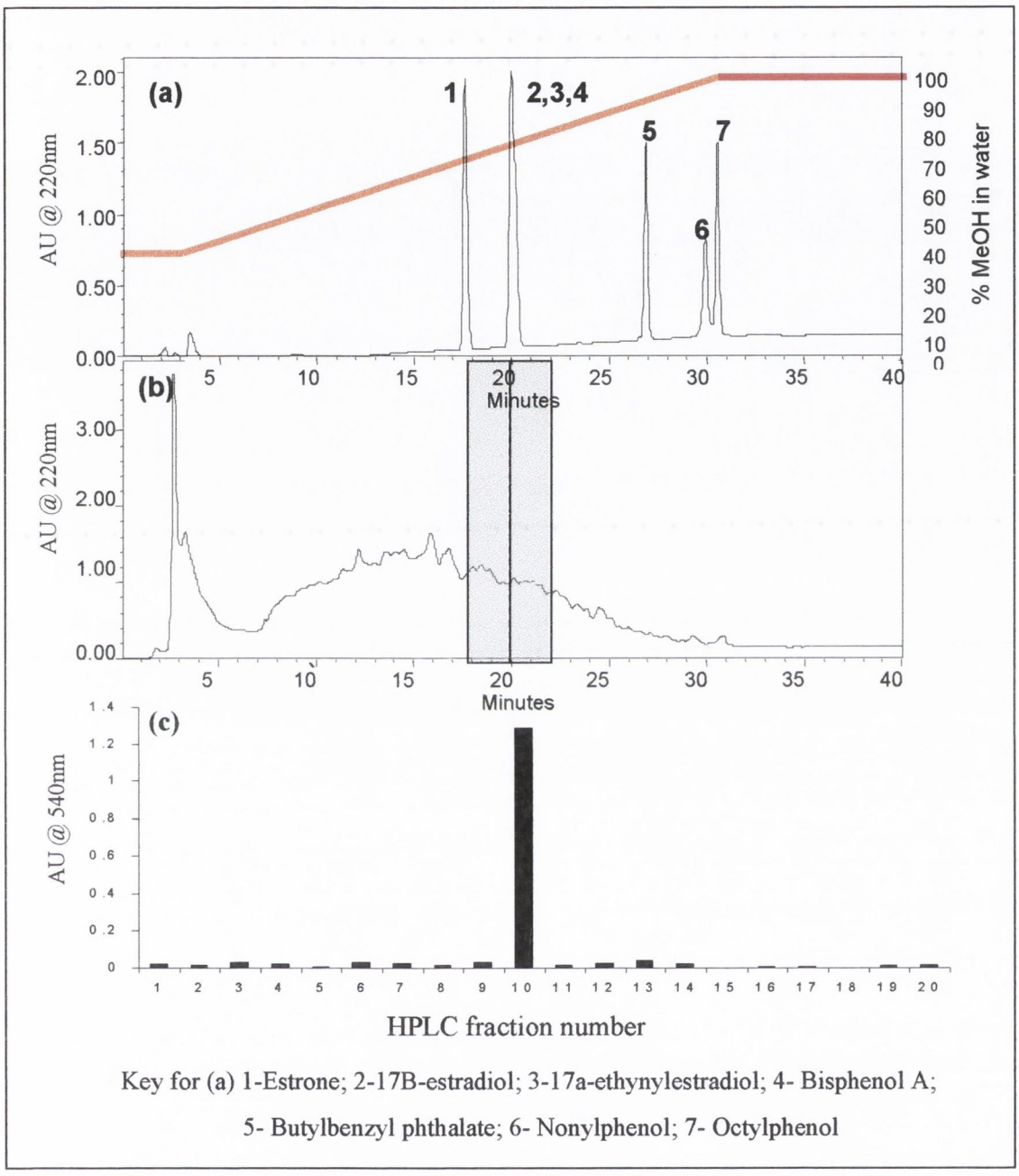


Figure 3.12. Fine fractionation of the tertiary treated effluent exposed to the zebra mussels *in situ* by semi-preparative reverse phase HPLC. **(a).** UV absorbance (AU) profile of a calibration standard showing HPLC gradient program. **(b).** UV absorbance (AU) profile of effluent showing timed fractions collected (20 x 20min fractions). **(c).** Results of qualitative YES bioassay analysis – estrogenic activity in the yeast screen following incubation of a 10 µl sample aliquot of each collected HPLC fraction.

Lake water taken from the zebra mussel population site in Quigley's marina, Lough Ree was also subjected to fine fractionation by semi-preparative reverse phase HPLC as above. The sample showed similar results to the tertiary treated effluent with the same HPLC fraction showing estrogenic activity using the YES bioassay (Figure 3.13). These results indicate that the water in the marina has a similar estrogen concentration to the tertiary treated effluent from the STW.

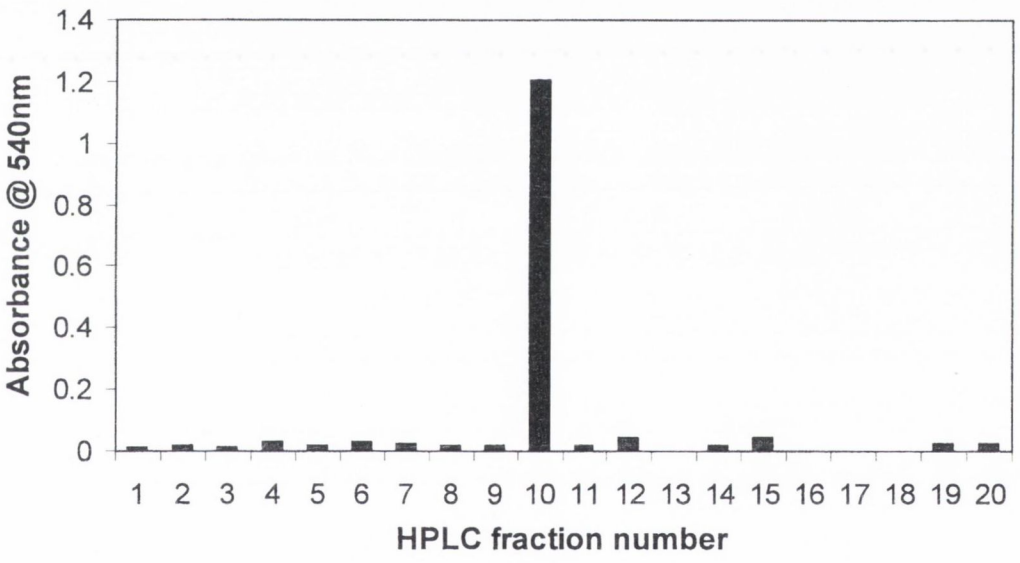


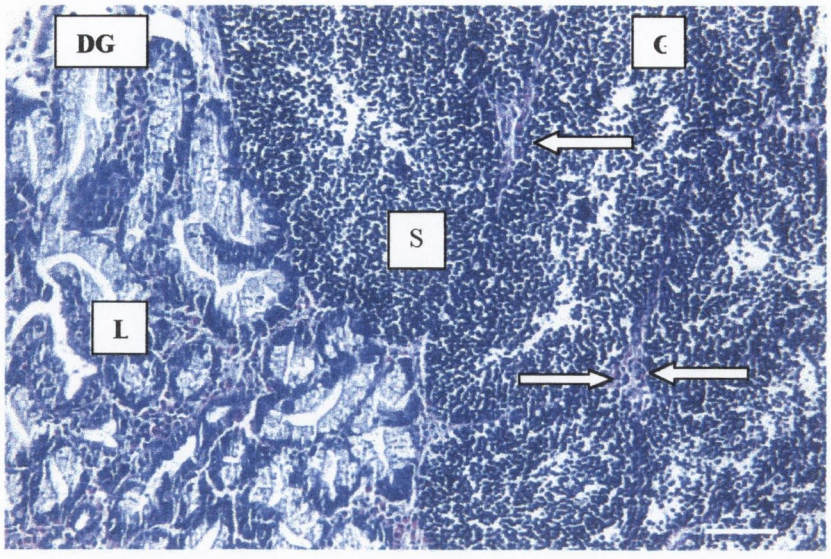
Figure 3.13. Results of the qualitative YES bioassay analysis of a sample of lake water taken from Quigley's marina. Estrogenic activity is measured by UV absorbance in the yeast screen following incubation of a 10 µl sample aliquot of each of the collected HPLC fractions.

Histological analysis

Light microscopy

In situ exposure

Quite severe effects were seen in tissues of mussels exposed to tertiary treated effluent, where general toxicological responses such as extreme vacuolation and enlargement of the lumen were seen in the tubules of the digestive gland (Figure 3.14(b&d)). There was also a morphological effect observed in the gonad as male mussels exposed to effluent showed a large increase in interstitial tissue between the seminiferous tubules (Figure 3.14). This was consistently larger in male mussels exposed to municipal effluent than in control mussels. Although both exposed and control male and female mussels were in the early active (stage B) phase of gonad development dominated by spermatogonids and spermatocytes in male and ovogonites and ovocytes in pre-vitellogenesis in female (Wang & Denson, 1995), exposed males appeared to contain fewer and less developed spermatogonids and spermatocytes than the controls, with a reduction in the area of the spermatocyte producing seminiferous tubules (Figure 3.14(b&d)). These photographs clearly show evidence of disruption of the male gonad after prolonged (112 day) exposure to tertiary treated effluent.



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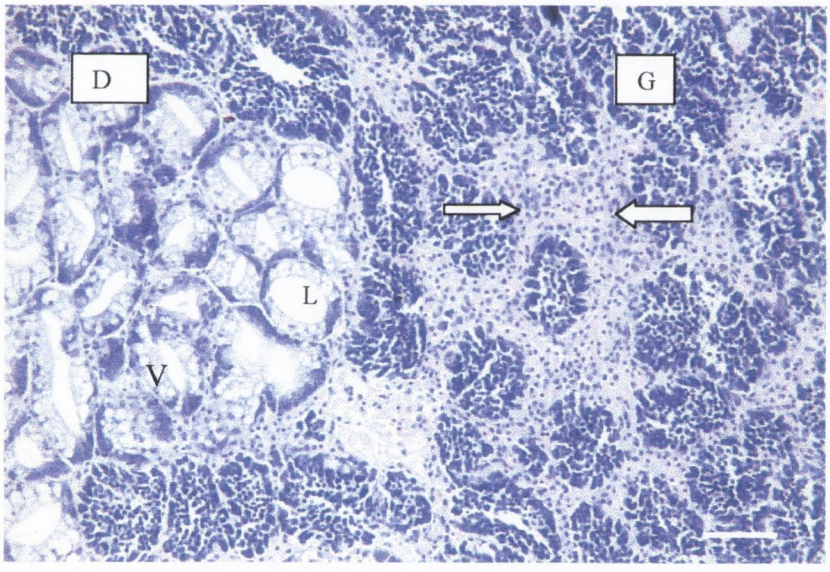
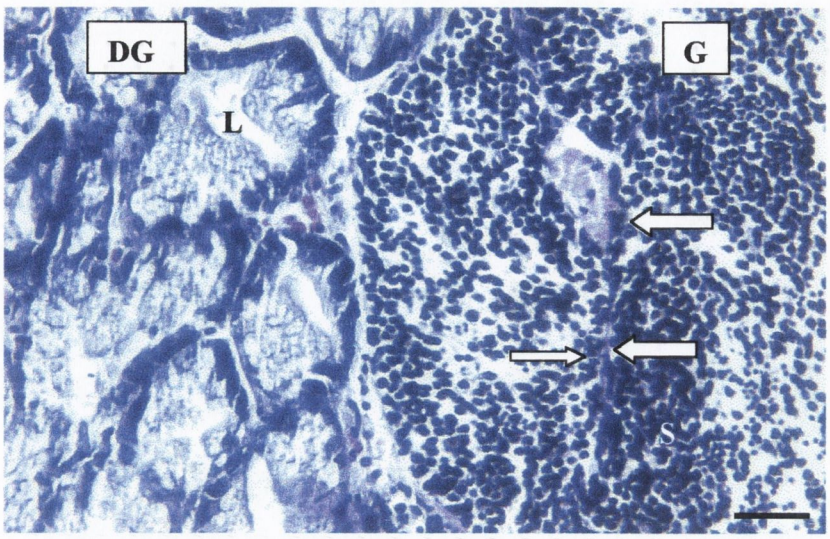
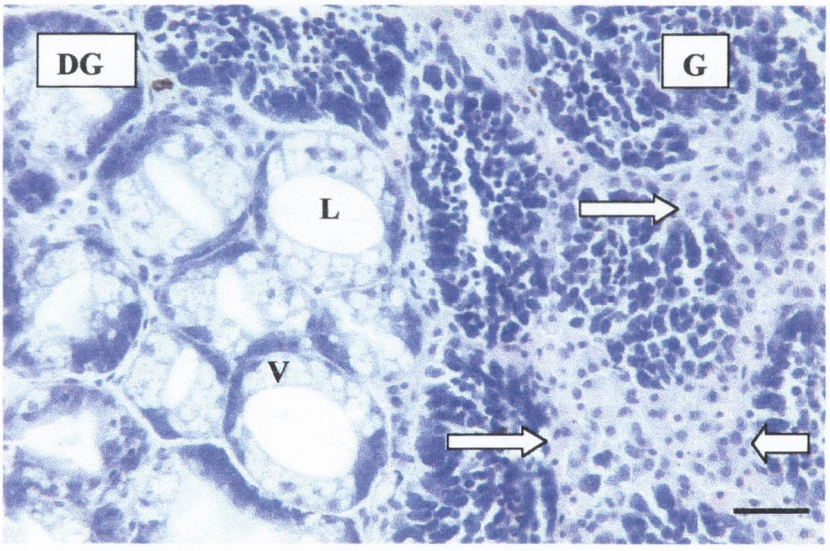


(b).

Figure 3.14. Series of photographs showing the effect of exposure to tertiary treated effluent on the development of the male gonad in the zebra mussel. **(a).** Visceral mass containing gonad (G) and digestive gland (DG) in a control male from Lough Ree. The tubules of the digestive gland have a regular shape with a narrow lumen (L). The seminiferous tubules of the gonad contain spermatocytes (S) and a narrow band of interstitial tissue (arrows). **(b).** Viseral mass of male mussel exposed to effluent for 112d. The digestive gland tubules have a greatly enlarged lumen and increased vacuolisation. The seminiferous tubules in the gonad are reduced in size with a large increase in the interstitial tissue (arrows). X 200 haemotoxylin and eosin. Scale bar = 100 μm.

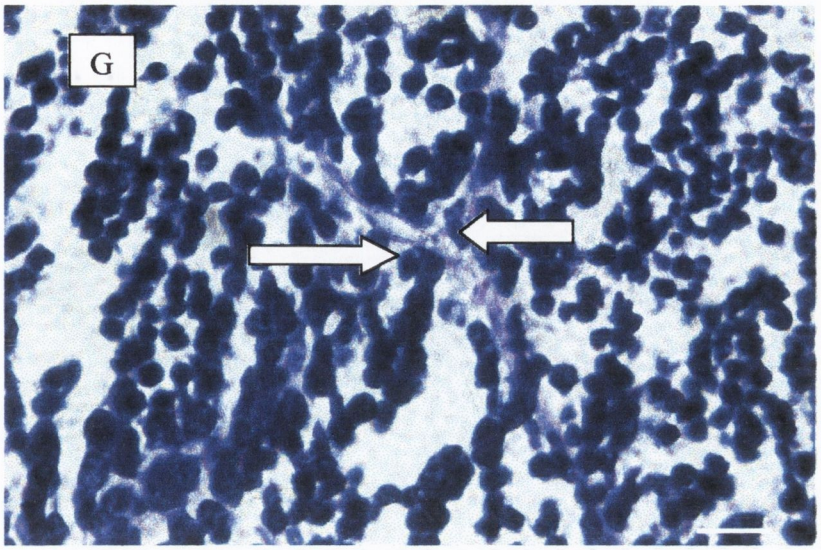


(c).

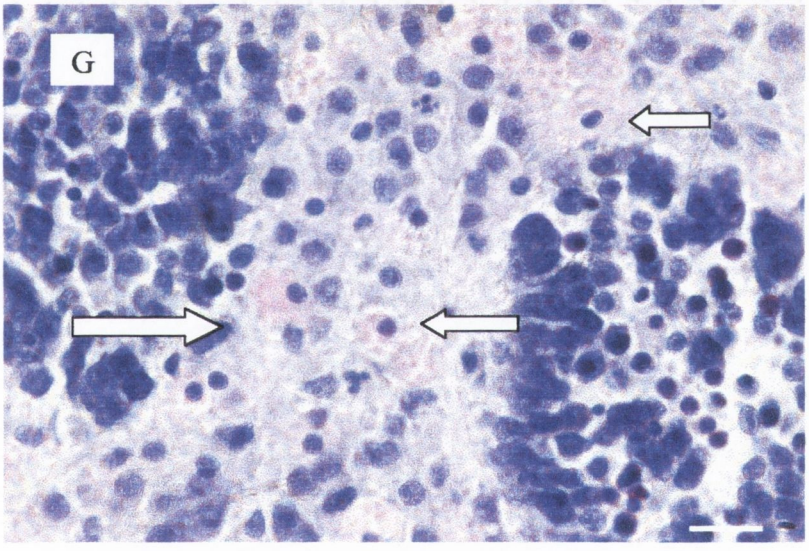


(d).

Figure 3.14. (c). Control mussel from Lough Ree showing male gonad (G) containing spermatocytes (S) and a narrow band of interstitial tissue (arrows). The tubules of the digestive gland (DG) have a narrow lumen and a regular shape. **(d).** Exposed male mussel showing enlarged interstitial tissue (arrows) and a reduction in the size of the seminiferous tubules in the gonad. The tubules of the digestive gland have greatly enlarged lumens (L) and contain many vacuoles (V). X 400 haemotoxylin and eosin. Scale bar = 40 μm



(e).

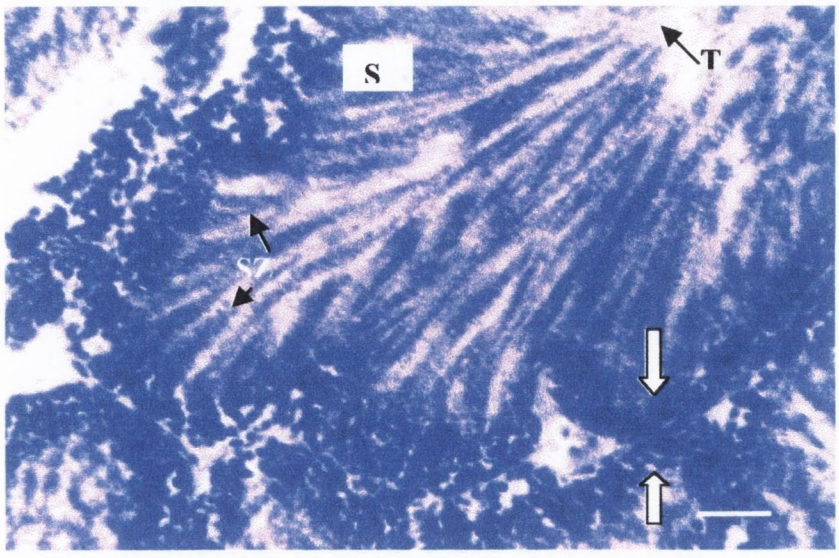


(f).

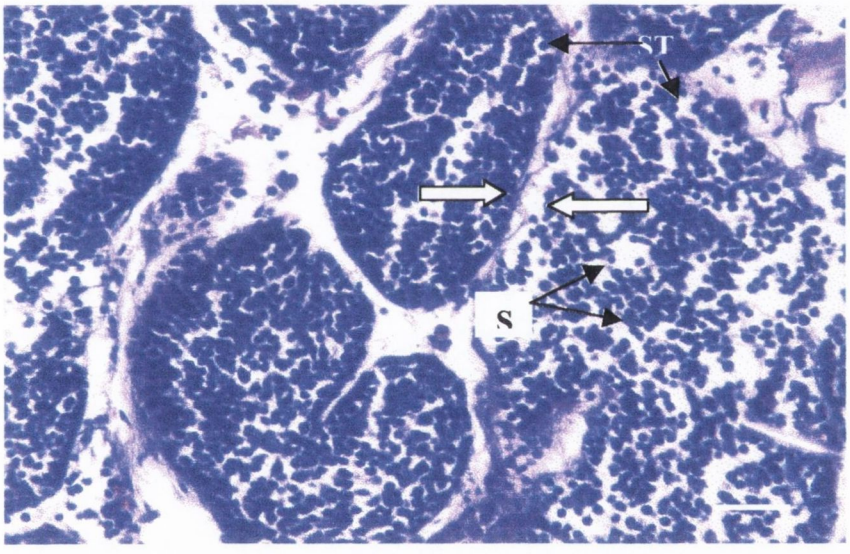
Figure 3.14. (e). Gonad of control male mussel showing spermatocytes and a thin interstitial tissue between the seminiferous tubules (arrows). **(f).** Exposed male with greatly enlarged interstitial tissue reducing the size of the spermatocyte producing seminiferous tubules. X 1000 haemotoxylin and eosin. Scale bar = 15 μm.

In vivo NP exposure

The gonads of the mussels used in the *in vivo* laboratory exposure were less developed than those in the *in situ* exposure, despite being a month older and further into their reproductive cycle. It would appear that gonad development slowed down in the mussels kept under laboratory conditions. This has been noticed in other experiments (Bielefeld, 1991; Cajaraville, et al., 1991) where male gonad development has been delayed and female mussels were noticed to reabsorb developing oocytes and maintain developed oocytes in the gonads. Figure 3.15 shows the developed spermatozoids in a control mussel taken from Lough Ree, stage C1 in development. In comparison to the gonad from the control mussel used in the *in vivo* exposure showing only stage B development with only developing spermatocytes being present. These mussels were the same age, were from the same population and should have the same stage of reproductive development.



(a).



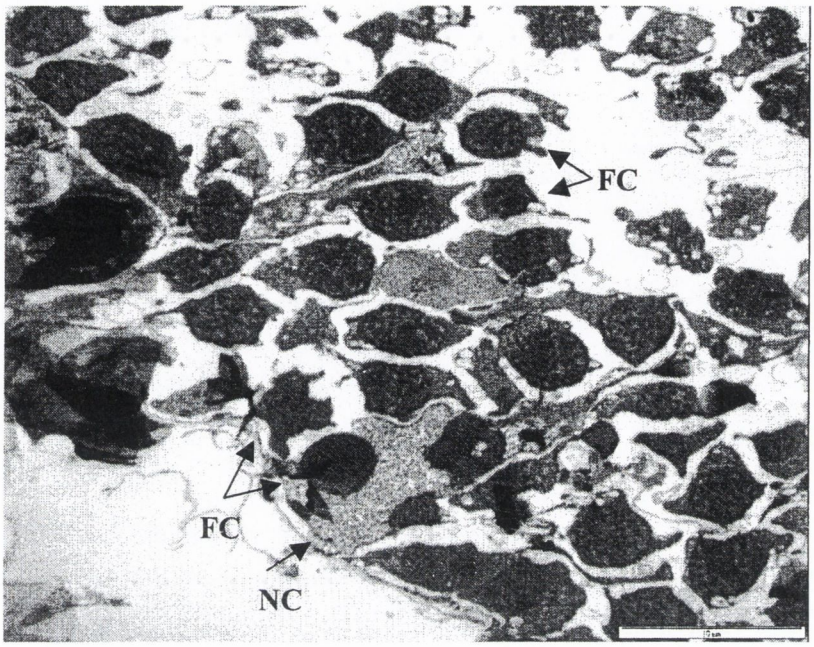
(b).

Figure 3.15. The effect on gonad development of the maintenance of zebra mussels in laboratory *in vivo* conditions. **(a).** Control mussel taken from Lough Ree. The seminiferous tubules (ST) in the gonad contain well developed spermatozoids (SZ) with their tails (T) pointing into the lumen of the tubule. The follicle cell wall is thick and well developed (arrows), stage C1 of development. **(b).** Gonad of control mussel used in the *in vivo* exposure after 112d. The seminiferous tubules (ST) look empty, containing few spermatocytes (S). The wall of the seminiferous tubule is very thin (arrows), stage B gonad development. X 400 haematoxylin and eosin. Scale bar = 40μm.

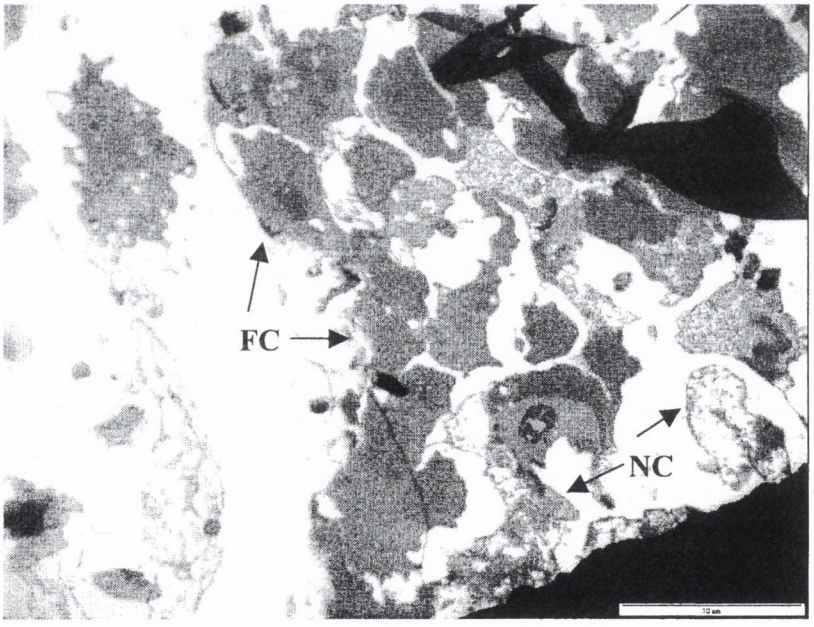
Transmission Electron Microscopy

The gonad and digestive gland of mussels exposed to both tertiary treated effluent and NP for 112 d were investigated using electron microscopy (EM). As with the light microscopy above changes in tissue morphology were observed after exposure to both tertiary treated effluent and NP *in vivo*, particularly in the male gonad. In the control mussel for the *in situ* exposure the follicle cells containing the developing spermatogonies were surrounded by nurturing cells (Figure 3.16(a&c)). However in the effluent exposed mussels both the nurturing cells and the follicle cells appear to be breaking down and the spermatogonies appear less developed (Figure 3.16(b&d)). There is also the development of what appear to be lipid yolk granules in the nurturing cells of the exposed males and an increase in interstitial tissue (Figure 3.16(f)).

Effects were also seen on the male gonad after exposure to NP for 112d. The *in vivo* control shown below was taken from the wild population in Lough Ree (Figure 3.17(a)) and shows well developed spermatozoids in the male gonad, stage C1 in gonad development (Wang & Denson, 1995). However developing spermatocytes were observed in the gonads of the mussels used in the *in vivo* experiment (Figure 3.17(b)), indicating stage B gonad development. The gonads of the males in the *in vivo* exposure resemble those of the mussels used in the *in situ* experiment that were a month behind in the gonad development cycle.

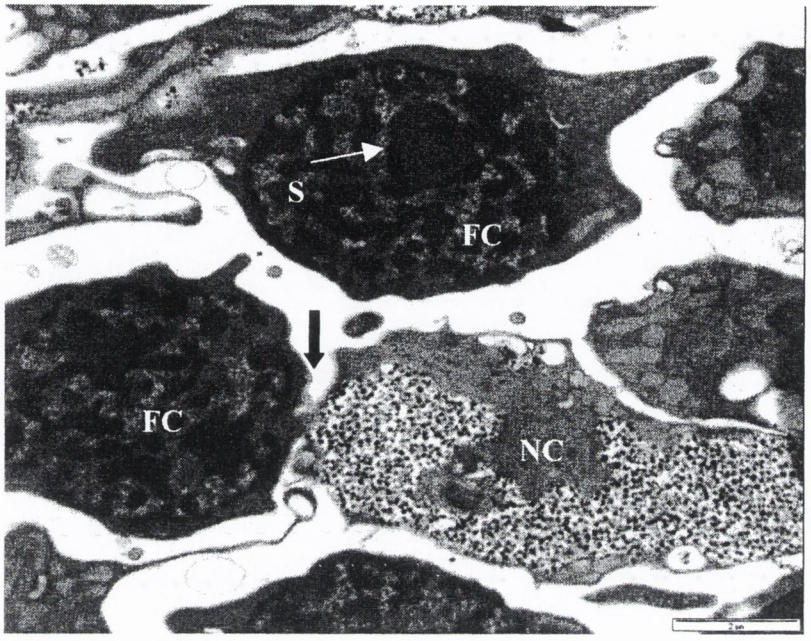


(a).

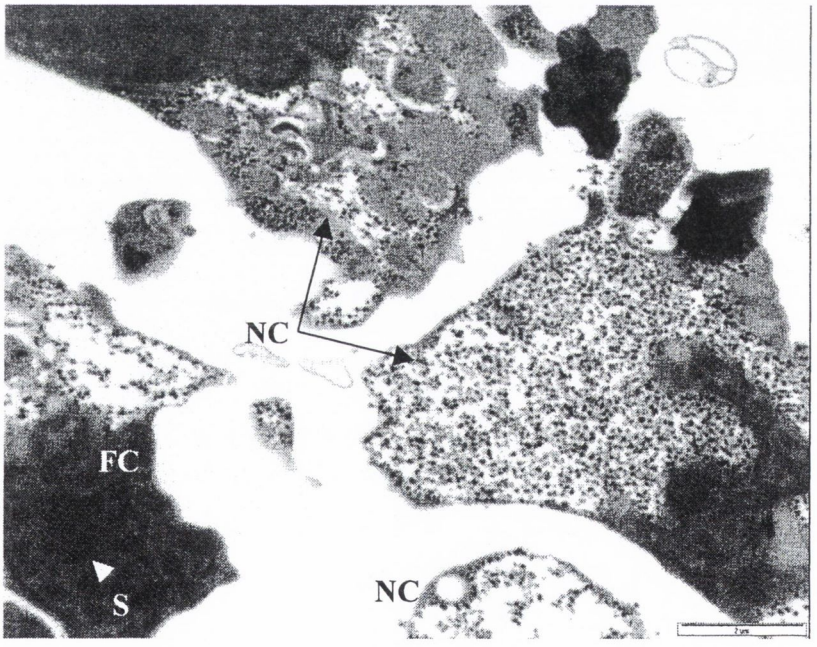


(b).

Figure 3.16. Series of electron micrographs investigating the effect of 112 d exposure to tertiary treated effluent on the gonad of the male zebra mussel. **(a).** Gonad of a control male mussel taken from Lough Ree. The gonad has a uniform appearance with follicle cells (FC) containing developing spermatogonies surrounded by nurturing cells (NC). **(b).** Gonad of exposed male mussel showing the tissue of the gonad breaking down. Nurturing cells (NC) appear to be empty and the follicle cells (FC) appear to be underdeveloped or breaking down. X 2500 magnification. Scale bar = 10 μm.

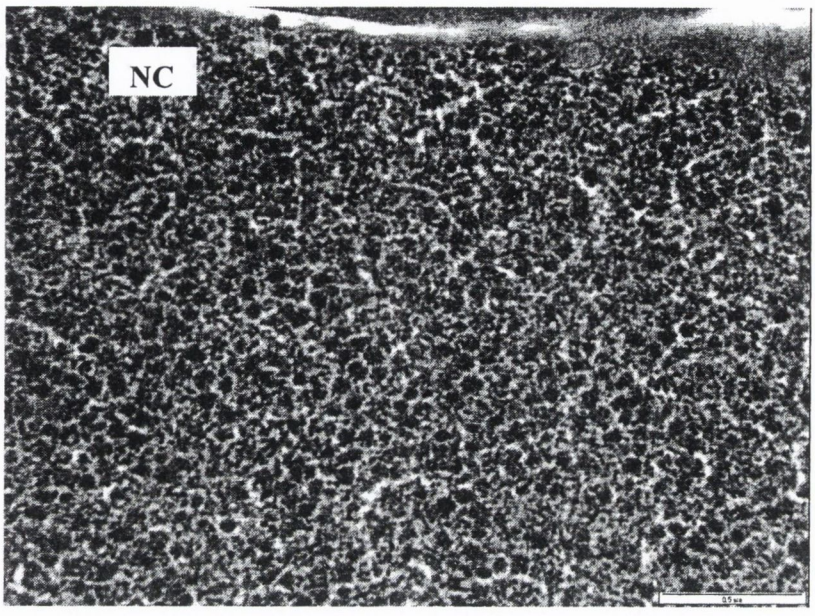


(c).

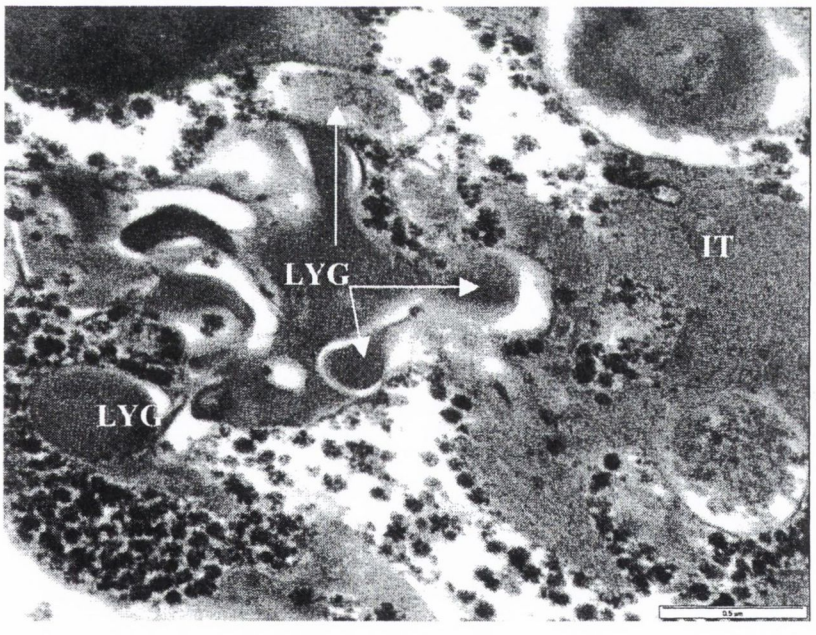


(d).

Figure 3.16. (c). Male gonad of control mussel showing the nurturing cells (NC) in close contact (arrow) with the follicle cells containing the developing spermatogonies (S). **(d).** Gonad of male mussel exposed to effluent showing the apparent breakdown of the nurturing cells (NC) and underdeveloped spermatogonies (S). X 10,000 magnification. Scale bar = 2 μm.

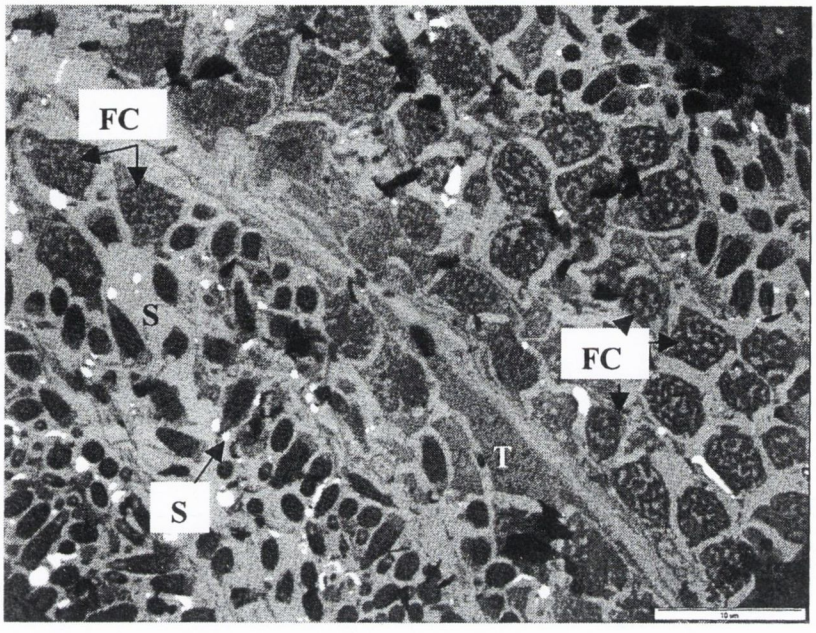


(e).

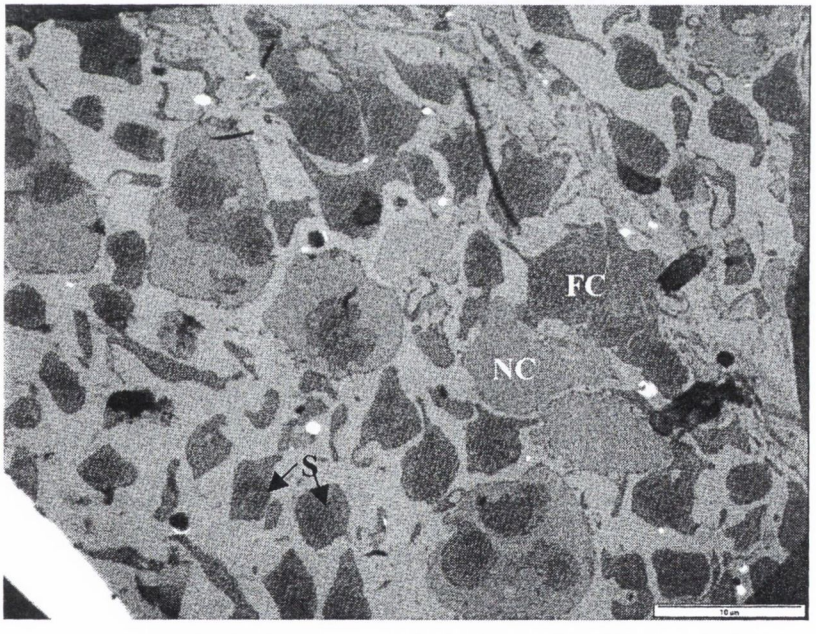


(f).

Figure 3.16. (e). Nurturing cell (NC) from the gonad of control male showing the uniform distribution of tissue within the cell. **(f).** Nurturing cell from the gonad of exposed male showing the development of interstitial tissue (IT) and a reduction in the number and uniform pattern of cells. Nurturing cells also show the development of what appear to be lipid yolk granules (LYG). X 40,000 magnification. Scale bar = 0.5 μm.



(a).

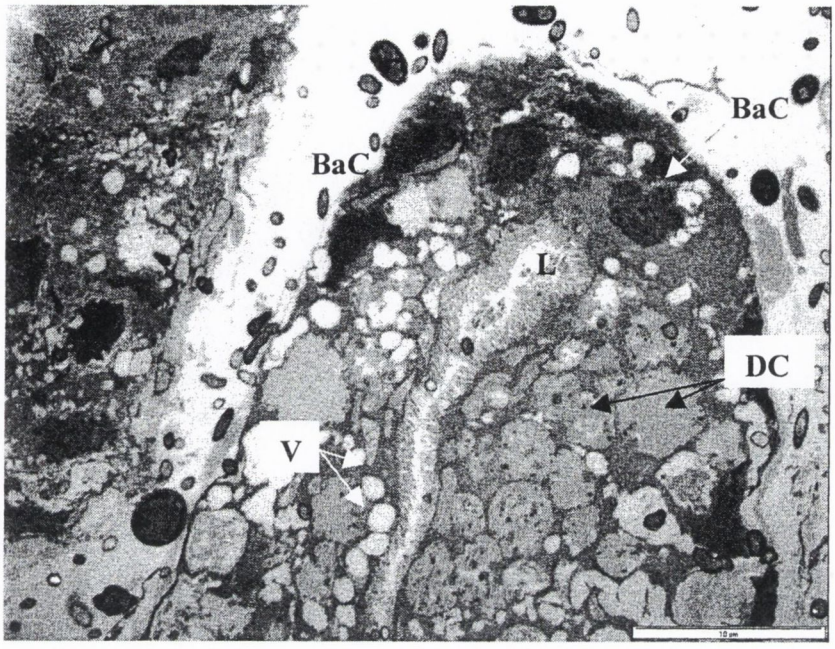


(b).

Figure 3.17. Electron micrographs investigating male gonad development in the 500 μg l^{-1} NP exposed mussels compared to a control mussel taken from Lough Ree. **(a).** Male gonad development in control mussel showing the well developed spermatozoids (S) and follicle cells, C1 development stage. T represents the tubule wall. **(b).** Gonad development in a male exposed mussel showing developing spermatocytes and nurturing cells, B stage of gonad development. Scale bar = 10 μm.

General toxicological effects of exposure to sewage effluent were also seen at the EM level of examination. In the digestive gland increased vacuolisation was evident with an increase in heterophagosomes and a breakdown of the digestive cells (Figure 3.18) when compared with the control (Figure 3.18). Surprisingly the lumen of exposed mussels became thinner than the controls. Gaps also appeared in the epithelium indicating where cells have degenerated (Figure 3.18 (a&b)). These effects can also be seen in mussels exposed to 500 $\mu g\ l^{-1}$ NP in the in vivo exposure (Figure 3.19).

(a).



(b).

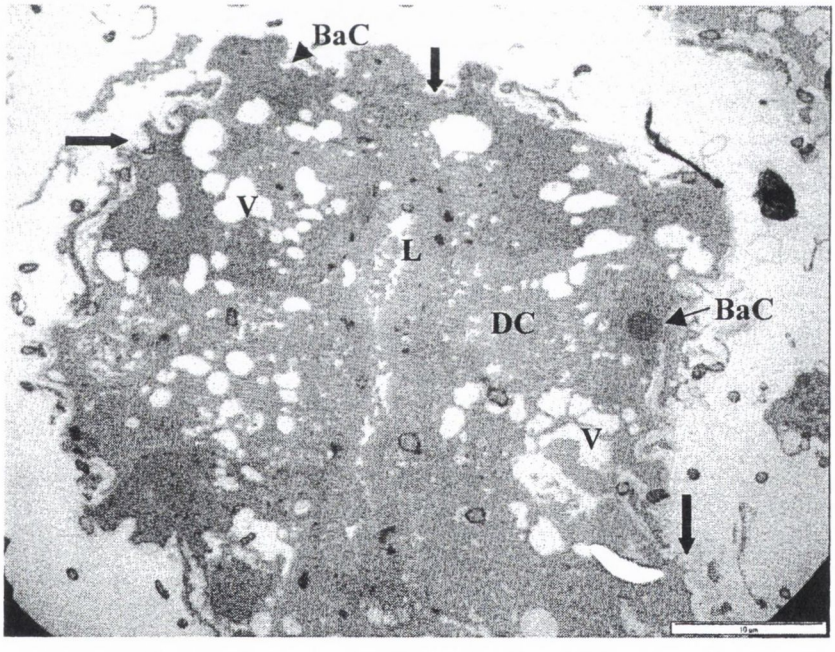
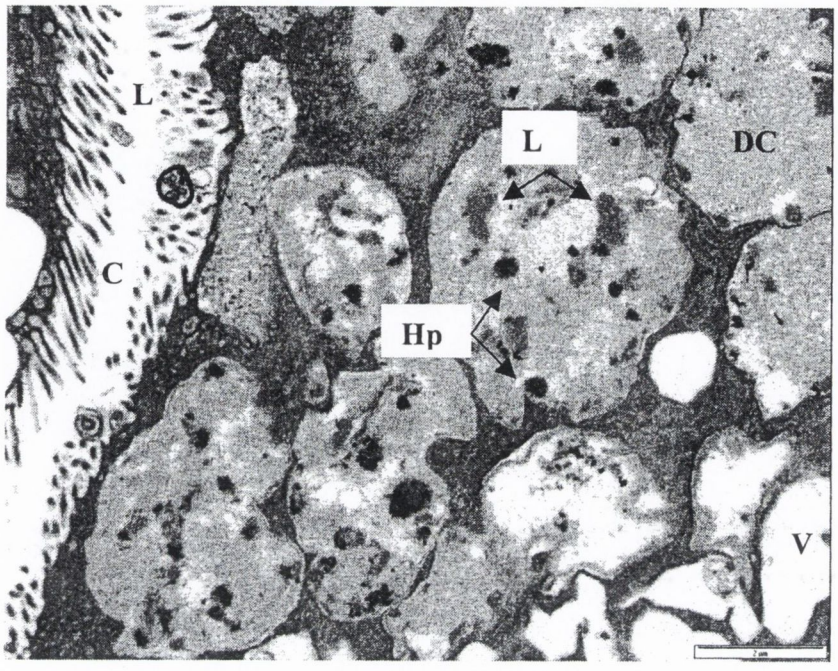
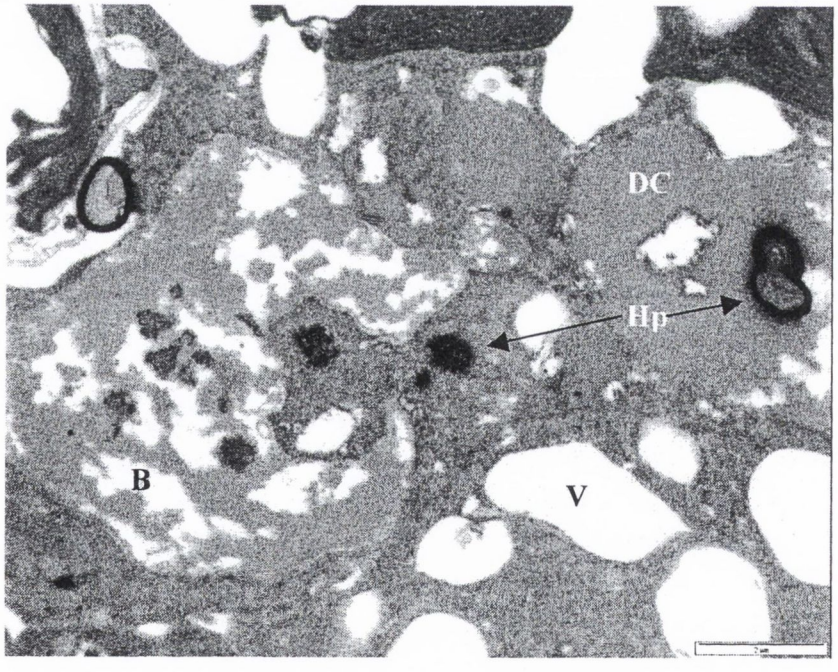


Figure 3.18. Electron micrographs of tubules from the digestive gland in zebra mussels exposed to tertiary effluent and a control mussel from Lough Ree. **(a).** Digestive gland tubule from control mussel showing normal thin lumen (L), with a regular tubule shape containing basophilic cells (BaC), digestive cells (DC) and vacuoles (V). **(b).** Tubule from mussel exposed to effluent showing increased vacuolisation (V) around both the basophilic (BaC) and digestive (DC) cells, a narrow lumen (L) and gaps in the epithelium (arrows). X 2500 magnification. Scale bar = 10μm.

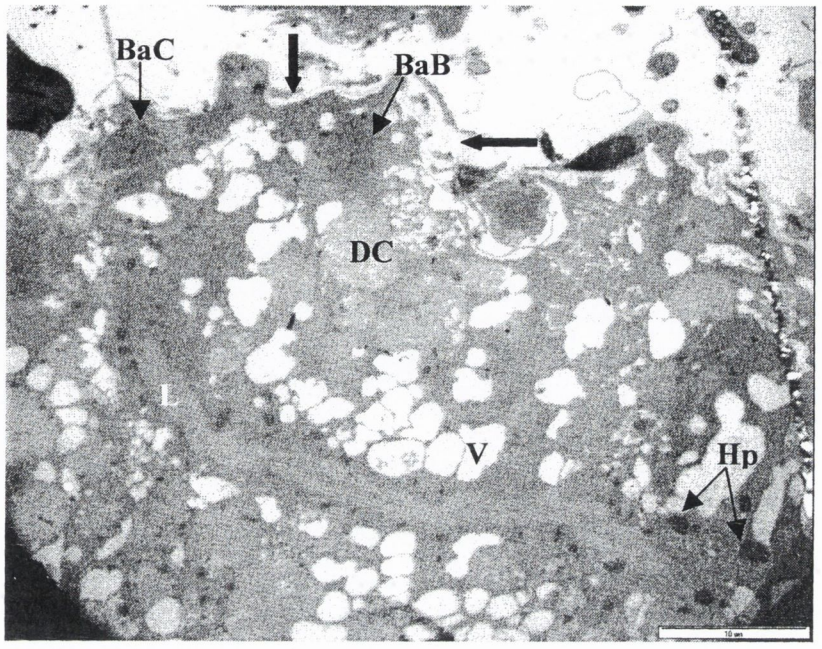


(c).

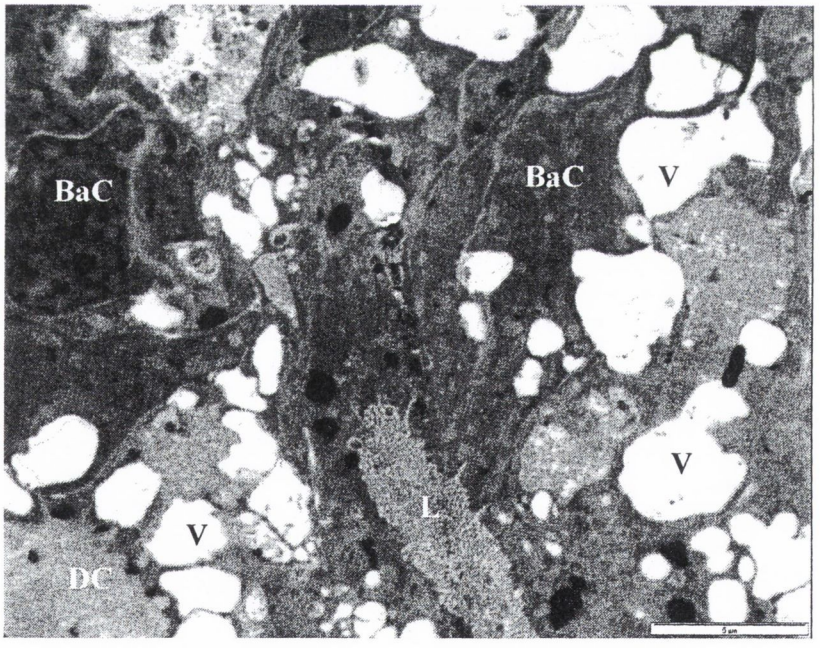


(d).

Figure 3.18 (c). Digestive cells (DC) in the digestive gland of the control mussel containing heterophaosomes (Hp) and lipid cells (L). Cilia (C) are found in the lumen (L). Vacuoles (V) have a regular shape. **(d).** Digestive cells deteriorating in effluent exposed mussel with areas of the cell broken down (B) and increased heterophagosomes (Hp) and vacuoles (V). X 10,000. Scale bar = 2 μm.



(a).



(b).

Figure 3.19. Digestive gland tubule from zebra mussel exposed to 500 μg l^{-1} NP *in vivo* for 112 d. **(a).** Digestive gland tubule showing increased vacuolisation (V) around the basophilic cells, a reduced lumen (L), increased heterophagosomes (Hp) and gaps in the epithelium (arrows). X 2000 magnification. Scale bar = 10 μm. **(b).** Increased vacuolisation around the basophilic cells (BaC) and digestive cells (DC). The lumen (L) has become closed in. X 5000 magnification. Scale bar = 5 μm.

DISCUSSION

The endocrine disrupting effect of prolonged exposure to tertiary treated effluent and nonylphenol on zebra mussels was primarily investigated by measuring levels of Vn-like proteins using the indirect alkali-labile phosphate assay and confirmed by gel electrophoresis analysis. This study confirmed that a significant increase in the levels of Vn-like proteins in zebra mussels occurred after prolonged exposure to both tertiary treated effluent *in situ* and NP under controlled laboratory conditions. Although the response to both exposures was greater in females, there was a significant increase in the protein levels in both male and female mussels exposed to effluent and NP when compared against controls, indicating that endocrine disruption had occurred. Although similar results have been found for other bivalve species (*Elliptio complanata* and *Mya arenaria*) exposed to secondary treated effluent (Blaise et al., 1999; Gagne et al., 2001 and 2001b) this is the first report of such a response in invertebrates exposed to tertiary treated municipal effluent.

In both exposures control male and female mussels were found to contain similar amounts of Vn-like proteins. This has been previously observed in natural wild populations of male and female zebra mussels (Lafontaine et al., 2000). However these similar levels of Vn-like protein changed after exposure to both effluent and NP as female mussels produce significantly more Vn-like proteins than the males. A larger response by females had been previously observed in the freshwater *Elliptio complanata* and was thought to suggest that female mussels were more sensitive to estrogen stimulation than

males (Gagne et al., 2001b). This response can be partially explained by the increased numbers of estradiol binding sites found in females of another freshwater mussel *Ellipito complanata* (Gagne et al., 2001b). Despite having a smaller increase in Vn levels, male zebra mussels do respond to xenoestrogen exposure with increased production of Vn-like proteins in a response similar to the widely observed phenomenon of vitellogenin production in male fish (Jobling et al., 1995; Giesy et al., 2000) (see Chapter 1).

This response of increased Vn-like protein production observed in the zebra mussels after exposure to xenoestrogens implies the possession of specific estrogen binding sites in the mussel. Attempts were therefore made to characterise and measure the binding capacity of the estrogen receptors (ER) in the zebra mussel. The idea was to measure the estradiol binding ability of gill cytosols from mussels exposed to effluent and NP. Reduced binding in the exposed mussels would indicate that these binding sites were already filled by a xenoestrogen. If optimised this technique could be used to indicate exposure to xenoestrogens, with the idea of developing the gill of the zebra mussel into a potential screen for estrogenic substances. The technique used was developed for gonad cytosolic proteins from the freshwater mussel *Elliptio complanata* (Gagne et al., 2001a) and needs to be optimised for the zebra mussel. Also fresh samples should be used rather than frozen tissues. This preliminary experiment shows that binding occurs and that NP has the ability to displace this binding. Saturation of the ER with estradiol was not reached, indicating that the apparent dissociation constant (K_d) value obtained was an overestimation. A K_d value of around 1.26 nM was found for the zebra mussel. This K_d is more consistent with the results for rainbow trout (1.5 nM) (Maitre et al., 1985) than for

other freshwater bivalves (0.4 nM) (Gagne et al., 2001a) also indicating that our K_d is an overestimation. If mixed with the ability to culture zebra mussel gills *in vitro* (see Chapter 4) this technique could provide a useful tool for the screening of xenoestrogens. However characterisation of the ER in the zebra mussel has yet to be determined.

In the *in vivo* laboratory exposure to NP the ALP data showed an unexpected decrease in the lipophosphoprotein levels in male mussels exposed to 500 μg l^{-1} NP. This decrease in phosphorylation was also shown when the ALP proteins were expressed per unit of Vn-like protein as measured by GE (ALP/VnGE) for both the mussels exposed to 500 μg l^{-1} NP and to sewage effluent. Changes in the state of phosphorylation of Vn have been reported in freshwater mussels in the wild (Gagne et al., 2001a). This response could result from the toxic effect of exposure to the effluent and to this relatively high concentration (500 μg l^{-1}) of NP. In a previous experiment zebra mussels exposed to 1 mg l^{-1} NP showed a significant ($p<0.05$) decrease in mortality, attachment and siphon extension (indicating feeding activity) (Chapter 2). Sub-lethal effects such as decreased byssal strength and change of scope for growth were observed for *Mytilus edulis* at 56 μg l^{-1} NP (Granmo et al., 1989). Although mortality was not significant ($p=0.1$) at exposure to 500 μg l^{-1} NP, a significant ($p<0.05$) reduction in the animals condition as measured by the dry weight : shell weight ratio. This could account for the lack in production of lipophosphoproteins by these mussels as phosphorylation is likely to produce major energy demands on the mussels. If the mussels are already negatively affected by the toxic effect of exposure, phosphorylation is likely to be reduced. Nutritional levels have also

been shown to affect levels of Vn-like proteins in the clam, *Mya arenaria* (Blaise et al., 1999).

Another possible ED effect observed in mussels exposed to both the effluent *in situ* and the laboratory exposure to NP was the effect on steroid composition. Although not extensively studied the endocrine system of most invertebrates seems to be based on a mixture of vertebrate type steroids (i.e. progesterone, testosterone and estradiol), with strong evidence indicating that these play a functional role (Reis-Henriques et al., 1990; Spooner et al., 1991; Li et al., 1998). In molluscs, cholesterol was identified as the main component of this steroid mixture (Reis-Henriques et al., 1990) and was found to be the parent compound for sexual steroids, being converted by a number of steps into testosterone and estrone (De Longcamp et al., 1974). By using HPTLC the present study showed cholesterol levels more than double after exposure to sewage effluent and that a clear dose response exists between exposure to NP and an increase in cholesterol levels in the zebra mussel. A similar response in cholesterol levels was found in gonad homogenates of the freshwater *Elliptio complanata* after exposure to effluent (Gagne et al., 2001c). The R_f value found for the zebra mussel (0.45) compares well with the R_f of 0.49 for cholesterol in the freshwater *Elliptio complanata* previously found by Gagné et al., (2001c). Since progesterone (the precursor of androgens and estrogens) is produced from cholesterol in *Mytilus edulis* (De Longcamp et al., 1974), it is possible that an increase in cholesterol after exposure to NP or sewage effluent could affect steroid levels in the zebra mussels, which may in turn lead to some endocrine disruption. Increasing levels of cholesterol may lead to the production of more estrogens and could therefore be

another mechanism of action for NP. This mechanism could potentially be exploited as a biomarker for ED. This increase in estrogen production could help explain why, based on the Vn results, female mussels were found to be more responsive than males.

These experiments have provided evidence of endocrine disruption on a protein and steroid level, but the most striking evidence came from the histological examination of the gonads of male mussels exposed to tertiary treated effluent. In these mussels a large increase in the interstitial tissue between the seminiferous tubules of the gonad was repeatedly observed. This increase in the interstitial undifferentiated tissue has led to a reduction in the size of the seminiferous tubules, reducing the effective sperm producing area of the gonad. The gonad in the control males appeared to be at a later stage of development containing more developed spermatogonies and spermatzoids in a later stage of gonad development, providing evidence of an interruption in the mechanism of sperm production in exposed males. These morphological effects indicate a reduction in fecundity of the exposed male mussels, which could have an effect at the population level. Effects were also seen in the male gonad at the electron microscopic level with a reduction in the development of spermatogonies in the follicle cells and the apparent breakdown of the tissue and the formation of what appear to be multi-membranous forms of lipid yolk granules in the nurturing cells.

Gonad development appeared to be altered in the mussels used in the *in vivo* experiment. When compared to mussels from the wild gonad development in the control mussels kept *in vivo* was greatly reduced. It has been reported that despite being an extremely robust

pest species in the wild, zebra mussels are surprisingly difficult animals to maintain for long periods in the laboratory (Wright et al., 1996). It is felt that due to poor nutrition and a stressful environment the gonads of the mussels used in the laboratory exposure did not develop beyond their early active stage (stage B) even in the control mussels. The gonads are the primary target tissue for de-growth when the reserves from other storage sites, such as the digestive gland are depleted (Sprung & Borcherding, 1991) as appears to have occurred here. In this experiment a prolonged (112 days) exposure to NP was chosen in order to examine the potential morphological endocrine disrupting effects on the gonad of zebra mussels exposed to NP. However it would appear that the present exposure regime did not encourage gonad growth in the zebra mussels, despite their showing elevated Vn levels after NP exposure. These animals showed a similar stage of development as the *in situ* male mussels, despite their gonads being a month further into the development cycle.

Other morphological effects such as ovotestis have been previously observed in male fish exposed to these EDCs along with elevated levels in Vtg resulting in a general feminisation of these male fish as previously described (Chapter 1). Initially it was thought that only the xenoestrogens such as NP in the municipal effluent caused these effects, and these compounds were identified as potentially the most potent source of EDCs in the Irish freshwater environment (Dempsey and Costello, 1998). However although xenoestrogens are proven effective estrogen mimics (Jobling et al., 1996; Giesy et al., 2000) and despite being found in municipal effluents and receiving waters at concentrations well above the threshold of response of fish (10-50 $\mu g\ l^{-1}$ for NP) (Gray & Metcalfe, 1997), phenol levels reportedly only accounted for a low percentage of the total

estradiol equivalent concentrations found in municipal sewage effluents (Korner et al., 2000). The majority of environmental estrogens result from municipal effluents and the majority of the effluents estrogenic activity is thought to result from other substances, most notably natural and synthetic hormones.

In STWs the microbial degradation of synthetic estrogens (17α-ethinylestradiol (EE2), diethylstilbestrol (DES)) and progestogens (levonorgestrel, norethindrone) used in contraceptive pills and medicines, releases the deconjugated biologically active form into the effluent (Routledge et al., 1998). When mixed with natural endogenous estrogens (17β-estradiol (E_2), estrone and estriol) these compounds are much more potent (by a factor of 10^3 to 10^6) than nonylphenolic compounds (Metcalfe et al., 2001) and are found in effluents of primarily domestic origin well within the threshold of response for fish (1-10 ng l^{-1}) (Desbrow et al., 1998; Ternes et al., 1999). HPLC analysis of the effluent used in the present *in situ* exposure revealed a complex mixture of compounds that could elicit estrogenic activity when tested using the YES assay. The fraction having the vast majority of the estrogenic activity contained E2, EE2 and bisphenol A. Similar findings were observed in wastewater treatment plant effluents in the UK and the U.S.A using very similar TIE methods, where single fractions were found to contain the majority of the estrogenic activity. In these earlier studies estrone (E1), EE2 and E2 were the most commonly found active compounds (Desbrow et al., 1998). Although found in lower quantities NP was also identified in the effluent by GC/MS.

NP was also found in the homogenate of both the mussels used in the *in vivo* laboratory exposure and those exposed to effluent *in situ* using HPTLC resulting from its ability to bioconcentrate and its lipophilic character (Ekelund et al., 1990). Mussels exposed to 500 $\mu g\ l^{-1}$ NP in the lab had a relatively high amount (11.7 μg NP g^{-1} protein) of NP in their soft tissue. This was considerably higher than the 1.1 μg NP g^{-1} protein found in mussels exposed to 5 $\mu g\ l^{-1}$ NP. These results are relatively consistent with other studies where up to 12.4 $\mu g\ g^{-1}$ tissue of non-ionic surfactants were found in mussels exposed to river water (Cathum & Sabik, 2001) and up to 265 ng g^{-1} NP was found in *Mytilus galloprovincialis* in the Adriatic sea (Ferrara et al., 2001). HPTLC offers a relatively easy method of measuring NP concentrations in tissue homogenate. However it was not expected to find NP in a measurable quantity in the control mussels for the *in situ* exposure, taken from Lough Ree. These results suggest that the control mussels may also have been exposed to estrogenic chemicals in their natural environment.

The wild mussels used as a control for the *in situ* experiment were taken from the same population as those used in both exposures. They were collected from their natural environment attached to floating buoys in a relatively large marina (Quigley's Marina) containing 100 moorings on Killenure Lough, one of 3 inner lakes located in the south east corner of Lough Ree (Figure 1.5). L. Ree receives effluent discharges from 21 municipal STWs serving a sewered population of approximately 25-30,000 in what is principally an agricultural catchment area. The overall trophic status of the lake for 1999 (based on the classification scheme of lake waters proposed by the OECD, 1982) was borderline between mesotrophic and eutrophic (EPA, 2000b). Killenure lough is

considered to have relatively good water quality with mean values for 1999 of dissolved oxygen (93% saturation), chlorophyll (2.7 μg Chl l^{-1}), total phosphate (12 μg P l^{-1}) and total oxidised nitrogen (840 μg N l^{-1}) regarded as satisfactory and within regulatory limits. Excessive colonisation by zebra mussels was noted in L. Ree, resulting in a significant increase in water transparency and a reduction in phosphates by the filtering of algae and cyanobacterial cells (EPA, 2000b). A 1996 report on L. Ree noted that discharges from pleasure craft could have a deleterious impact on water quality in harbours and other confined areas (EPA, 1996). This appears to be the case as water samples taken from this marina were found to elicit an estrogenic response using the YES assay. However no evidence of ED was observed in these control mussels.

During the *in situ* experiment zebra mussels proved to be very resilient animals as prolonged exposure to municipal effluent had little effect on mortality or on the sub-lethal parameters of attachment and adductor muscle activity (gaping). As the exposure took place *in situ* during winter/spring (December to April) the mussels were subjected to effluent temperatures ranging from -8 to 20°C, with the largest variation (18°C) occurring between mid to late January. On several occasions the flow of effluent into the exposure tanks was blocked, leaving the mussels in stagnant effluent for unknown periods. All this appeared to have little outward effect on the mussels during the exposure making them ideal animals for this type of experiment. However quite marked effects were seen at a histological level with severe vacuolation and enlarging of the lumen apparent in the digestive gland of the exposed mussels. The digestive gland is the most important energy source for the mussel during gametogenesis (Gabbott & Bayne, 1973) and it is therefore

likely that its degradation could also have an effect on the animals energy balance and could effect reproduction. The histopathology of zebra mussels has been previously reported and is recognised as a sensitive measure of environmental conditions (Bowmer et al., 1991). A highly significant ($p=0.0001$) reduction in condition of the exposed mussels was also observed when compared with the controls, indicting that effluent exposure was detrimental to the mussels health.

CONCLUSION

This is the first study to look at the ED effect of tertiary treated effluent on invertebrates and the first report to measure the levels of EDCs in both tertiary treated effluent and lake water in Ireland. The zebra mussel appeared to be a suitable bioindicator to ED having significant effects at a protein, steroid and histological level. The measurement of cholesterol levels in invertebrates could provide a relatively easy and quick method of measuring ED. The *in vivo* exposure of 112 d may have been too long to maintain the mussels in the laboratory as gonad development appeared to be reduced. However zebra mussels are very well suited for *in situ* experiments to measure endocrine disruption.

Appendix 3.1.

Fixative for electron microscopy:

25% Glutaraldehyde (Sigma, St. Louis, USA) diluted to 2.5% in 0.1M Sorensen's phosphate buffer. Sorensen's phosphate buffer:

40.5 ml of 0.2M disodium hydrogen orthophosphate (Na_2HPO_4)

(Sigma, St. Louis, USA).

9.5 ml of 0.2M sodium dihydrogen orthophosphate dihydrate ($Na_2H_2PO_4.2H_2O$)

(Sigma, St. Louis, USA).

Added to 50 ml distilled H_2O

Adjust pH to 7.4

Appendix 3.2.

Comparison of the intra exposure condition between mussels from the 3 exposure tanks used in the in situ exposure to tertiary treated effluent. P values calculated using one way ANOVA. Level of significance $p<0.05$. T1 = tank 1; T2 = tank 2; T3 = tank 3.

Dry/Wet Weight		**Dry/Shell Weight**	
Conditions	p value	Conditions	p value
T1:T2	0.778	T1:T2	0.533
T1:T3	0.762	T1:T3	0.458
T2:T3	0.982	T2:T3	0.792

Appendix 3.3.

Comparison of intra exposure differences in condition between each of the 3 tanks for the control mussels and those exposed to 5 and 500 μg l^{-1} NP *in vivo*. **(a).** Using the dry : wet weight ratio. **(b).** Using the dry : shell weight ratio. P values calculated using one way ANOVA. Level of significance p=0.05. C = control; SC = solvent control; 1 = tank 1; 2 = tank 2; 3 = tank 3.

(a). Dry : Wet Weight

Conditions	P value	Conditions	P value	Conditions	P value	Conditions	P value
C1 : C2	0.24	SC1:SC2	0.266	5μg l^{-1}1: 5μg l^{-1} 2	0.669	500μg l^{-1}1: 500μg l^{-1}2	0.615
C1 : C3	0.66	SC1:SC3	0.336	5μg l^{-1}1: 5μg l^{-1}3	0.957	500μg l^{-1}1: 500μg l^{-1}3	0.574
C2 : C3	0.4	SC2:SC3	0.09	5μg l^{-1}2: 5μg l^{-1}3	0.795	500μg l^{-1}2: 500μg l^{-1}3	0.83

(b). Dry : Shell Weight

Conditions	P value	Conditions	P value	Conditions	P value	Conditions	P value
C1 : C2	0.06	SC1:SC2	0.06	5μg l^{-1}:5 μg l^{-1}2	0.258	500μg l^{-1}1: 500μg l^{-1}2	0.538
C1 : C3	0.079	SC1:SC3	0.45	5μg l^{-1}: 5μg l^{-1}3	0.749	500μg l^{-1}1: 500μg l^{-1}3	0.466
C2 : C3	0.89	SC2:SC3	0.27	5μg l^{-1}: 5μg l^{-1}3	0.625	500μg l^{-1}2: 500μg l^{-1}3	0.835

Appendix 3.4.

Inter exposure comparison of the condition of mussels exposed to 5 μg l^{-1} and 500 μg l^{-1} NP and the control mussels using **(a).** the dry : wet weight ratio and **(b)** the dry : shell weight ratio. P values calculated using one way ANOVA. Level of significance $p<0.05$. C = control; SC = solvent control; 1 = tank 1; 2 = tank 2; 3 = tank 3.

(a). Dry : Wet Weight

Conditions	P value	Conditions	P value	Conditions	P value
C:SC	0.541	SC:5μg l^{-1}	0.141	5μg l^{-1}:500μg l^{-1}	0.899
C:5μg l^{-1}	0.3	SC:500μg l^{-1}	0.118		
C:500μg l^{-1}	0.25				

(b). Dry : Shell Weight

Conditions	P value	Conditions	P value	Conditions	P value
C:SC	0.885	SC:5μg l^{-1}	0.91	5μg l^{-1}:500μg l^{-1}	0.05
C:5μg l^{-1}	0.963	SC:500μg l^{-1}	0.07		
C:500μg l^{-1}	0.05				

Appendix 3.5.

Comparison of mussels used in the *in vivo* exposure with wild control mussels taken from Lough Ree for both condition indices. P values calculated using one way ANOVA. Level of significance $p<0.05$. WC = wild control; C = control

Dry : Wet Weight		**Dry : Shell Weight**	
Conditions	p value	Conditions	p value
WC:C	0.009	WC:C	0.00002
WC:SC	0.119	WC:SC	0.00006
WC:5μg l^{-1}	0.004	WC:5μg l^{-1}	0.00003
WC:500μg l^{-1}	0.003	WC:500μg l^{-1}	0.0000005

Chapter 4.

DEVELOPMENT OF AN *IN VITRO* CULTURE METHOD FOR TISSUES FROM THE ZEBRA MUSSEL (*DREISSENA POLYMORPHA*).

INTRODUCTION

The phrase '*in vitro*' literally means in glass, referring to the technique of studying biological processes or reactions that occur outside the body of an organism in an artificial environment. The idea behind this technique is simple, to grow or maintain tissues or cells of an organism in an artificial, controlled environment. This strict control of environmental conditions is one of the main advantages of *in vitro* cellular models. The ability to nurture and grow cells from a particular organ or tissue in an isolated environment, removed from the processes governing metabolism, provides an ideal opportunity to study the molecular, cellular and physiochemical mechanisms of that organ or tissue (Quinn & Mothersill, 2000).

In vitro studies provide a mechanistic basis for toxicity testing (Fentem & Balls, 1994). They can allow the detailed study of the biotransformation of chemicals and the investigation of mechanisms of chemical induced toxicity (Blaauboer et al., 1994). This method is also generally more economical than *in vivo* testing and reduces the need for

whole organism tests, solving the problem of ethical issues related to animal testing. A major application of cell culture is in the understanding of mechanisms involved in cellular and molecular responses to environmental change. This is particularly true for invertebrate cell culture as invertebrates represent >90% of all species, are found in all environments, and are already frequently used as monitors of environmental change. Other uses of invertebrate tissue cultures would be in the area of pathology, to investigate host-pathogen responses and the study of cellular biochemistry.

Cell culture methods have been in use for over a century, but have concentrated on vertebrate, particularly mammalian tissues. Despite this in 1907 the first reference of aquatic invertebrate cell culture using sponges appears (Wilson, 1907) and the first active long-term cell cultures from a crustacean were developed in 1968 (Quiot et al., 1968). However very little progress has been made in the area of invertebrate *in vitro* tissue culture, particularly in the area of molluscan bivalve tissue culture where it has not yet been possible to maintain growing cells *in vitro* (Mothersill & Austin, 2000). The phrase 'cell and tissue culture' requires the demonstration of growth of the relevant cells *in vitro* from tissue fragments or suspensions. Although readily achieved in mammalian tissue culture this has yet to be achieved in the culture of invertebrate tissues. In comparison with mammalian cell culture, invertebrate culture methods are very underdeveloped (Mothersill & Austin, 2000).

Although tissue culture appears to be a relatively straightforward technique, there are an enormous number of variables, each of which has to be tested individually and together as

they often act cumulatively. The tissues to be cultured are put through an array of techniques including dissection, decontamination and dissociation before being placed in culture, any of which could have a deleterious effect on the tissue. The constituents of the media also need to be carefully chosen as different media and supplements can be toxic to different cells. It is for this reason that invertebrate tissue culture has only met with limited success. Another important factor hindering success is the very limited amount of knowledge about the nutrition of invertebrate cells. Although primary cultures of bivalves have been maintained for up to several weeks, their functionality is uncertain and their growth is limited (Mulcahy, 2000).

There are several different types of *in vitro* culture. Initially dissected pieces of tissue known as an explant were added untreated to the culture media in a culture vessel. The tissue may also be digested with enzymes or chemicals to give a suspension of single cells or cell aggregates, know as the cell suspension method. Explants may also be subjected to digestion to aid cell migration from the tissue. The cells or tissue to be cultured are introduced into a culture vessel where they are allowed to attach, which may take up to several days. The cells may require special supplements to the media in order for them to attach and grow in culture. However as little is known about the nutritional requirements of invertebrate cells this is often achieved by trial and error, with the use of much guesswork to enable cells to grow *in vitro*. The osmolarity and pH of the media are fixed to resemble as closely as possible the haemolymph of the animal, to try to recreate the cells *in vivo* environment. Generally antibiotics are added to the media to control bacterial contamination. Previous attempts at culturing tissues of freshwater mussels failed due to

fungal and bacterial contamination (Sengel, 1964) resulting from their being in direct contact with the environment. The decontamination of bivalves before culture generally uses a multistep approach involving the depuration of the animal, the cleaning and disinfecting of the shell and the dissection and immersion of the tissue to be cultured in antibiotic solution (Stephens & Hetrick, 1979; Domart-Coulon, et al., 1994; LeMarrec, 1995). However a balance is needed as some antimicrobials were found to be toxic to cells in culture (Gardiner et al., 1991).

There are many reports of successful short-term cultures of molluscan cells (Table 4.1) but few reports of secondary cultures. One molluscan cell line has been developed, the *Bge* cell line developed by Hansen (1976) from embryonic tissues of the freshwater snail *Biomphalaria glabrata*. Most work has concentrated on commercial species used in aquaculture e.g. oysters, mussels and scallops. Research has also concentrated on oysters used in the pearl industry in Asia. However research is starting to concentrate on mussels used as environmental indicator species (*Mytilus edulis* and *Anadona*) (Mulcahy, 2000). Bivalve molluscs are particularly useful as bioindicators due to their sessile life style, living in close contact with benthic substrates and sediments, filtering large volumes of water for feeding (see Chapter 1). In many instances it is favourable to isolate and culture a single cell type. However in environmental toxicology where the ultimate goal is to use an animal as a representative of an entire ecosystem, heterogeneous cell cultures may be more representative of the whole animal.

The cells and tissues to be cultured have been largely chosen based on their relevance to the particular problem under study. Different cells that have been cultured include embryonic and larval cells, cells from the heart, gills, mantle and digestive gland and haemic tissue. Successful cultures have been obtained from heart cells of *Pecten maximus* that were maintained in culture for 1 month (Le Marrec-Croq et al., 1998) and of *Crassostrea gigas* (Wen et al., 1993) (Table 4.1). Primary culture of gill cells from *Ruditapes decussatus* were maintained for 45 days and from larvae of *Haliotis rufescens* remaining viable for up to 12 weeks (Naganuma et al., 1994). Cells from the digestive gland were cultured by explant from *Mytilus galloprovincialis*, although bacterial contamination was often a problem (Robledo & Cajaraville, 1997). Although these cells can be maintained in culture for extended periods, cell proliferation/reproduction has yet to be reported. The digestive gland in molluscs is a multifunctional organ involved in xenobiotic metabolism and is a popular tissue for culturing. For this reason the digestive gland from the zebra mussel was chosen for culture. Other organs chosen were the gill and the mantle, due to their constant contact and exposure to the environment and their obvious relevance in environmental toxicology.

Table 4.1. Summary of some of the aquatic invertebrate species and their tissues used for *in vitro* tissue culture. DG = digestive gland. * indicates suspected thraustochytrid contamination (see Discussion).

Test species	Tissue	Comment	Reference
Crassostrea virginica	Larvae Heart	Contamination after 2 weeks Maintained up to 6 months	Brewster & Nicholson, 1979
Crassostrea virginica *Mercenaria mercenaria*	Larvae	Maintained up to 8 months *	Ellis & Bishop, 1989
Pinctada fucata *Pinctada maragaritifera*	Mantle	Cell migration from explant	Machii & Wada, 1989
Mizuchopecten yessoensis	Larvae	Maintain for 4 months *	Odinstova & Khomenko, 1991
Ruditapes ducussatus	Gill	Maintained for 45 days	Auzoux et al., 1993
Crassostrea gigas	Heart	Proliferation and sub-cultured 6 times *	Wen et al., 1993
Crassostrea gigas	Heart	Cell proliferation *	Domart-Coulon, 1994
Mytilus edulis *Patinopecten yessoensis*	Larvae Mantle DG	Primary cell culture survive 4-6 months *	Odinstova et al., 1994
Crassostrea gigas *Mytilus galloprovincialis*	larvae	Maintained for 2 weeks * Secondary cultures	Takeuchi et al., 1994
Haliotis rufescens	larvae	Viable up to 12 weeks and cell division observed	Naganuma et al., 1994
Mytilus edulis	Mantle	Short-term cultures for chromosome spreads	Cornet, 1995
Mya arenaria	Heart	subcultured after 6 weeks*	Kleinschuster et al., 1996
Mytilus galloprovincialis	DG	Bacterial contamination	Robledo & Cajaraville, 1997
Pecten maximus	Heart	Maintained for 1 month	Le Marrec-Croq et al., 1998

Once a basic culture method has been established, the challenge is to develop techniques to maintain the culture *in vitro* (Mothersill & Austin, 2000). The level of maintenance may vary depending on the end use of the culture. The ideal is to maintain the *in vivo* function of an organ so as to allow the examination of toxicological responses *in vitro*. In invertebrates it is not yet possible to maintain growing cells *in vitro* as cell culture of aquatic invertebrates is in its infancy. The ultimate aim of this research was to establish an *in vitro* technique to maintain isolated cells and tissues from the zebra mussel in culture for use as a tool in toxicology. More specifically in order to study the effects of the endocrine disrupting chemical nonylphenol (NP) on the individual tissues and at a cellular level. This work was carried out both in the laboratory in Dublin and in the Unite de Culture Cellulaire, University de Bretagne Occidentale, Brest, France under Dr. Germaine Dorange.

MATERIALS AND METHODS

All procedures were carried out under sterile conditions in a laminar flow hood (Class II microbiological safety cabinet) exclusively used for cell-culture purposes and are summarised in Table 4.2. This protocol is described in detail below.

Table 4.2. Summary of the *in vitro* tissue culture technique developed for the explant and cell suspension of tissues of the zebra mussel (*Dreissena polymorpha*). For details on techniques see below.

Summary of Tissue culture technique for zebra mussel

- Scrape mussel clean under running water. Place in sterile water.
- Place in antibiotic sol. (2X) for 2 hr in laminar flow partially on ice.
- Rinse mussel in ethanol 70°. Allow to dry.
- Set up binocular microscope and light in laminar flow (if needed).
- In laminar flow, rinse mussel with 10 ml sterile H_2O, using microscope dissect tissue.
- Place tissues in sterile buffer solution.
- Trim tissue to ensure as pure a sample as possible.
- Rinse in petri dish of sterile buffer solution. Cut tissue into 1-2 mm^2 pieces.
- Antibiotic sol. (separately) - X 4 (10 ml of anti-b sol.) 30 min
  - X 2 (5 ml of anti-b sol. + 5 ml buffer sol.) 20 min
  - X 1 (2.5 ml anti-b sol. + 7.5 ml buffer sol.) 10 min
- Rinse in sterile buffer sol.

DISSOCIATION WITH PRONASE

- Add tissue to 0.025% pronase in buffer sol. (12.5 mg in 50 ml) with antibiotic (X1).
- Keep separate organs from different animals in separate tubes.
- Store at slight angle at 4°C for specified time.
- Filter sample liquid through autoclaved gauze 60 μm (slowly) into centrifuge tube.
- Rinse tube and gauze with buffer sol.
- Centrifuge filtered liquid for 3 min at 1200 rpm.
- Remove liquid, add buffer sol. and re-centrifuge, 3 min at 1200 rpm. X2
- Remove supernatant.
- Add media, mix cells up using pipette. Calculate cell density. Adjust media if necessary.
- Place cell suspensions in culture, 1 ml in petri and 0.3 ml in multiwell plate. Add 0.5 and 0.2 ml after 24 hr incubation at 15°C.

EXPLANT (after dissociation)

- Take tissue material from gauze and place in petri dish with buffer sol.
- Cut up tissue into 1mm^2 using scalpel.
- Place 10-12 explants on small petri dish. If drying place drop of media on explant.
- Leave for 10 min.
- Slowly add 1ml of media. Drag around explant. Add 0.5ml after 24 hr incubation.
- Incubate at 15°C

TRYPAN BLUE

- 100 μl of trypan blue is mixed with 100 μl of cell suspension, mix.
- Using haemacytometer count number of blue and viable cells per square and find the % cell viability.
- Find cell density using number of live cells only. Adjust media volume if necessary.

Zebra mussels

The zebra mussels used for *in vitro* tissue culture were maintained in as sterile an environment as possible. For the *in vitro* work carried out in Dublin mussels were collected from Lough Ree (see Chapter 1). This was the same mussel population as was used for the *in vivo* exposures. The mussels were brushed clean under running water to remove any foreign material. They were maintained in 10 l glass tanks containing autoclaved de-chlorinated tap water in a room with constant temperature (15°C) and a 12 hour light/dark cycle. Mussels were fed a commercial bivalve food Phytoplex, and new mussels were collected as often as possible (every 2 weeks) to ensure healthy animals for culturing.

In France mussels were posted to the laboratory from the river Rance, near St. Malo in the north of Brittany. Initially mussels were maintained in autoclaved ultrapure water in a controlled room (15°C, 12 hour light/dark cycle). However ultrapure water was discovered to be toxic to the animals. Autoclaved stream water was used instead. Mussels were fed a commercial bivalve food and were replaced every 3 weeks. Tanks were cleaned twice a week, with fresh water added.

Tissues

The tissues chosen for culture *in vitro* were the gills, mantle and the digestive gland (also known as the digestive diverticulata, midgut gland and the hepatopancreas). The position

of these organs in the zebra mussel is shown in Figure 4.1. The gills were chosen as they come into direct contact with the environment as the animal filters large volumes of water. They are large independent organs that can be easily dissected offering a pure tissue sample. The mantle is also in contact with the environment and provides a relatively pure tissue sample. The Digestive gland is particularly relevant for environmental toxicology as it is the main site of detoxification in the zebra mussel. However it is more difficult to dissect as it is mixed with the gonad and stomach in the visceral mass (Figure 4.2). Although the digestive gland was dissected using a binocular microscope it was impossible not to include some gonad and stomach. Therefore these cultures must be considered mixed tissue cultures. All tissues were dissected in the laminar flow using aseptic technique and once dissected they were placed in sterile buffer solution (Table 4.3). Samples were trimmed to remove all traces of other tissues, ensuring wherever possible a pure tissue sample.

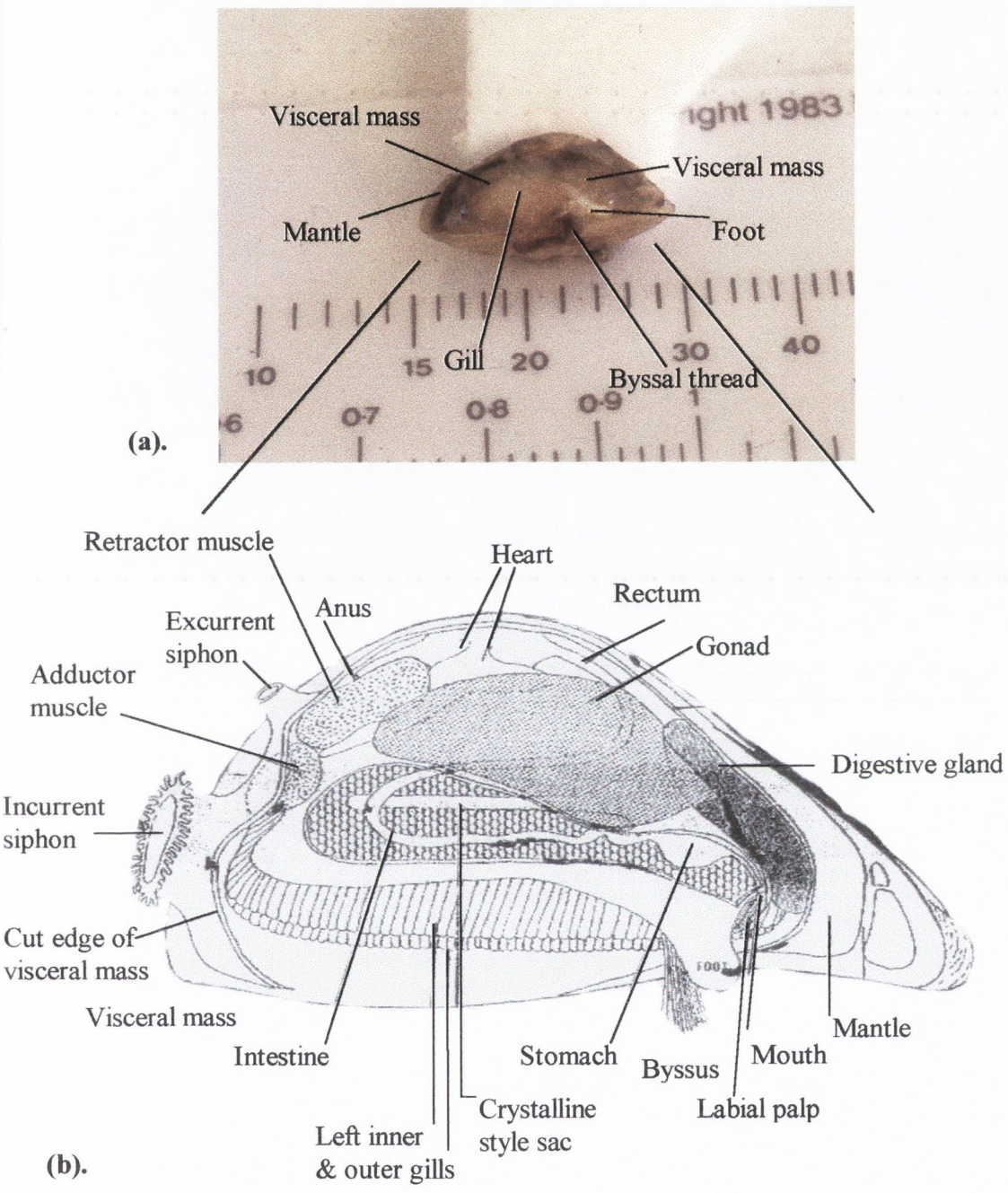


Figure 4.1. Transverse section of the zebra mussel showing the location of the major internal organs. **(a).** Photograph of zebra mussel dissected for tissue culture. **(b).** Diagram showing location of major internal organs of the zebra mussel. Note, diagram shows digestive gland, gonad and intestine as separate organs whereas they are all mixed up in the visceral mass (Figure 4.2).

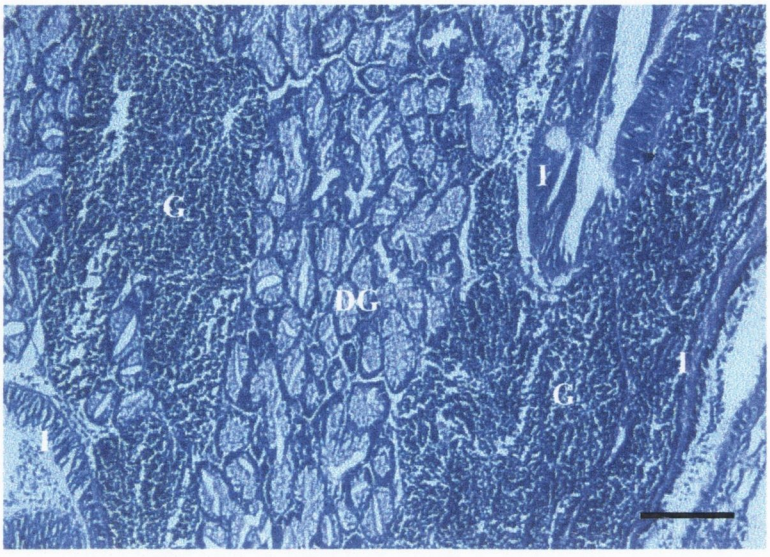


Figure 4.2. Slide of a cross section taken through the visceral mass of an adult male zebra mussel preserved in paraffin wax showing the digestive gland (DG), gonad (G) containing spermatocytes and intestine (I) mixed into the visceral mass. Haemotoxylin and eosin, X100. Scale bar = 100μm.

Decontamination

Decontamination procedure

The final decontamination procedure that was developed is as follows (for summary see Table 4.2): Using a scalpel the outside of the shell was scraped clean under running water and placed in sterile water. Mussels were transferred to an antibiotic solution (X2) partially on ice (to maintain a cool temperature) in the laminar flow and are left for 2 hours to filter the antibiotic solution. The mussels were then rinsed in 70° ethanol and allowed to dry. Once the bivalve shells were opened the mussels were rinsed with 10 ml of sterile

of sterile buffer solution (Table 4.3). The tissues were dissected out and rinsed in sterile buffer solution. Different tissues were kept separate throughout the procedure. Tissues were cut up into 1-2 mm^2 pieces and placed into X4, X2 and X1 antibiotic solution for 30, 20 and 10 minutes respectively and were finally rinsed in sterile buffer solution.

Table 4.3. Sterile buffer solution used during the *in vitro* culture of tissues from the zebra mussel.

Sterile buffer solution:	**1L**
Sterile cell culture water (Sigma Aldrich)	1L
NaCl (Sigma Aldrich)	2.32 g
Osmolarity	80-100 mOSM
pH	7.5
Lasts: 1 month at 4°C or 6 months at -20°C.	

The final antibiotic solution that was developed and used in all subsequent experiments is shown in Table 4.4. This antibiotic solution was the most concentrated (X4) and was subsequently diluted in half (X2) and to a quarter (X). The osmolarity was fixed at 80-100 mOSM (the same osmolarity as the mussel heamolymph) by adding the relevant concentration of NaCl, and the pH maintained at 7.5. Initially tissues were exposed to this antibiotic solution at a concentration of X4, X2 and X1 for 10 minutes each. However this was later changed to X4 for 30 min, X2 for 20 min and X1 for 10 min. Each tissue was exposed to the antibiotic solution separately.

Table 4.4: Final antibiotic solution used for the decontamination of tissues cultured *in vitro* from the zebra mussel.

Antibiotic solution (X4):	**1 L**
Sterile water, (Sigma)	972 ml
Penicillin - Streptomycine (5000 IU ml^{-1}-5000 $\mu g\ ml^{-1}$)(Gibco)	20 ml
Gentomycine (50 mg ml^{-1})(Gibco)	80 $\mu g\ ml^{-1}$
Kanamycine (759 $\mu g\ ml^{-1}$)(Sigma)	40 $\mu g\ ml^{-1}$
Osmolarity	80-100 mOSM
pH	7.5
Filtration	0.2 μm
Aliquot	50 ml
Lasts: 1 month at 4°C or 6 months at -20°C.	

Several other decontamination methods were tested for tissues from the zebra mussel before culturing. These include:

Bleach and Ethanol

Initially after dissection tissues were placed in 5, 10 and 20% bleach (Milton, containing 2% sodium hypochlorite) diluted with PBS, for a specified period of time (10, 30, 60 and 120 seconds). 70° ethanol was also used with exposure times of 10, 30, 60 and 120 seconds to disinfect tissues.

Antibiotic solution

Other antibiotic mixtures were also tested. The first of these had been previously developed for use with rainbow trout (*Oncorhynchus mykiss*) (Mothersill et al., 1995) and contained:

PBS	500 ml
Fungizone Amphotericin B (250 $\mu g\ ml^{-1}$)(Gibco)	10 ml
Penicillin - Streptomycine (5000 IU ml^{-1}-5000 $\mu g\ ml^{-1}$) (Gibco)	20 ml
Gentomycine (50 mg ml^{-1}) (Gibco)	15 ml

Tissues were exposed to this antibiotic mixture for 0.5, 1, 2, 3, 5, 10 and 30 minutes. A mixture of the two methods was tried with tissues placed in bleach for 10 or 30 seconds, rinsed in PBS and placed in antibiotic solution for up to an hour. The antibiotic solution was made up in both PBS and HBSS to both full strength and half this strength.

DTT

In order to increase the effectiveness of the antibiotic solution on the gills, they were first immersed in DTT (1,4 dithiothreitol) (Euromedex), a mucolytic agent used to decrease the bacteria trapped in the mucus in the gills. Once dissected and rinsed with buffer solution, the gills were immersed in 50 mM DTT for 15 minutes, before being added to the antibiotic solution.

Dissociation

Pronase

The final dissociation method that was subsequently adopted for use in all experiments involved enzymatic dissociation using Pronase (from *Streptomycea griseus*, Sigma Aldrich, St. Louis, USA) at a concentration of 0.025% in buffer solution with antibiotic X1 (Table 4.5). After antibiotic treatment the different tissues were placed separately in tubes containing pronase and left at 4°C for a specified time (12, 16 and 40 hours for the digestive gland, gills and mantle respectively).

Table 4.5. Optimum dissociation solution using Pronase (*Streptomycea griseus*) to dissociate tissues of the zebra mussel for culture *in vitro*.

Pronase dissociation solution:	**100 ml**
Sterile buffer solution	75 ml
Antibiotic solution (X4)	25 ml
Pronase (4 units ml^{-1})(Sigma Aldrich)	25 mg
Osmolarity	80-100 mOSM
pH	7.5
Filtration	0.2 μm
Lasts: 1 week at 4°C.	

Other methods of tissue dissociation attempted include:

Collagenase and Trypsin

Tissues were added to a solution containing the enzymes Collagenase (from *Clostridium histolyticum* type IV, Sigma Aldrich) and trypsin (2.5%, Gibco) at a concentration of 5 mg collagenase per 1 ml trypsin. The digestive gland, gills and mantle were exposed for 10, 15 and 20 minutes respectively. Collagenase was also mixed with PBS (6 mg per 1 ml of PBS) with exposure lasting 15 minutes.

Collagenase and trypsin were also added to the culture media to aid tissue dissociation in culture. Collagenase was added at a concentration of 1 and 0.5 mg ml^{-1}, with Trypsin being added at a concentration of 0.025 % and 0.05%.

EDTA

Chemical dissociation was undertaken using EDTA (ethylenediaminetetraacetic acid, Sigma Aldrich), which works by the formation of complexes with Mg^{++} and Ca^{++} used in the gap junctions in tissues. As it was not toxic to cells variable concentrations were tested, generally around 5 mM. This solution was made up in sterile water, with the osmolarity and pH fixed to 80-100 mOSM and 7.5 respectively. After decontamination with antibiotic solution the dissected tissues were added to the EDTA solution and placed on an agitator for 1 hour.

Tissue culture technique

Once the tissues had been dissociated, the pronase buffer solution containing all of the dissociated tissue in suspension is slowly filtered through autoclaved gauze with a diameter of 60 μm into a centrifuge tube and rinsed with sterile buffer solution.

Explant method

The clumps of tissue left on the gauze after rinsing with buffer solution were taken and cut into 1 mm^2 explants. Several explants were added to each petri dish (10 to 12) and well of the 24 well miltiwell plate (3 to 4). They were left to attach for 10 minutes without the addition of media. However if the explants were drying out a drop of media was added to the tissue. A small quantity of media (0.5 ml for petri dish or 0.3 ml for multiwell dish) was added to one edge of the dish and dragged around the explants using a pipette. This was done so as not to dislodge the explants. More media was added (0.5 ml for petri dish) until the explants are covered and they were placed in the incubator. After 24 hours the final amount of media was added to each vessel, 0.5 ml for petri dish and 0.2 ml for multiwell plate for a total volume of 1.5 ml and 0.5 ml for the petri dish and 24 multiwell plate respectively.

Cell suspension method

The gauze was rinsed with sterile buffer solution and the liquid containing the cells in suspension was centrifuged for 3 minutes at 1200 g. The supernatant was pipetted off and the cells washed in buffer solution. This was repeated twice. The supernatant was

removed and culture media added. A small sub-sample was taken and the concentration of cells per ml calculated using a haemacytometer. The media concentration was adjusted to give a cell density of 2 million cells per ml. Only 1 and 0.3 ml of media with cells in suspension was added to the petri dish and 24 multiwell plates respectively. As with the explant method a further 0.5 and 0.2 ml (respectively) was added after 24 hours incubation (total volume 1.5 ml and 0.5 ml for the petri dish and 24 multiwell plate respectively).

Cell viability

Cell viability was tested using the trypan blue exclusion test. Typan blue can either be made from powder form (Gibco, 0.2 g in 100 ml sterile water) or bought ready to use (Gibco). A sample of the media containing the cells in suspension was added to the trypan blue dye (50:50) and left standing for a few minutes. Using an inverted microscope and a haemacytometer the number of healthy cells and those that have taken up the blue dye (indicating cell death) were counted (Table 4.2). The average of 3 counts is taken and the percentage viability calculated.

Media

Final media composition

The osmolarity of the haemolymph from the zebra mussel was measured using an Osmomat 030 osmometer (Gonotec, Germany) and was found to be around 80 mOSM. Media with a similar osmolarity was developed using the commercial media Leibovitz L-

15. This was the most successful media developed and was subsequently used in all experiments. To reduce the osmolarity the Leibovitz L-15 media was diluted by 85% using sterile cell culture water (Sigma Aldrich). If necessary the osmolarity can be increased by adding NaCl. The supplements added to this media are listed in Table 4.6. The media was sterile filtered (0.2 μm, Nalgene) before use, and can be stored for up to 1 month at 4°C and for up to 6 months at -20°C. Foetal calf serum (FCS) and L-glutamine should only be added after the media is defrosted. Once added check osmolarity and adjust pH if necessary. Media must be used at ambient temperature and should be taken out of the fridge before use. Initially no HEPES was added to the media, but after a few days in culture the media started to turn yellow, indicating an increase in acidity. Therefore HEPES buffer was added to maintain the pH at around 7.5.

Table 4.6. Culture media based on the commercial Leibovitz L-15 media developed for the maintenance of cell and tissues of zebra mussel *in vitro*.

15% Leibovitz L-15	**1L**
Leibovitz L-15, (Gibco)	150 ml
Penicillin - Streptomycine (5000 IU ml^{-1}-5000 $\mu g\ ml^{-1}$) (Gibco)	5 ml
Gentomycine (50 mg ml^{-1}) (Gibco)	2 ml
Kanamycine (759 $\mu g\ ml^{-1}$)(Sigma)	0.01 g
Phenol Red (Sigma)	0.01 g
Sterile water (Sigma)	843 ml
HEPES (Gibco)	2.38 g
Foetal calf serum (10% final volume) (Gibco),	100 ml
L-Glutamine (200 mM)(Gibco)	10 ml
Osmolarity	80-100 mOSM
pH	7.5
Filtration	0.22 µm
Aliquot	100 ml
Lasts: 1 month at 4°C or 6 months at -20°C	
***Note:** Add FCS and Glutamine after media defrosted. Add separately.*	

Media development

Several types of media, enriched with various supplements were used to encourage the growth and maintenance of cells from the zebra mussel in culture. Dissected tissues were cultured in supplement enriched commercial media. RPMI 1640 (Sigma Aldrich) was the first media tried as it had previously been successfully developed for the primary culture

of explants from the rainbow trout (*Oncorhynchus mykiss*) (Mothersill et al., 1995; Kilemade et al., 2001). Supplements added to this media to encourage cell growth were:

Foetal calf serum (FCS) (Gibco),	13%,
Horse serum (Gibco),	7%,
Human recombinant insulin (Novo Nordisk A/S),	0.05 IU ml^{-1}
Hydrocortisone (Sigma Aldrich),	1 $\mu g\ ml^{-1}$
Penicillin - Streptomycine (Gibco),	50-50 IU ml^{-1} ($\mu g\ ml^{-1}$)
L-Glutamine (Gibco),	20 mmol l^{-1}
Fungizone - Amphotericin B (Gibco),	1 $\mu g\ ml^{-1}$

The next media under examination was Leibovitz L-15 (Gibco). This media was first used undiluted and was later diluted to 50% and 10% using both Hanks balanced salt solution (HBSS) and phosphate buffer solution (PBS) and supplemented with:

FCS (heat inactivated),	10%
L-Glutamine,	20 mmol
Penicillin - Streptomycine (Gibco),	50-50 IU ml^{-1} ($\mu g\ ml^{-1}$)
Fungizone - Amphotericin B (Gibco),	1 $\mu g\ ml^{-1}$

Other supplements added to the culture media include 5 $\mu l\ ml^{-1}$ & 15 $\mu l\ ml^{-1}$ 0.1% lipid mixture solution (Sigma Aldrich) and 10 $\mu l\ ml^{-1}$ & 100 $\mu l\ ml^{-1}$ 0.1% yeast extract solution (Sigma Aldrich). These stock solutions were made in HBSS. A combination of 10 $\mu l\ ml^{-1}$ & 100 $\mu l\ ml^{-1}$ of both solutions together was also added to the media.

Media change

After the first four days in culture half of the media was changed in the culture vessel. This was done using a pipette and care was taken not to infect the culture or to disturb cell attachment. The media was fully changed every four days after that. This has the added advantage of removing all dead and floating cells.

Culture vessels

Initially tissues were cultured in 25 cm^2 polystyrene and collagen coated polystyrene flasks (NUNC) (Table 4.7). These flasks have the advantage of being air tight when sealed, helping to reduce contamination. Media volumes of 1, 1.5 and 2 ml were added to these flasks used for the culture of tissue explants only.

Small polystyrene petri dishes (NUNC and Falcon) and 24 well multiwell plates (NUNC and Falcon) were also used to culture both explants and cell suspensions, with 1.5 and 0.5 ml of media added respectively. All culture vessels were sterile before use.

Table 4.7. Culture vessels and media volume used for the *in vitro* culture of tissues and cells from the zebra mussel.

Type	Make	Surface area	Media volume Tested	Media volume (optimal)
NUNCOLN polystyrene flasks	NUNC	25 cm^2	1, 1.5 & 2 ml	2 ml
NUNCOLN collagen coated polystyrene flasks	NUNC	25 cm^2	1, 1.5 & 2 ml	2 ml
Petri dish	NUNC & Falcon	8.8 cm^2	1.0, 1.5 & 2 ml	1.5 ml
24 well multiwell plate	NUNC & Falcon	1.9 cm^2	0.3, 0.4 & 0.5 ml	0.5 ml

Incubation

In keeping with the mussel's natural environment a temperature of 15°C was chosen as the optimal for the culture of zebra mussel tissues *in vitro*. Temperatures of 10 and 20°C were also investigated. The cells were incubated in normal air, using a refrigerated incubator (Leec, Nottingham, U.K.).

Cell proliferation

Cell proliferation *in vitro* was investigated using immunocytochemical analysis. Expression of the monoclonal mouse Anti-Proliferating Cell Nuclear Antigen antibody (PCNA) (Clone PC-10, DAKO, Cambridge, UK) was used to detect cell replication in the

cultured cells from the tissues of the zebra mussel. The immunogen used to raise the antibody was rat PCNA made in the protein A expression vector pR1T2T. This antibody cross-reacts with PCNA from all vertebrate species and numerous invertebrate species including the zebra mussel (Chapter 2.). Positive controls used were sections of normal human tonsil.

Immunoreactivity was investigated using the avidin-biotin complex (ABC) method of immunoperoxidase staining, using a mouse monoclonal vectastain Elite ABC kit (Vectastain Corporation Burlingame, USA). This method was based on the standard indirect immunoperoxidase technique as described in Chapter 2. After culturing the media was pipetted off and cells were fixed in 10% formalin for 1 hour. Cells were then covered in PBS and left for 24 hr at 4°C. The culture vessel with cells attached were placed in a moisture chamber and washed with PBS. The immunocytochemical procedure was described in Chapter 2. Concentrations of the antibody used were 1:200; 1:400; 1:800; 1:1200; 1:1600; 1:2400; 1:3200. The cells were counterstained with filtered Mayers haematoxylin (30 seconds), blued in running hot water and coverslipped and mounted in glycergel. Brown staining was indicative of a positive reaction.

As cell adhesion was proving to be a problem attempts were also made to test PCNA on suspended cells in a monolayer attached onto glass slides after centrifugation at 800 g for 3 min using a Shandon cytospin 3. The same immunocytochemical procedure as above was applied.

Cell characterisation

Cells were characterised based on their morphology identified using light microscopy at X200, X400 and X1000 (oil immersion). Attempts were also made to identify cells in culture using electron microscopy. Cells and tissues in culture were washed with PBS and fixed using 0.1M Sorensen's phosphate buffer (Appendix 3.1). However after a subsequent PBS rinse all cells in culture became detached from the culture substrate and could therefore not be examined.

RESULTS

Tissue culture

Tissue samples were taken from the gill, digestive gland and mantle of the zebra mussel and cultured *in vitro* as both explants and cell suspensions.

Explant

The first attempts at *in vitro* cell culture involved the culture of gill and mantle explants from the zebra mussel. These first attempts using the initial media, decontamination and dissociation techniques were unsuccessful with cells not adhering to the culture vessel and the explants dying after only a few days. However as the method began to develop with the optimisation of the culture media, disinfection and dissociation techniques, the

the optimisation of the culture media, disinfection and dissociation techniques, the explants started to adhere to the culture vessel (Figure 4.3) and were maintained in culture.

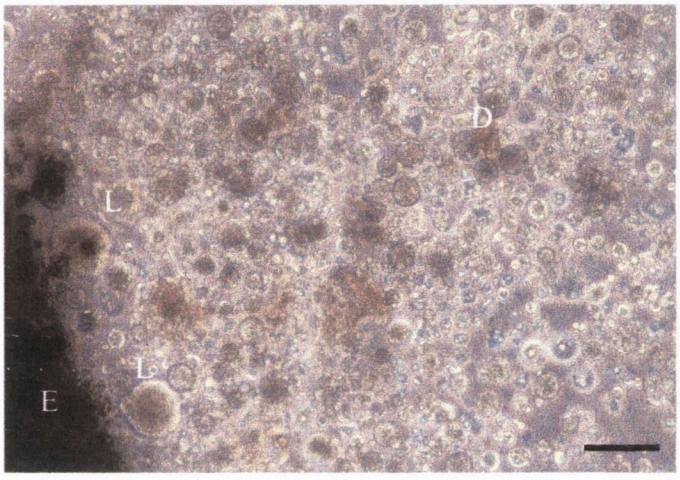


Figure 4.3. Photo showing initial explant technique with trypsinised cells from the gonad and digestive gland attaching to the culture vessel. L = large lipid cells; E = explant; D = digestive cells. Magnification X 200. Scale bar = 100 μm.

When the final method had been developed viable tissue explants were maintained in culture for up to 2 weeks after which time viability decreased (Table 4.8). Cells migrated out of the explant to form a matrix of cells around the tissue (Figure 4.4.) These cells are described as elongated or fibroblast-like as they have attached and spread out onto the culture vessel so that they are no longer ball shaped (Figure 4.4). These elongated cells form connections between one another forming a matrix of cells. When cultured according to the final protocol, mantle explants formed a good matrix of cells around the explant (Figure 4.4).

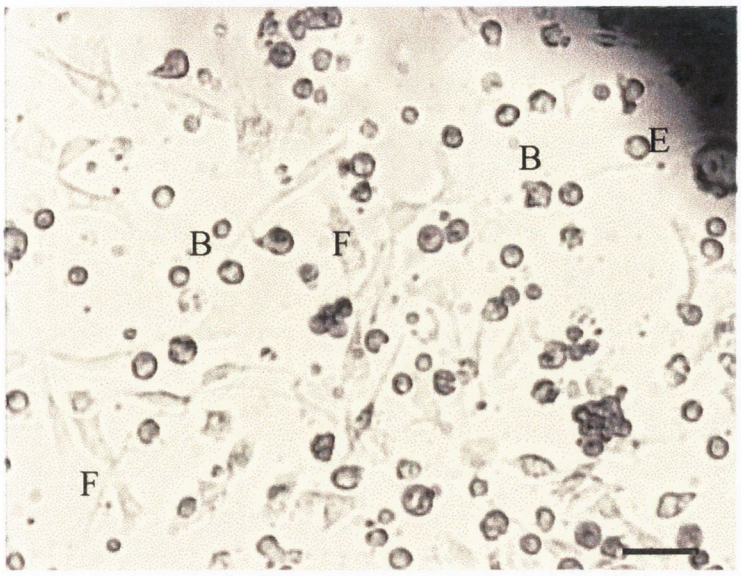


Figure 4.4 (a). Mantle explant after 5 days in culture. The explant (E) in the top right of picture with fibroblast-like cells (F) and ball shaped cells (B) migrating from the explant and attached to the surface. Magnification X 200. Scale bar = 100 μm.

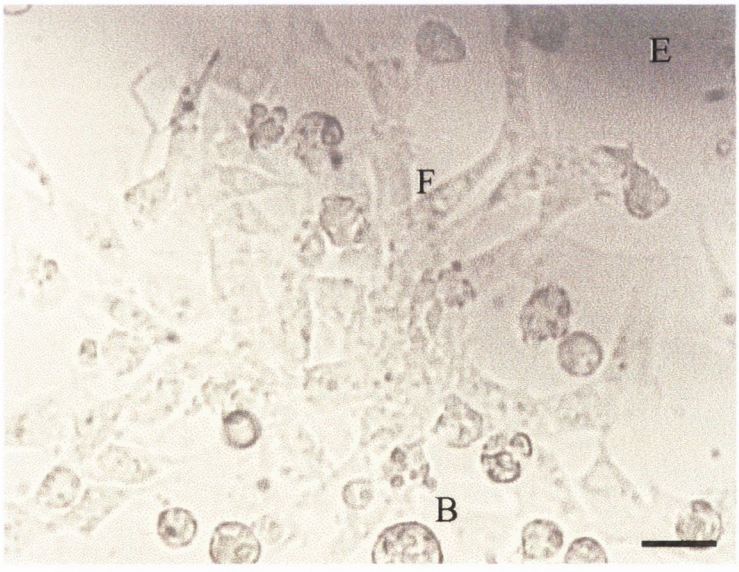


Figure 4.4 (b). Cell matrix consisting of elongated fibroblast-like cells (F) and ball shaped cells (B) migrating from the mantle explant (E) after 4 days in culture. Magnification X 400. Scale bar = 40 μm.

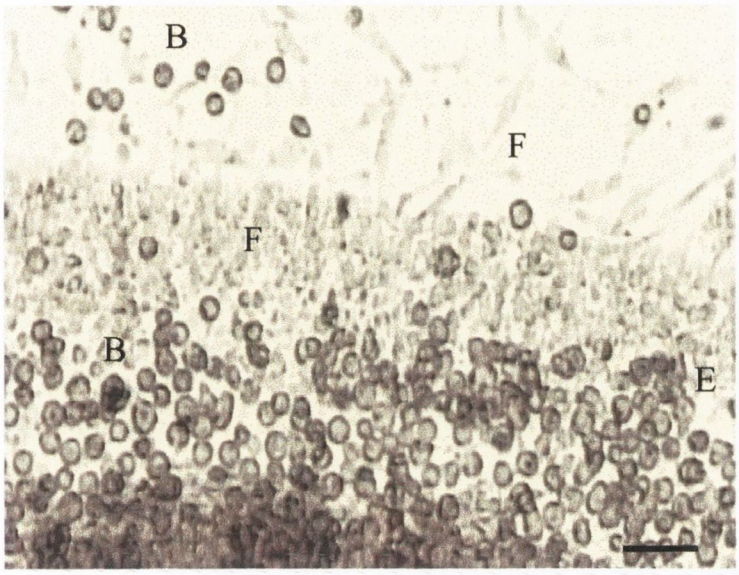


Figure 4.4 (C). Mantle explant after 6 days in culture showing migration of fibroblast-like (F) and ball shaped (B) cells from the explant (E). Picture shows layers of cells migrating out from explant. Magnification X 200. Scale bar = 100 μm.

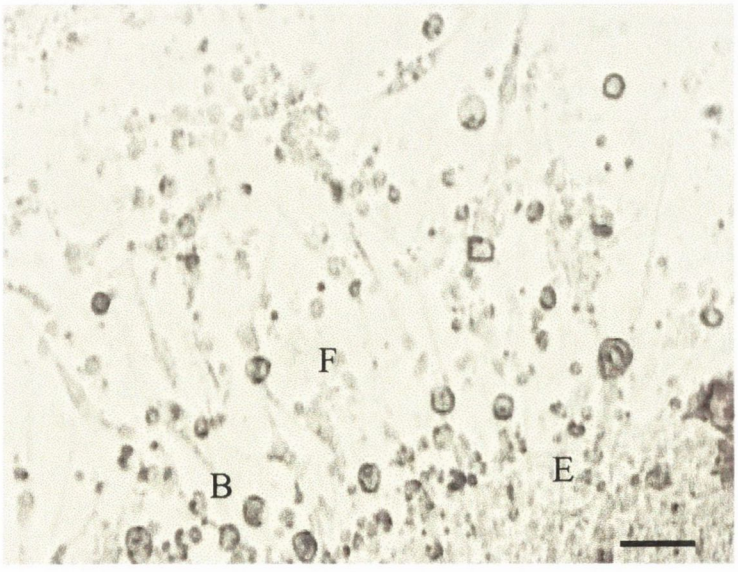


Figure 4.4 (d). Mantle explant after 11 days in culture. Elongated fibroblast-like cells (F) and ball shaped cells (B) still attached to the substrate and migrating from explant (E). Magnification X 200. Scale bar = 100 μm.

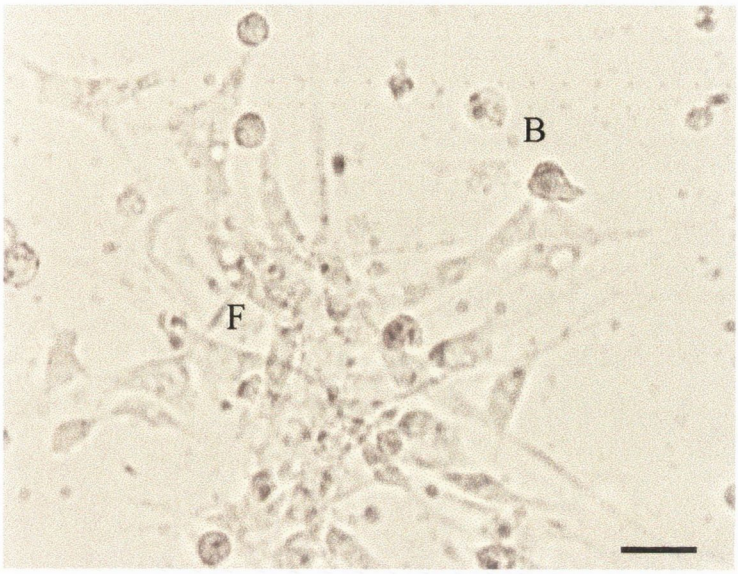


Figure 4.4 (e). Cell matrix of fibroblast-like cells (F) and ball shaped cells (B) still attached to the substrate after 14 days in culture. Magnification X 400. Scale bar = 40 μm.

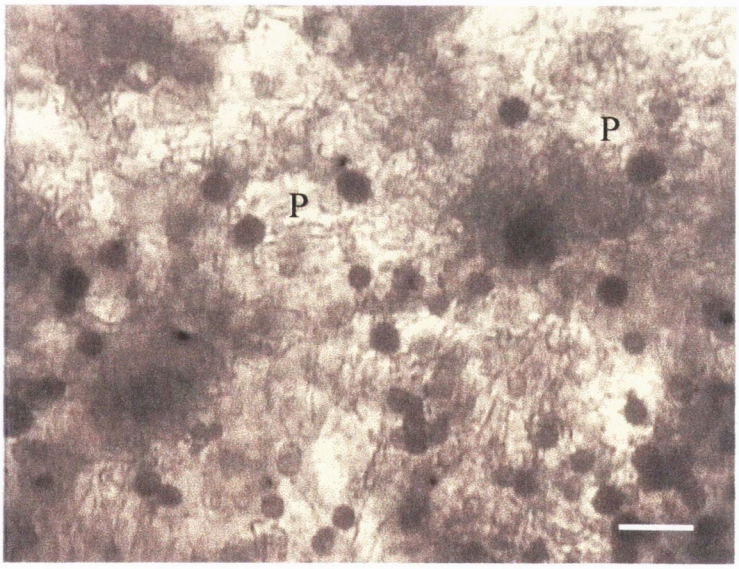


Figure 4.4 (f). Explant of mantle tissue after 12 days in culture. Tissue partially dissociated with cells forming dense clumps around darkly coloured pigment cells (P). Magnification X 200. Scale bar = 100 μm.

Although gill and digestive gland explants were maintained in culture, cell migration from these explants was not a good as with the mantle explants (Figure 4.5). As can be seen from Table 4.8, after 4 days in culture cells from the mantle explant started to adhere to the culture vessel, elongate and migrate out from the tissue. This continued and by 8 days in culture a good matrix of cells was found around the explant. These cells looked healthy and remained attached up to 14 days. However after 16 days these cells became unattached and returned to a ball shape. Digestive gland explants also show good cell adherence, but not as much elongation as mantle cells. Zebra mussel gills do not form good explants (Table 4.8) and show more promising results when cells are cultured in suspension.

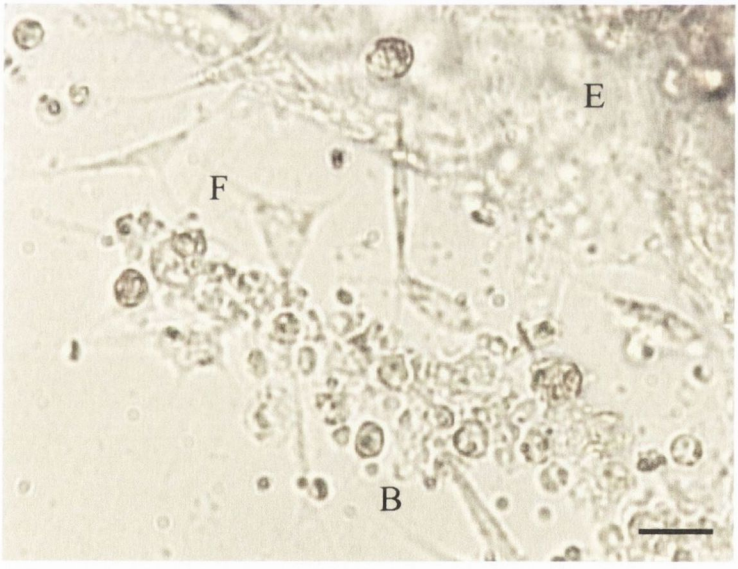


Figure 4.5. Digestive gland explant after 7 days in culture with fibroblast-like cells (F) and ball shaped cells (B) migrating out from the explant (E). Magnification X 200. Scale bar = 100 μm.

Table 4.8. Results from the culture of various tissues of the zebra mussel as tissue explants. + = mediocre; ++ = good; +++ = very good; - = not good. DG = digestive gland.

Tissue	4 days			8 days			14 days		
	Attach	Elongate	Matrix	Attach	Elongate	Matrix	Attach	Elongate	Matrix
Mantle	+	+	-	+++	+++	+++	++	++	++
Mantle	+	+	-	+++	+++	+++	++	++	+
Mantle	++	++	+	+++	+++	+++	++	++	+
Mantle	++	++	+	+++	+++	+++			
				(Cilia + flagella moving)					
DG	+	+	-	++	++	+	++	++	+
				(flagella moving)					
DG	+	-	-	++	+	+	++	+	+
Gill	+	-	-	+	+	+	-	-	-
							(looks unhealthy)		
Gill	+	-	-	-	-	-			
				(looks unhealthy)					

Cell suspension

Generally tissues from the zebra mussel were maintained best in culture as a cell suspension rather than as an explant. Fewer lipid cells are found in cell suspensions as they are removed by the centrifugation step. Cell suspensions were best maintained in a culture vessel with a smaller surface area, with the 24 well multiwell plate giving the best results. Cell attachment and elongation was better in areas where the cell density was lower (Figure 4.6), for example around the edges of the culture vessel where cells were not all tightly packed together.

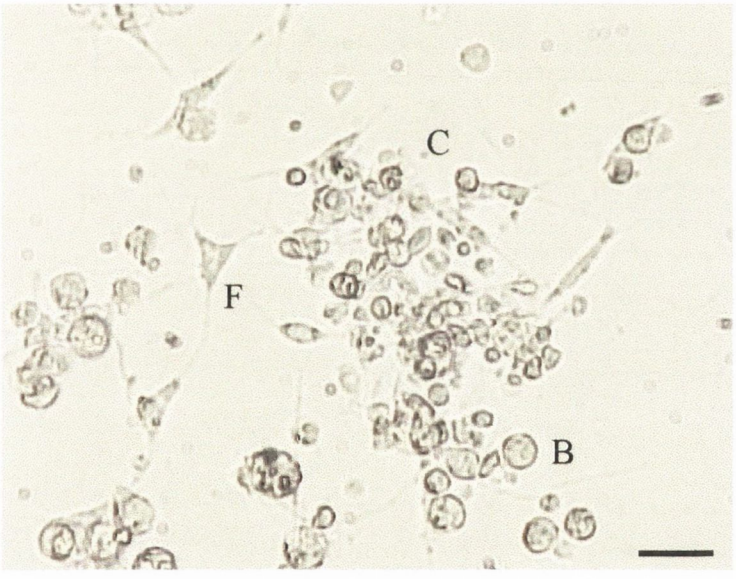


Figure 4.6 (a). Dissociated gill cells after 4 days in suspension. Cells have started to aggregate into a cell clump (C) containing ball shaped cells (B) and interconnecting fibroblast-like cells (F). Magnification X 200. Scale bar = 100 μm.

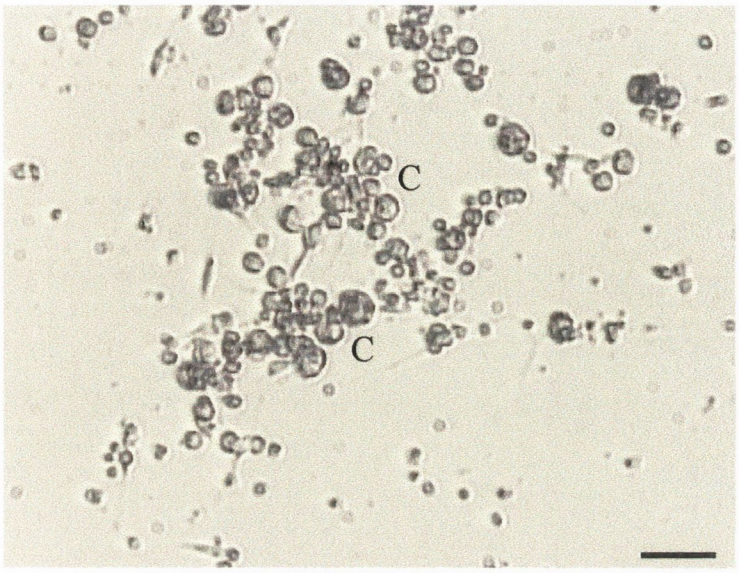


Figure 4.6 (b). Dissociated gill cells after 4 days culture attaching to the substrate forming clumps of cells (C). Magnification X 100. Scale bar = 200 μm.

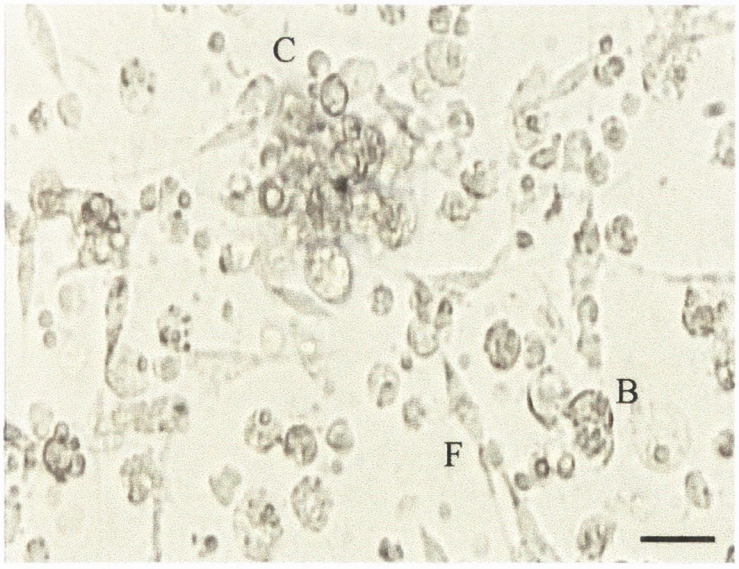


Figure 4.6 (c). Gill cells in suspension after 10 days in culture. Cells have aggregated into cell clumps (C) with fibroblast-like cells (F) and ball shaped cells (B) attached to the substrate. Magnification X 400. Scale bar = 40 μm.

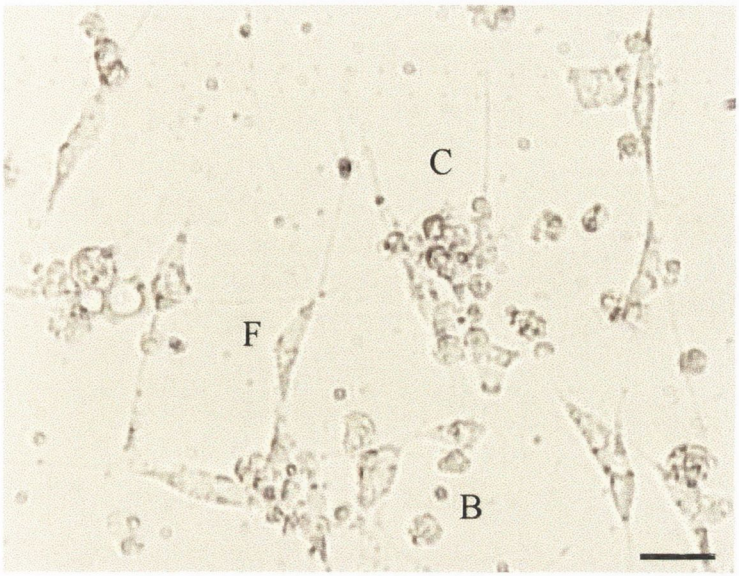


Figure 4.6 (d). Gill cells in suspension after 13 days in culture. Cell clumps (C) consist of fibroblast-like cells (F) and ball shaped cells (B) still attached to substrate. Magnification X 200. Scale bar = 100 μm.

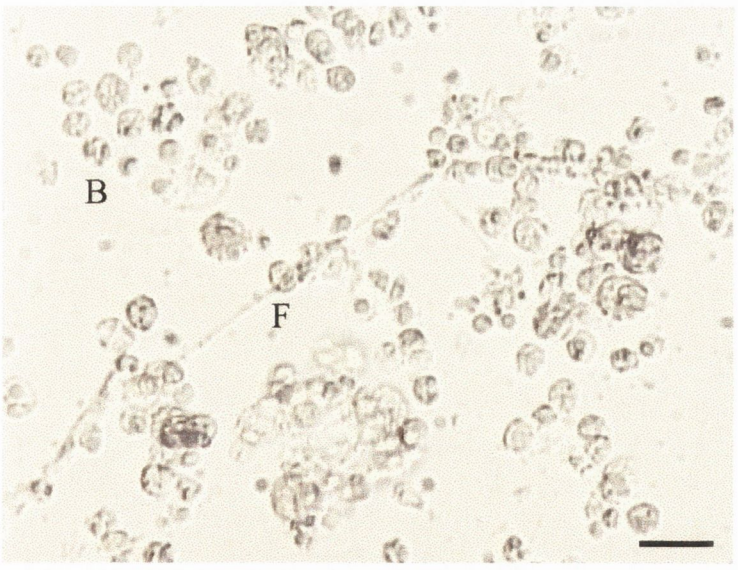


Figure 4.6 (e). After 17 days in suspension the gill cells begin to detach from the substrate. The number of fibroblast-like cells (F) reduces and more ball shaped cells (B) are evident. Magnification X 200. Scale bar = 100 μm.

After 4 days in culture cells in suspension showed good cell attachment with many elongated cells. In contrast with the explant method, gill cells cultured in suspension were the most successful, having good cell attachment and elongation after 4 days (Figure 4.6(a))(Table 4.9). Cilia and flagella were also seen to beat up to 8 days in culture. Cell elongation improved up to 8 days and continued up to 14 days in culture when cells could be seen to form connections and a cell matrix has developed (Table 4.9) (Figure 4.6(c&d)). Gill cells were still attached and elongated after 14 days in culture. Digestive gland cells were also maintained well in suspension (Figure 4.7), taking longer than the gill cells to attach and elongate (8 days) (Table 4.9). However after 14 days in culture these cells still looked healthy and remained elongated. The cells dissociated from the mantle were not as well maintained in suspension as they were when explanted. These cells generally did not attach well or elongate when in suspension, even after 8 to 14 days in culture.

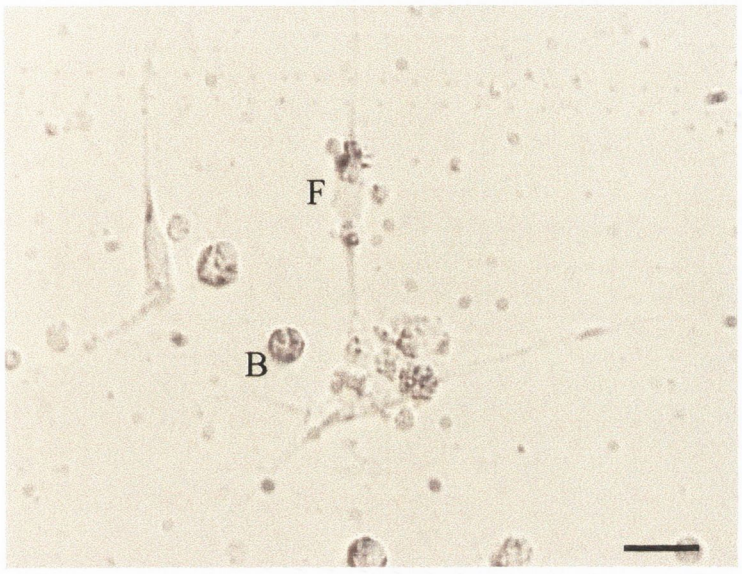


Figure 4.7. Digestive gland cells in suspension after 5 days in culture. Fibroblast-like cells (F) begin to attach to the substrate and form a cell matrix with ball shaped cells (B). Magnification X 400. Scale bar = 40 μm.

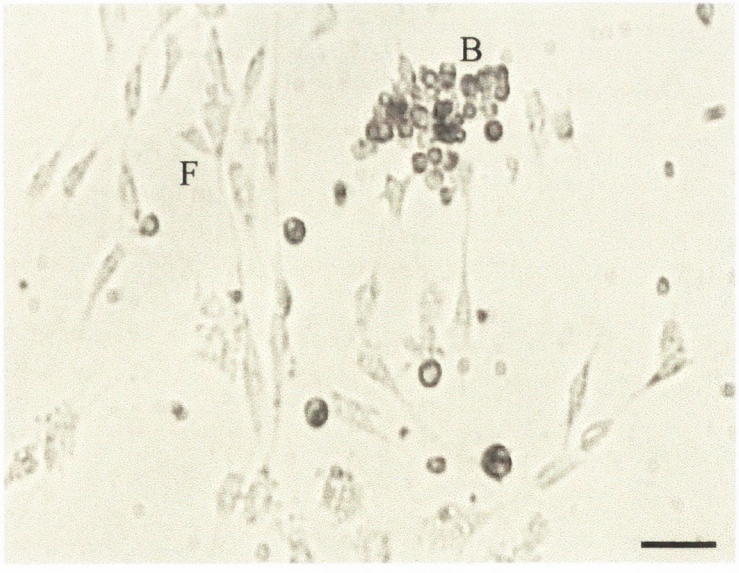


Figure 4.8. Mantle cells in suspension after 8 days in culture. As with the other tissues cells attach to the substrate forming clumps of ball shaped cells (B) and fibroblast-like cells (F). Magnification X 400. Scale bar = 40 μm.

Table 4.9. Results of cell suspensions from various tissues of the zebra mussel.

+ = Mediocre; ++ = Good; +++; Very good; - = Not good; DG = digestive gland.

Tissue	4 days			8 days			14 days		
	Attach	Elongate	Matrix	Attach	Elongate	Matrix	Attach	Elongate	Matrix
Gill	++	++	+	+++	+++	++	+++	+++	++
Gill	+++ (Cilia + flagella moving)	+++	+	+++	+++	+++	++	++	++
DG	+	+	-	++	++	++	++	++	+
DG	++	+	-	++	++	++	++	+	+
DG	+	+	-	++	++	+	++	++	+
Mantle	+	+	-	+	+	-	+	+	-
Mantle	+	+	-	Fungi					
Mantle	++	+	-	++	++	+	++	+	-

Overall the most successful cell culture method developed was for dissociated gill and digestive gland cells cultured in suspension in 24 mulitwell plates. These cells showed maximum attachment to and elongation on the culture substrate.

Cell viability

Cell viability could only be tested on cells in suspension and was measured using the trypan blue exclusion test. Cell viability was initially measured when the media was added to the cells before seeding. It was subsequently measured after different time intervals in culture. The minimum requirement for the use of cells in culture is 80% cell viability. Generally the average cell viability from 3 counts was around 85-90%, but could vary between 80% to 95%. Cell viability was affected by the mussel's health. Therefore only healthy fresh mussels were used for tissue culture. The initial viability when seeding cells was consistently >80%. Measured after 4 days in culture, digestive gland cell viability was found to have dropped to 65%, while gill cells maintained 85% viability (Table 4.10). After 11 days in culture cell viability is halved and remains constant up till day 14.

Table 4.10. Viability of gill and digestive gland (DG) cells in suspension after various periods in culture measured using the trypan blue exclusion test.

Tissue	Initial viability	4 d viability	6 d viability	11 d viability	14 d viability
Gill	85%	82%			
DG	85%	65%			
Gill	80%		73%		
DG	81%		64%		
Gill	88%			41.5%	
DG	85%			40%	
Gill	87%				40%
DG	86%				50%

Decontamination

Bleach and ethanol

Bacterial and fungal contamination was found to persist after short exposures (10 and 30 seconds) to both bleach and 70° ethanol. However although the longer exposure times (60 and 120 seconds) were successful at preventing contamination (Table 4.11), they were detrimental to tissue viability as few cells or explants were found to attach to the culture vessel.

Antibiotic solution

Although exposure for 15 minutes to the initial antibiotic solution (developed for use with fish cells) did prevent bacterial and fungal contamination cells did not attach and elongate

on to the culture vessel and no cell matrix were formed. This is probably due to the antibiotic solution being too strong for the cells. No difference was noticed between the use of PBS or HBSS. Shorter exposures and exposure to half strength solution resulted in bacterial and fungal contamination.

Only when the final antibiotic solution was introduced was cell migration observed from explants and a matrix formed by cells in suspensions (Figures 4.4 to 4.8). Using this technique cell viability was maintained (>80%) while bacterial and fungal contamination was reduced. With an initial 10 minute exposure at each antibiotic concentration (X4, X2 and X1) bacterial contamination persisted. Therefore exposure times were changed to 30, 20 and 10 minutes for the X4, X2 and X1 antibiotic concentrations respectively. Although infrequent contamination did persist (contamination with gram negative bacteria, protists and fungi), it was not a major problem and generally contamination was confined to one or two wells in the multiwell plate.

DTT

Initially when tissues were being exposed whole to the antibiotic solution exposure to DTT helped to reduce the occurrence of bacterial contamination. However once the tissues were cut into 1-2 mm^2 pieces before antibiotic treatment, no difference in contamination was noticed between tissues exposed and not exposed to DTT. This step was therefore removed from the procedure.

Table 4.11. Summary of the decontamination techniques used during the development of tissue culture technique for the zebra mussel and their effect on cell viability. + = Good disinfection/viability; - = Bad disinfection/viability

Disinfectant	**Disinfection**		**Cell viability**	
	<30 sec	*60 sec*	*<30 sec*	*60 sec*
Bleach				
5%	-	-	+	-
10%	-	+	+	-
20%	-	+	-	-
Ethanol 70°	-	+	+	-
	<10 min	*>15 min*	*<10 min*	*>15 min*
Initial anti-b	-	+	+	-
	10 min	*30, 20, 10 min*	*10 min*	*10, 20, 30 min*
Final anti-b	-	++	+	++
	Whole tissue	*Cut tissue*	*Whole tissue*	*Cut tissue*
DTT	+	+	+	+

Tissue dissociation

Initially tissues were not dissociated and were just cut into small pieces and explanted. However as few cells were seen in the culture vessel it became evident that some degree of dissociation was necessary.

Collagenase and trypsin

Collagenase and trypsin were the first dissociation enzymes to be used. Trypsin was too strong and over dissociated the tissues. It was detrimental to cell viability with non-trypsinised tissues having higher cell viability. Collagenase dissolved in PBS was less harmful to the tissues but cell attachment was low and no cell elongation was observed. The addition of collagenase and trypsin to the culture medium did seem to aid attachment although cells did not elongate.

EDTA

After chemical dissociation using EDTA cell attachment and limited cell elongation did occur. However after continued experimentation no increase in cell elongation was observed, and it was therefore decided to try pronase.

Pronase

Using this method of enzymatic dissociation tissues were found to be appropriately dissociated while cell viability remained consistently high (>80%). However if left too long in pronase (e.g. gills left for 24 hours) tissues will dissociate completely leaving few

explants or cell aggregates, but a lot of cells in suspension. Pronase was not as toxic to the tissues as trypsin. The optimum dissociation times were 12, 16 and 40 hours for the digestive gland, gill and mantle respectively.

Table 4.12. Summary of different methods of tissue dissociation and their effect on cell viability on tissues of the zebra mussel in culture. + = Good dissociation/viability; - = Bad dissociation/viability.

Dissociation method	**Dissociation**		**Cell viability**	
No dissociation	-		-	
Collagenase	*6 mg ml^{-1} PBS*	*5 mg ml^{-1} trypsin*	*6 mg ml^{-1} PBS*	*5 mg ml^{-1} trypsin*
	+	+	+	-
Trypsin	*2.5% in PBS*	*0.25% in media*	*2.5% in PBS*	*0.25% in media*
	+	+	-	-
EDTA	*5 mM*		*5 mM*	
	+		+	
Pronase	*0.025% in buffer*		*0.025% in buffer*	
	++		++	

Media

RPMI 1640

Explants and cells from the zebra mussel maintained in the RPMI 1640 media did not attach to the culture vessel, and were often found floating in the media. Cells that did attach remained round and did not elongate.

Leibovitz L-15

Although attachment did improve using the initial Leibovitz L-15 medium, cells remained round with few cells migrating from the explants. No difference was noticed between the use of HBSS or PBS to dilute the L-15. The use of 0.1% lipid solution did seem to increase attachment, but still no cell migration or elongation was observed. Addition of the yeast solution to the media showed no obvious effect, nor did the addition of 10 $\mu l\ ml^{-1}$ and 100 $\mu l\ ml^{-1}$ 0.1% lipid and 0.1% yeast solution.

With the development of the 15% Leibovitz L-15 media, cells in culture started to attach and elongate on the culture vessel as described in the tissue culture section above. Cell attachment, elongation and formation of a cell matrix were all observed using this media (Figure 4.4 to 4.8). The cilia on gill cells was seen to beat rhythmically after up to 8 days in culture and cells remained viable for up to 14 days in culture.

Table 4.13. Effects of various cell culture media and supplements on cells of the zebra mussel in culture. + = Mediocre; +++ = Very good; - = Not good.

	Cell attachment	Cell elongation	Cell viability
RPMI 1640	-	-	-
Leibovitz old	+	-	-
Yeast supplement	-	-	-
Lipid supplement	+	-	-
Leibovitz new	+++	+++	+++

Media change

The addition of fresh media after every 4 days had a noticeable effect on the cells. In one experiment the media was changed in only half of the gill cells in suspension in a 24 multiwell plate. A huge increase in cell attachment and elongation was observed in the cells where the media had been changed.

Culture vessels

The surface area of the 25 cm^2 flasks was too great. Even when several explants were cultured, few explants attached and no cell migration was observed. No difference between collagen coated and normal flasks was found. Better results were found with the reduced surface area of the petri dish and 24 well multiwell plate (Table 4.14). The NUNCLON surface coated vessels (NUNC) were found to produce better cell adhesion than the Falcon petri dish. For cell suspensions the 24 well multiwell plate gave the best

results, while the Petri dish containing 10-12 explants was best for tissue explants. Although good results were also obtained when 3-4 explants were cultured in each well of the 24 multiwell plate.

Table 4.14. Summary of the effectiveness of culture vessels used for *in vitro* culture of tissues of the zebra mussel. + = Mediocre; ++ = Good; +++ = Very good; - = Not good.

Type	Surface area	Optimal vol.	Cell attachment	Cell elongation	Cell viability
NUNC flask	25 cm^2	2ml	-	-	-
NUNC collagen coated flask	25 cm^2	2ml	-	-	-
NUNC petri dish	8.8 cm^2	1.5 ml	++	++	++
NUNC 24 multiwell dish	1.9 cm^2	0.5 ml	+++	+++	+++

Incubation

Of the 3 incubation temperatures tested (10, 15 and 20°C), 15°C was found to be best suited for the culture of tissues from the zebra mussel.

Cell proliferation

Various problems were encountered when using the immunocytochemical technique to measure PCNA in the cells in culture. The technique was found to be too harsh on the

cells and all cells became dislodged and were lost during the process. The cells centrifuged onto the glass slides using a cytospin also became dislodged. Therefore cells and tissues from the zebra mussel are not attached to the culture vessel well enough to allow the use of this technique.

Cell characterisation

Attempts at using electron microscopy for cell characterisation were unsuccessful as neither the cells nor the explants in culture were adhered to the surface of the culture vessel after fixation in Sorensen's buffer. Cells were identified by light microscopy. Fibroblast-like cells were easily identified as they were the cells that elongated and spread out along the culture surface with a long thin process (Figure 4.4 to 4.8). All other types of cell maintained a round shape and were difficult to identify. Lipid cells were also identified by light microscopy (Figure 4.3). These were large cells found in explants, particularly from the digestive gland. Having been removed by centrifugation, they were not generally found in cell suspensions. Sex cells were also identified. Oocytes were identified as large globular cells with a large nucleus. Spermatocytes were also easily identified as small triangular shaped cells with a long flagella. As these cultures were developed before sexual maturity these cells are not shown in the figures provided.

In the literature regarding invertebrate cell culture the cells found in culture are described as fibroblast-like, epithelial-like/epithelioid, round, ball shaped, large or small cells. Very rarely is an effort made to actually identify the cell type. From the literature it is known

that bivalve gills mostly consist of ciliated epithelial cells. Numerous cell types have been identified in the digestive gland with digestive, basophilic and epithelial cells found in *Mytilus edulis* (Livingstone et al., 1992).

DISCUSSION

Although some areas of invertebrate tissue culture have been successful with the establishment of over 200 cell lines from insects and ticks (Mitsuhashi, 1989), most efforts to develop permanent and proliferative cell cultures from marine (aquatic) invertebrates have been unsuccessful (Rinkevich et al., 1994). Relatively little work on the *in vitro* culture of aquatic invertebrates has been published, largely owing to the fact that experimental failures are generally not suitable for publication in most scientific journals (Rinkevich, 1999). Invertebrate tissue culture has been based on the idea that all the cells from different animal taxa are basically the same, having similar nutrient requirements, are controlled by the same developmental and physiological biochemical pathways and are under the expression of identical genes (Rinkevich, 1999). Therefore techniques developed for the growth and maintenance of vertebrate, particularly mammalian cells have been adapted for use with invertebrate tissues. Although having been successful with other invertebrates (e.g. insects), this technique does not seem to have worked with aquatic invertebrates.

In the present study a technique for the maintenance of cells from the gill, digestive gland and mantle of the zebra mussel was developed. Initially explants of the tissues proved unsuccessful and several methods of tissue dissociation were attempted, the most successful of which involved a prolonged exposure to low concentrations of the enzyme pronase. Trypsin was found to be too harsh on zebra mussel cells reducing viability, a problem also experienced by other authors (Brewster & Nicholson, 1979; Le Marrec, 1995). However in the literature trypsin is the most popular of the enzymatic dissociation techniques and is the most common form of dissociation for invertebrate tissues (Naganuma et al., 1994; Takeuchi et al., 1994; Domart-Coulon et al., 1994). Although not successful here the addition of dissociating enzymes in the culture media has reportedly increased cell attachment and viability (Wen et al., 1993). In some cases dissociation was found to decrease cell viability and the explant method was preferred (Brewster & Nicholson, 1979). However cell suspensions have also been commonly used with reported success (Brewster & Nicholson, 1979; Odinstova et al., 1994; Takeuchi et al., 1994; Renault et al., 1995). Once in suspension cells from the zebra mussel, particularly from the gill were noticed to aggregate and attach to the surface of the culture vessel in clumps, a phenomenon that has been previously observed (Odinstova et al., 1994; Renault et al., 1995).

Being filter feeders bivalves come into direct contact with micro-organisms such as bacteria, fungi and protists and can harbor such infectious agents in their tissues, particularly the gills. For this reason the culture of molluscan hearts has been popular, as they are surrounded by a pericardium membrane and are maintained in a relatively sterile

environment (Odinstova & Khomenko, 1991; Wen et al., 1993; Domart-Coulon et al., 1994; Naganuma et al., 1994; Odinstova et al., 1994; Takeuchi et al., 1994; Kleinschuster et al, 1996). However recently a standard protocol for decontaminating tissues from various bivalve species has developed and has been used with success on the zebra mussel. This protocol involves surface decontamination, depuration, aseptic removal of tissues and a series of antibiotic washes (Stephens & Hetrick, 1979; Odinstova & Khomenko, 1991; Le Marrec, 1995). Commonly used antimicrobials include penicillin, streptomycin, gentamycin, and the fungicide amphotericin B (Am-B). Am-B has been reported to be toxic to cells in culture (Ellis and Bishop, 1989) and has not been included in the present antibiotic formulation. The use of antibiotics and other decontaminates can negatively effect the cells health (Odinstova & Khomenko, 1991) and a balance between decontamination and maintaining cell viability must be found. Other chemicals used to aid decontamination include serotonin, which increases epithelial ciliary activity in the gill (Gardiner et al., 1991) and DTT, used to reduce mucous in the gills. In the current study DTT was not found to significantly reduce contamination.

The media developed for the maintenance of tissues of the zebra mussel in the present study was based on the commercial Leibovitz L-15 media with the addition of supplements and antibiotics. Modifications were made to the osmolarity and pH of the media in order to make it resemble more the haemolymph of the mussel. Although developed for use with mammalian cells, Leibovitz L-15 has been extensively used in aquatic invertebrate culture (Table 4.15) and has been reported as the most successful media to date (Nadala et al., 1993; Domart-Coulon et al., 1994; Kleinschuster, et al.,

1996). L-15 is though to be the most suitable commercial media for molluscan cell culture owing to its high amino acid content, an important component of the molluscan diet (Renault et al., 1995). Other commercial media used with varying degrees of success in the culture of bivalve molluscs include Eagle's MEM, Hanks 199 and RPMI 1640 (Table 4.15). Recommendations on how often media should be changed vary from every 2 days (Gardiner et al., 1991; Takeuchi et al., 1994), 4 days (Kleinschuster et al., 1996; Machii & Wada, 1989) to every 3 weeks (Brewster & Nicholson, 1979). It was found that for the zebra mussel a media change every 4 days was suitable.

Supplements added to the medium for the culture of zebra mussel tissues included L-glutamine, foetal calf serum (FCS) and a series of antibiotics (Table 4.6). There is considerable contradiction in the literature regarding the addition of supplements to culture media of aquatic invertebrates. Several authors recommend the use of serum in the culture media to increase cell viability and aid attachment to the culture vessel (Li & Stewart, 1966; Brewster & Nicholson, 1979; Domart-Coulon et al., 1994; Takeuchi et al., 1994; Kleinschuster et al., 1996). However serum content above 2% (Odinstova et al., 1994) and above 20% (Domart-Coulon et al., 1994) has been reported to be toxic to some invertebrate cells, particularly larval cells. Epithelial growth factor (EGF)-like growth factors have been found in tissues of *mytilus edulis* (Odinstova & Khomenko, 1991). Growth factors were found by Domart-Coulon et al. (1994) to have a significantly positive effect on oyster heart cell cultures. However Wen et al. (1993) did not observe any benefit from the addition of these same growth factors to the same tissues. This is one example of many conflicting results published regarding the formulation of media for use

with aquatic invertebrates. Therefore the best policy when formulating a media for the culture of tissues of a previously unstudied animal is trial and error.

Regarding cell culture, molluscs are probably the most intensively studied group of aquatic invertebrates, with most of this research occurring during the 1970's. It was during this time that the only molluscan cell line, the *Bge* cell line was established from embryos of the freshwater snail *Biomphalaria glabrata* by Hansen (1976). However a few successes in the area of primary culture of aquatic invertebrates has been reported (Table 4.1). Oyster amebocytes were reportedly successfully maintained *in vitro* for periods of up to 6 months (Brewster & Nicholson, 1979). Takeuchi et al, (1994) published a method for primary and secondary cultures of larval cells of the pacific oyster (*Crassostrea gigas*), resulting in a monolayer of epithelioid cells that could be disssociated, subcultured and propagated into confluent monolayers. However this is one of several publications of long-term cell cultivation from a variety of mollusc tissues that are suspected to have been contaminated by thraustochytrid species, common marine and freshwater heterotrophic protists (Rinkevich, 1999) (Table 4.1). Infection was found in approximately 30% of oyster cultures and 27% of clam cultures (Rinkevich, 1999). It is possible that many of the best reports of invertebrate cell culture have described the development and maintenance of thraustochytrids rather than the original animal cultures. This means that for aquatic invertebrates it has still not yet been possible to maintain growing cells in culture.

When work on the *in vitro* culture of tissues of the zebra mussel started it was initially hoped to use these techniques to study endocrine disruption in the zebra mussel by looking at the effect of endocrine disrupting chemicals at a cellular level. In order for this to be able to occur fully differentiated and functional proliferating cells would need to be maintained in culture. But as aquatic invertebrate tissue culture is still in its infancy this was not possible. However a technique for the short-term culture of the gills, mantle and digestive gland of the zebra mussel was successfully developed, producing explants and cell suspensions that remain viable in culture for up to two weeks suitable for use in short term toxicological studies.

CONCLUSION

One of the main problems in establishing primary cultures from marine invertebrates is due to the difficulty of finding the optimal growth medium to promote cell proliferation. This is due to the lack of basic information on the nutritional requirements and metabolism of the animals. It is therefore necessary to take a step back, and to study the biochemical requirements and make up of the animal cells. In most studies haemolymph was the basis for the culture media. L-15 has had the most reported success. Invertebrate tissue culture is still very much a developing science and a new researcher to the field faces a myriad of problems. It is important to note that it is the combination of all of these factors described above (decontamination, dissociation, media and culture parameters) that act cumulatively to produce a successful tissue culture. The development of a new *in*

vitro method could be described as opening a can of worms, owing to the huge number of variables involved, any one of which could prove detrimental to the culture. This is one of the reasons why aquatic invertebrate tissue culture is still very much in its developmental stage.

Table 4.15. Summary of various media formulations used in the *in vitro* culture of tissues from aquatic invertebrates. DG = digestive gland.

Medium	Suppliments	Species	tissue	Reference
Hanks 199	50% salt solution 10% BAF 10% HS	*Crassostrea virginica*	Heart	Li & Stewart, 1966
Balanced salt solution	1% FCS 1% yeast extract 0.5% lactalbumin hydrolysate	*Crassostrea virginica*	Amoebocytes	Tripp et al., 1966
Medium 199	FCS Beef embryo extract Oyster hear extract	*Crassostrea Virginica*	Heart & Embryo	Brewster & Nicholson, 1979
Leibovitz L-15	Mollusc haemolymph Embryo bovine extract Taurine	*Mizuchopecten Yessoensis*	Embryo	Odinstova & Khomenko, 1991
Eagle's medium	HBSS Egg yolk (6%)	*Mytilus edulis*	DG, gill Mantle	Cornet, 1995
Leibovitz L-15	10% FBS Neomycin sulfate	*Meretrix lusoria*	Heart	Wen et al., 1993
Leibovitz L-15	10% FP1 10% FCS PDGF & EGF	*Crassostrea gigas*	Heart	Domart-Coulon, 1994
Leibovitz L-15	FCS Distilled water	*Crassostrea gigas*	Larvae	Takeuchi et al., 1994
Leibovitz L-15	2% FCS Insulin Taurine	*Mizuchopecten yessoensis*	Embryo	Odinstova et al., 1994
Leibovitz L-15	3% L-15 in sea water 10% FCS Oyster haemolymph	*Ostrea edulis*	Heart	Renault et al., 1995
Leibovitz L-15	10% FCS Sea water	*Pecten maximus* *Crassostrea gigas*	Gill Heart Labial palp	La Marrec, 1995
MEM Eagle	10% FBS 10% haemolymph 2% Insulin	*Mya arenaria*	Heart	Kleinschuster et al., 1996

Chapter 5.

DETERMINATION OF THE *IN VITRO* LC_{50} FOR CELLS FROM THE DIGESTIVE GLAND AND GILL OF THE ZEBRA MUSSEL (*DREISSENA POLYMORPHA*) EXPOSED TO NONYLPHENOL.

INTRODUCTION

4-n-Nonylphenol (NP) is the degradation product formed after the breakdown of alkylphenol ethoxylates, non-ionic surfactants used in a variety of industrial processes and cleaning products and released into the environment in wastewater treatment plant effluent (Ahel et al., 1984; Giger et al., 1987). NP is a widespread pollutant and there has been much concern regarding the endocrine disrupting (ED) effect of this chemical (for summary see Chapters 1 and 3). Although much research has concentrated on the endocrine distupting (ED) effect less work has been carried out to study the toxic effect of NP on animals particularly at a cellular level. The zebra mussel (*Dreissena polymorpha*) has been widely used as both a model species in freshwater toxicology and as a sentinel species in pollution monitoring (Mersch et al., 1992; Busch & Schuchardt, 1991; Comba et al., 1996; Dauberschmidt et al., 1997). Cell cultures from vertebrates have long been shown to provide convenient tools for the evaluation of potential toxicants (Zahn & Braunbeck, 1993). This chapter describes the use of the previously developed method

(Chapter 4) of maintaining cells from the zebra mussel in culture as cell suspensions to study the toxicity of NP at a cellular level.

In a previous experiment the toxicity of NP to zebra mussels *in vivo* was established (Chapter 2). However such *in vivo* exposures involving whole animals are costly, time consuming and ethically questionable. According to EU directive 86/609/EEC, the use of animals in toxicity testing should be avoided provided that a scientifically satisfactory alternative is available and encourages research in developing these alternative methods. Advantages of *in vitro* tests compared to *in vivo* tests are that the experimental procedures are easier, cheaper, allow for small-scale multiple experiments, standardisation, reduction in intra-specific variability and the reduction of waste (Lilius, 1997; Olabarrieta et al., 2001). These experiments are less time consuming allowing more compounds to be tested. They are useful in aquatic toxicology screening and allow more control of experimental conditions regarding both the cellular environment and the exposure conditions (Baksi & Frazier, 1990).

The principal assumption in *in vitro* toxicology is that a compound exerts its toxicity at the cellular level. The cytotoxic concentration established *in vitro* should therefore predict the toxic blood or tissue concentration *in vivo* (Bondesson et al., 1989). When this data is combined with a model predicting the toxicokinetics of the compound it should be possible to determine the acute oral toxicity of the chemical (Ekwall, 1983, Bondesson et al., 1989). Cellular toxicity can be defined as the adverse effect on such structures or functions that are essential for cell survival and proliferation (Seibert et al., 1996). Basal

or general toxicity is where chemicals act by interfering with the basal functions fundamental to all cells i.e. energy metabolism, plasma membrane integrity, etc. (Ekwall, 1983). Cell suspension cultures are suitable for use to detect basal toxicity and have the advantage of having less tedious and more economical procedures.

Most toxicity tests developed are based on cell lines with endpoints measuring cell proliferation or death, the basal toxicity of a compound (Walum et al., 1992). The main disadvantage of immortalised cell lines is that they have dedifferentiated and therefore do not have all the characteristics of the tissue of origin. However freshly isolated cells and primary cultures represent cellular models with functions corresponding to those in the tissue of origin. Hepatocytes are frequently used in toxicological research because of their central function in the metabolism of toxicants. For example fish hepatocytes have been widely used for cytotoxicity studies (Reader et al., 1993; Braunbeck, 1993; Zahn & Braunbeck, 1995). Isolated cells from marine invertebrates have been shown to be an effective system for studying pathways and mechanisms of xenobiotic-mediated effects. They have been used to study lysosomal latency (Lowe et al., 1995), the uptake of heavy metals and polycyclic aromatic hydrocarbons (Zaroogian & Anderson, 1995) and in studies on pesticide metabolism and enzyme effects (Bermelin et al., 1998). The use of isolated cells from various molluscan species in toxicological studies including digestive gland and blood cells of *Mytilus edulis* (Lowe & Pipe, 1994; Lowe et al., 1995), brown cells of the clam *Mercenaria mercenaria* (Zaroogian & Anderson, 1995) and the heart cells of the oyster *Crassostrea gigas* (Burgeot et al., 1995) has been reported. Although

the toxicity of NP to invertebrates has been previously investigated (see Chapter 2), no studies looking at the toxicity of NP to invertebrate cells in culture have been undertaken.

In this work an *in vitro* model for studying the cytoxicity of chemicals on various tissues of the zebra mussel is reported. This model was used to measure the toxicity of the endocrine disrupting chemical NP on a heterogeneous cell population isolated from the gill and digestive gland of the zebra mussel *Dreissena polymorpha* and maintained *in vitro*. The digestive gland has been chosen for the development of a cell culture system for use in molluscan toxicology because it is the main organ of metabolism of organic xenobiotics and the main tissue site of biotransformation and antioxidant enzyme activities (Livingstone, 1991). The gills of freshwater mussels are complex organs that function in ion transport, respiration, food capture and sorting, storage, and maintenance of embryos during reproduction. They commonly come into contact with pollutants and were therefore also chosen for this cytotoxicity study.

Cell viability after exposure to various concentrations of NP was tested using the standard trypan blue exclusion test. Viability of cultured cells after exposure to NP was also tested using the 3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction test. This colorimetric assay is based on the reduction of the yellow tetrazolium salt (MTT) to blue formazan by mitochondrial enzymes and was first developed by Mosmann (1983) for screening lymphocyte stimulation. It has subsequently been used extensively in cytotoxicity testing of toxins in vertebrate cell culture systems (Martin & Clynes, 1993) and was adapted for use in measuring the viability of bivalve cells in

different culture conditions (Domart-Coulon, 1994; Auzoux-Bordenave, 1995). The toxicity of NP to cells from the chinook salmon embryo cell (CHSE) cell line were also tested using the MTT assay. The acute toxicity of NP was tested on cell suspensions maintained in culture from both the gill and digestive gland and conventional ecotoxicological endpoints (LC_{50}, NOEC, LOEC) were determined. These *in vitro* LC_{50} results were compared with the previously reported *in vivo* LC_{50} results (Chapter 2) in order to draw an *in vitro* / *in vivo* comparison.

MATERIALS AND METHODS

Zebra mussels

The zebra mussels used for *in vitro* cell culture in this experiment were collected from Lough Ree (see Chapter 1). This was the same mussel population that was used in the previous *in vivo* exposure to NP. Water temperature at time of collection was 16°C. Mussels were taken back to the laboratory thoroughly scrubbed and placed in autoclaved tap water in sterilised 15 L glass tanks kept in a controlled room at 15°C with a 12 hour light/dark cycle. The water was changed three times a week. To ensure healthy mussels for tissue culture, mussels used were replaced every 2 weeks with fresh mussels collected from Lough Ree.

Tissue culture technique

Tissues from the zebra mussel were cultured *in vitro* using a slightly modified version of the technique previously developed and described in detail in Chapter 4. This technique is summarised in Table 5.1. Using this technique cells from the digestive gland (DG) and gills were cultured as cell suspensions only. The cell suspension method was used as it consistently produced more viable cells in culture than the explant method. Cultures from the DG must be regarded as mixed tissue cultures as it was impossible to completely separate the DG from the gonad and intestine (see Chapter 4).

The number of cells in suspension was counted using a Maillaise haemacytometer and the cell density regulated with the addition of the appropriate quantity of media. Cells were seeded at a density of around 2 million cells per ml for the trypan blue exclusion test and 50,000 cells ml^{-1} for the MTT reduction assay. Once added to the culture vessel cells were placed in a refrigerated incubator (Leec, Nottingham, U.K.) at 15°C and left undisturbed for 6 days to encourage cell attachment onto the surface of the culture vessel. After this time the media was changed and a quantity of NP was added to the fresh media. Cells were left to incubate with NP for a specified period of 24, 48 or 96 hours (hr) after which time cell viability was measured.

Table 5.1. Protocol for *in vitro* culture of cells in suspension from the gill and digestive gland of the zebra mussel for use in the NP toxicity tests to determine the *in vitro* LC_{50}.

DISSINFECTION

- Scrape mussel clean under running water. Place in sterile water.
- Place in antibiotic sol (2X) for 2 hr in laminar flow partially on ice.
- Immerse mussel in ethanol 70°. Allow to dry.
- In laminar flow, rinse mussel with 5-10ml sterile H_2O, dissect DG and gills (X10-20).
- Trim tissue to ensure a pure tissue sample (where possible).
- Place organs in separate sterile buffer sol. and chop up tissue 1-2 mm^2.
- Rinse in petri dish of sterile buffer sol.
- Antibiotic Sol. - X 4 (10ml of anti-b sol.) 30 mins
  - X 2 (5ml of anti-b sol + 5ml buffer sol.) 20 mins
  - X 1 (2.5ml anti-b sol + 7.5ml buffer sol.) 10 mins
- Rinse in sterile buffer sol.

DISSOCIATION WITH PRONASE:

- Add tissue to 0.025% pronase in Buffer sol. (50ml - 12.5mg) with antibiotic (X).
- Keep separate organs from different animals in separate tubes.
- Place at slight angle in 4°C for 15 hr.
- Filter sample liquid through autoclaved gauze 60 μm (slowly) into centrifuge tube.
- Rinse tube and gauze with buffer sol.
- Centrifuge filtered liquid for 3 min at 1200 rpm.
- Remove supernatant, add buffer and re-centrifuge, 3 min at 1200 rpm. X2
- Remove supernatant.
- Add media and mix cells using pipette. Find concentration of viable cells using a hemocytometer and adjust volume if necessary.
- Place cells in culture into incubator at 15°C.

NONYLPHENOL EXPOSURE:

- After 6 days in culture, media change and add NP.
- Leave cells incubate for specified time (24, 48 or 96hr).
- Use trypan blue or MTT to check cell viability.

TRYPAN BLUE:

- Mix equal quantity of trypan blue and media containing cells.
- Use an inverted microscope and haemacytometer to count number of blue and viable cells per square in graticule and find % viability.
- Find cell density using number of live cells only. Adjust volume if necessary.

MTT

- Add 10 μl MTT to 100 μl cells in media and incubate for 6 hr at 15°C.
- Add 100 μl isopropanol. Shake for 10 min at 15°C.
- Measure absorbance at 580 nm using ELISA reader.

Media

The cell culture media used was the same as that described in Chapter 4. Different volumes of media were added to the different culture vessels in an attempt to optimise conditions for the cells in culture. The culture vessels used in these experiments and the optimum media volume are shown in Table 5.2. Only the 24 and 96 well multiwell plates were used in exposures to NP. Initially half of the media volume was changed after the first 4 days in culture with a full media change every 4 days after that. However this was later changed to a full media change after the first 6 days in culture. There was a problem with the culture vessels drying out in the incubator, which was overcome by wrapping the culture vessels in parafilm.

Table 5.2: Culture vessels and media volume used for the maintenance of cells in suspension for the *in vitro* toxicity tests to NP.

Culture vessel type	Make	Surface area	Media volume tested	Media volume (optimal)
Petri dish	NUNC	8.8 cm^2	1.0, 1.5 & 2 ml	1.5 ml
24 well multiwell plate	NUNC	1.9 cm^2	0.3, 0.4 & 0.5 ml	0.3 ml
96 well multiwell plate	NUNC	0.33 cm^2	0.1 & 0.2 ml	0.1 ml

Preparation of Nonylphenol solutions

The compound under investigation during this *in vitro* toxicity experiment was 4-NP (98+% purity, Lancaster Synthesis Ltd.). The chemical was added to the cells in culture at the concentration of 0.1, 0.5, 1.0, 5.0, 10.0 and 100.0 mg l^{-1} (nominal concentration). A control and solvent control were also included. These are the same concentrations as were used in the *in vivo* lethality exposure to NP (Chapter 2), with the addition of 0.5 mg l^{-1}. Stock solutions were prepared by dissolving the appropriate amount of NP into a known volume of solvent. Dimethylsulfoxide (DMSO) was used as the carrier solvent as it had previously been found not to be toxic to cells in culture at the concentration used (2.5% of the culture medium) (Kilemade & Mothersill, 2001), and has little or no activity in animal cell systems at low doses (Rubin, 1983). Dilutions were prepared from a stock solution of 100 mg l^{-1} NP, with 7.5 and 2.5 µl added to the 24 and 96 well plates respectively (2.5% of the culture medium).

Viability

Trypan blue

Cell viability was measured using the trypan blue exclusion test as described in Table 5.1. Viability was initially measured when the cells in suspension were added to the media (0 hr exposure) and was found to be consistently >80%. Viability was also measured after the cells were exposed to NP for a specified time in hours (24, 48 & 96 hr). Exposure took place in a 24 well multiwell plate containing 0.3 ml media. After exposure to NP 0.2

ml of the media were removed leaving 0.1 ml in well to which 0.1 ml of trypan blue was added and left to stand for a few minutes. Using an inverted microscope the number of healthy cells and those that had taken up the blue dye (indicating cell death) per square on a 1 mm^3 graticule were counted.

MTT

The method used in the MTT reduction test was adapted from Domart-Coulon et al. (1994). A stock solution was prepared by dissolving the MTT chemical (Sigma) into PBS at a 5 mg ml^{-1} final concentration. This solution was sterile filtered (0.22 μm filter, Nalgene) and stored at 4°C. For the assay 10 μl of this MTT stock solution was added directly to 100 μl of the cell suspension in wells of a 96 well microplate (Nunclon). The plate was incubated for 6 hours at 15°C. The formazan produced by the cells was then dissolved by adding to the wells 100 μl of isopropanol (Sigma) containing 0.04 N HCl (Sigma), and by shaking the plate for 10 minutes at 15°C. The absorbance at 580 nm was measured using an ELISA reader (Molecular devices, Spectra Max 250) with a reference wavelength of 630 nm. The ELISA reader was connected to a PC (Hewlett Packard) containing SOFTmax PRO software version 3.1.1 where data output was performed.

CHSE cell line

The MTT assay was also tested on the CHSE-214 fish cell line established from Chinook salmon embryo cells in 1964. Cells were maintained as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with FCS 12.5%, L-glutamine

200 mM, 5 ml penicillin/streptomycin 5000 IU ml^{-1} and HEPES buffer solution 0.025 mol l^{-1}. Once 80-100% confluent, the cells were sub-cultured following detachment from the culture vessel using a versene/trypsin mix (50:50) and were cultured at 20°C before exposure to NP. Cells were exposed to NP after 24 hr in culture at a density of around 70,000 cells per well in a 96 well multiwell plate.

Statistical analysis

For each exposure 3 cell counts were made and each exposure was undertaken in triplicate using different animals, therefore n=9. All data were expressed as the arithmetic mean +/- the standard error of the mean (SEM). Multiple comparison of treatment means with the control means was undertaken for each exposure time (24, 48 and 96 h) by the parametric multiple comparison test (Dunnett's test) using the DUNNETT computer programme, version 1.5 (US EPA, ToxTest computer programme) following Dunnett's procedure (Dunnett, 1955). This procedure indicates which toxicants means were statistically different from the control mean at the 5% level of significance by one way analysis of variance (ANOVA). If tested significant the p-value was determined using the student t-test ($p<0.05$) (SigmaPlot 7 computer program). Significant variation between exposure times (0, 24, 48 and 96 hr) for each dose was also calculated. LC_{50} data was calculated using the Trimmed Spearman-Karber (TSK) program version 1.5 (US EPA, ToxTest computer program) designed for the analysis of mortality data from acute and chronic toxicity tests. A toxicity threshold (TT) was calculated as $TT = (NOEC \times LOEC)^{1/2}$. Regression analysis was undertaken using the Pearson product moment

correlation coefficient, (r) a dimensionless index ranging from -1.0 to 1.0 inclusive and reflects the extent of a linear relationship between two data sets. Significance was set at p=0.05.

RESULTS

Tissue culture

An initial cell density of 3 million cells ml^{-1} for the 24 multiwell plate was found to be too dense with not enough room for cells to adhere to the culture surface. 2 million cells ml^{-1} was best. Bacterial and fungal contamination remained only a small problem. Of the 168 wells sown with cells used in the trypan blue exclusion assay, only a small proportion were infected (Table 5.3). The digestive gland cells were more commonly contaminated than the gill cells.

Table 5.3. Percentage of gill and digestive gland (DG) cells contaminated with bacteria and fungi during the trypan blue exclusion test (n=168).

	% Clear	% Bacteria	% Fungi	% Both
Gill	98.8	1.2	0	0
DG	89	6.1	2.5	2.4

Cytotoxicity

Trypan blue

Both the gill and digestive gland cell viability decreased with exposure to increasing concentrations of NP. Initial cell viability for both tissues was reduced from 70-80% down to 30-40% after NP exposure, with the longer exposure times further reducing cell viability (Figure 5.1 & 5.2). Statistically significant ($p<0.05$) differences in cell viability are shown in Table 5.4(a&b). Cell viability was also affected by the solvent used in the exposures with a significant decrease occurring in one or two instances. For this reason cell viability was tested for significance against both the control and solvent control exposed cells (Table 5.4(a&b)). For gill cells a significant reduction in cell viability was found after exposure to 5, 0.5 and 0.1/0.5 (when compared against the control and solvent control respectively) mg l^{-1} NP for 24, 48 and 96 hr respectively (Table 5.4(a)).

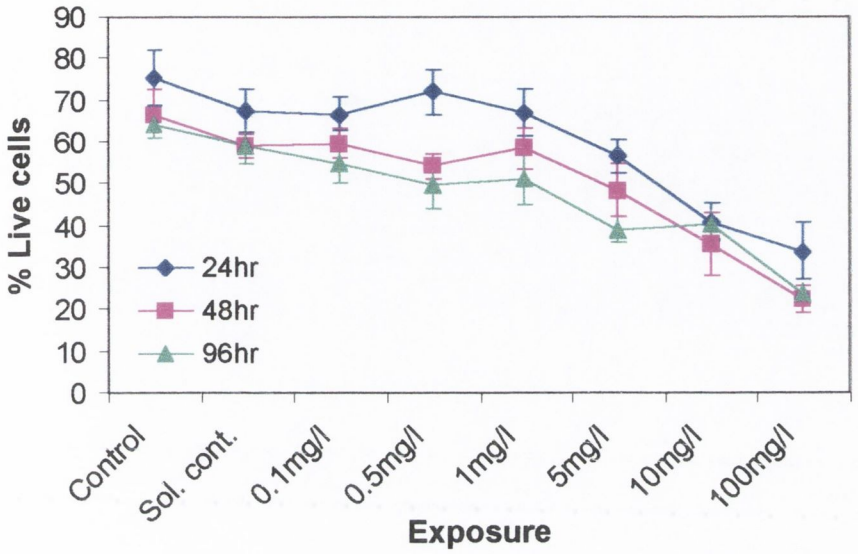


Figure 5.1. Percentage viability of gill cells from the zebra mussel in suspension exposed to various concentrations of NP for 24, 48 and 96 hr. Error bars represent the standard error of the mean (S.E.M.) for n=9 replicate cultures. Sol. cont. = solvent control.

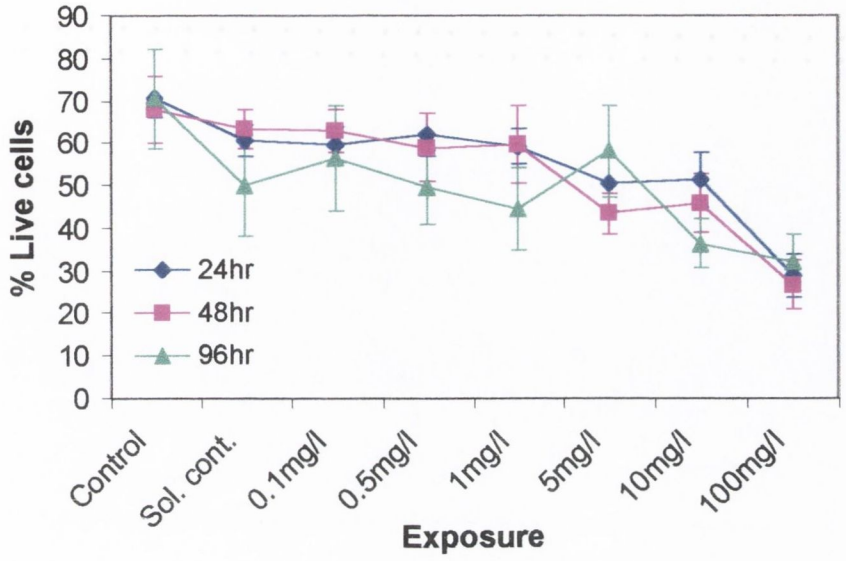


Figure 5.2. Percentage viability of digestive gland cells from the zebra mussel in suspension exposed to various concentrations of NP for 24, 48 and 96 hr. Error bars represent the standard error of the mean (S.E.M.) for n=9 replicate cultures. Sol. cont. = solvent control.

There was more discrepancy between the control and solvent control for the digestive gland cells with a significant reduction in viability occurring after 24 hr exposure (Table 5.4(b)). When compared to the control cells digestive gland cell viability was significantly reduced by 0.1, 5 and 0.5 mg l^{-1} NP exposure after 24, 48 and 96 hr respectively (Table 5.4(b)). However when compared against the solvent control these figures rose to 5, 5 and 10 mg l^{-1} NP for 24, 48 and 96 hr exposure.

Viability for both the gill and digestive gland cells was significantly reduced after the 3 exposure periods when compared with the initial 0 hour viability. Only the 24 hour control and 0.5 mg l^{-1} NP exposed gill cells, and all of the control, 96 hr solvent control and 0.1 mg l^{-1} NP exposed digestive gland cells did not show a significant reduction in viability (Table 5.5(a&b)). When compared with the 24 hr exposed cells, gill cells did show a significant reduction in viability for each exposure and controls at both 48 and 96 hr except for the 10 mg l^{-1} NP exposed cells and the 48 hr control, 1 and 5 mg l^{-1} exposed cells (Table 5.6(a)). In comparison the digestive gland cells only showed a significant reduction in viability after 96 hr exposure to 0.5, 1 and 10 mg l^{-1} NP when compared against the 24 hr cells (Table 5.6(b)).

Table 5.4(a). Comparison of zebra mussel gill cell viability using trypan blue after NP exposure with control and solvent control cells. Significance (p=0.05) tested using Dunnet's test (ANOVA). Actual p-values calculated using Student's t-test. # indicates not significant; * p=<0.05 significant; ** p=<0.01 highly significant; *** p=0.0001 very highly significant; #/* indicates result not significant using Dunnet's test but was significant using the Student t-test. SC. = solvent control.

	SC.	0.1mg l^{-1}	0.5mg l^{-1}	1mg l^{-1}	5mg l^{-1}	10mg l^{-1}	100mg l^{-1}
Control 24hr	#	#	#	#	***	***	***
SC. 24 hr		#	#	#	**	***	***
Control 48hr	# / *	#	**	#	***	***	***
SC. 48 hr		#	*	#	**	***	***
Control 96hr	#	**	***	**	***	***	***
SC. 96 hr		#	*	#	***	***	***

Table 5.4(b). Comparison of zebra mussel digestive gland cell viability using trypan blue after NP exposure with control and solvent control cells. Significance (p=0.05) tested using Dunnet's test (ANOVA). Actual p-values calculated using Student's t-test. # indicates not significant; * p=<0.05 significant; ** p=<0.01 highly significant; *** p=0.0001 very highly significant; #/* indicates result not significant using Dunnet's test but was significant using the Student t-test.

	SC.	0.1mg l^{-1}	0.5mg l^{-1}	1mg l^{-1}	5mg l^{-1}	10mg l^{-1}	100mg l^{-1}
Control 24hr	**	**	# / *	**	***	***	***
SC. 24 hr		#	#	#	**	*	***
Control 48hr	#	#	#	#	***	**	***
SC. 48 hr		#	#	#	***	**	***
Control 96hr	#	#	# / *	**	#	***	***
SC. 96 hr		#	#	#	#	*	*

Table 5.5(a). Gill cell intra-exposure differences in viability (trypan blue method) between initial seeding (0 hr) and after 24, 48 and 96 hr exposure for each NP concentration and controls. Significance (p=0.05) tested using Dunnet's test (ANOVA). Actual p-values calculated using Student's t-test. # indicates not significant; * p=<0.05 significant; ** p=<0.01 highly significant; *** p=0.0001 very highly significant. C = control; SC = solvent control.

	C. 0hr	SC. 0hr	0.1 mg l^{-1} 0hr	0.5 mg l^{-1} 0hr	1 mg l^{-1} 0hr	5 mg l^{-1} 0hr	10 mg l^{-1} 0hr	100 mg l^{-1} 0hr
C 24hr	#							
C 48hr	*							
C 96hr	***							
SC 24hr		*						
SC 48hr		***						
SC 96hr		***						
0.1 mg l^{-1} 24hr			**					
0.1 mg l^{-1} 48hr			**					
0.1 mg l^{-1} 96hr			***					
0.5 mg l^{-1} 24hr				#				
0.5 mg l^{-1} 48hr				***				
0.5 mg l^{-1} 96hr				***				
1 mg l^{-1} 24hr					*			
1 mg l^{-1} 48hr					***			
1 mg l^{-1}96hr					***			
5 mg l^{-1} 24hr						***		
5 mg l^{-1} 48hr						***		
5 mg l^{-1} 96hr						***		
10 mg l^{-1} 24hr							***	
10 mg l^{-1} 48hr							***	
10 mg l^{-1} 96hr							***	
100 mg l^{-1} 24hr								***
100 mg l^{-1} 48hr								***
100 mg l^{-1} 96hr								***

Table 5.5(b). Digestive gland cell intra-exposure differences in viability (trypan blue method) between initial seeding (0 hr) and after 24, 48 and 96 hr exposure for each NP concentration and controls. Significance (p=0.05) tested using Dunnet's test (ANOVA). Actual p-values calculated using Student's t-test. # indicates not significant; * p=<0.05 significant; ** p=<0.01 highly significant; *** p=0.0001 very highly significant. C = control; SC = solvent control

	C. 0hr	SC. 0hr	0.1 mg l^{-1} 0hr	0.5 mg l^{-1} 0hr	1 mg l^{-1} 0hr	5 mg l^{-1} 0hr	10 mg l^{-1} 0hr	100 mg l^{-1} 0hr
C 24hr	#							
C 48hr	#							
C 96hr	#							
SC 24hr		**						
SC 48hr		**						
SC 96hr		#						
0.1 mg l^{-1} 24hr			***					
0.1 mg l^{-1} 48hr			**					
0.1 mg l^{-1} 96hr			#					
0.5 mg l^{-1} 24hr				**				
0.5 mg l^{-1} 48hr				*				
0.5 mg l^{-1} 96hr				**				
1 mg l^{-1} 24hr					***			
1 mg l^{-1} 48hr					*			
1 mg l^{-1}96hr					**			
5 mg l^{-1} 24hr						***		
5 mg l^{-1} 48hr						***		
5 mg l^{-1} 96hr						*		
10 mg l^{-1} 24hr							***	
10 mg l^{-1} 48hr							***	
10 mg l^{-1} 96hr							***	
100 mg l^{-1} 24hr								***
100 mg l^{-1} 48hr								***
100 mg l^{-1} 96hr								***

Table 5.6(a). Gill cell intra-exposure differences in viability (trypan blue method) between 24 hr exposure and after 48 and 96 hr exposure for each NP concentration and controls. Significance (p=0.05) tested using Dunnet's test (ANOVA). Actual p-values calculated using Student's t-test. # indicates not significant; * p=<0.05 significant; ** p=<0.01 highly significant; *** p=0.0001 very highly significant. C = control; SC = solvent control.

	C. 24hr	SC. 24hr	0.1 mg l^{-1} 24hr	0.5 mg l^{-1} 24hr	1 mg l^{-1} 24hr	5 mg l^{-1} 24hr	10 mg l^{-1} 24hr	100 mg l^{-1} 24hr
C 48hr	#							
C 96hr	*							
SC 48hr		*						
SC 96hr		*						
0.1 mg l^{-1} 48hr			*					
0.1 mg l^{-1} 96hr			**					
0.5 mg l^{-1} 48hr				***				
0.5 mg l^{-1} 96hr				***				
1 mg l^{-1} 48hr					#			
1 mg l^{-1} 96hr					**			
5 mg l^{-1} 48hr						#		
5 mg l^{-1} 96hr						***		
10 mg l^{-1} 48hr							#	
10 mg l^{-1} 96hr							#	
100 mg l^{-1} 48hr								*
100 mg l^{-1} 96hr								*

Table 5.6(b). Digestive gland cell intra-exposure differences in viability (trypan blue method) between 24 hr exposure and after 48 and 96 hr exposure for each NP concentration and controls. Significance (p=0.05) tested using Dunnet's test (ANOVA). Actual p-values calculated using Student's t-test. # indicates not significant; * p=<0.05 significant; ** p=<0.01 highly significant; *** p=0.0001 very highly significant. C = control; SC = solvent control.

	C. 24hr	SC. 24hr	0.1 mg l^{-1} 24hr	0.5 mg l^{-1} 24hr	1 mg l^{-1} 24hr	5 mg l^{-1} 24hr	10 mg l^{-1} 24hr	100 mg l^{-1} 24hr
C 48hr	#							
C 96hr	#							
SC 48hr		#						
SC 96hr		#						
0.1 mg l^{-1} 48hr			#					
0.1 mg l^{-1} 96hr			#					
0.5 mg l^{-1} 48hr				#				
0.5 mg l^{-1} 96hr				*				
1 mg l^{-1} 48hr					#			
1 mg l^{-1} 96hr					*			
5 mg l^{-1} 48hr						#		
5 mg l^{-1} 96hr						#		
10 mg l^{-1} 48hr							#	
10 mg l^{-1} 96hr							**	
100 mg l^{-1} 48hr								#
100 mg l^{-1} 96hr								#

LC_{50}

In vitro LC_{50} values of 31.62, 21.45 and 21.19 mg l^{-1} were calculated for gill cells after 24, 48 and 96 hr (respectively) exposure to NP (Table 5.7). These values were considerably higher for the digestive gland with 50.65, 40.84 and 26 mg l^{-1} NP needed to kill 50% of the exposed cell population after 24, 48 and 96 hr respectively. The lowest observable effect concentration (LOEC) and no observable effect concentration (NOEC) were also considerably higher for the digestive gland than for the gill cells (Table 5.7). The Toxicity threshold (TT) was also estimated for these cells and was 2 and 3 orders of magnitude higher for the digestive gland cells exposed to 48 and 96 hr respectively.

Using the quadratic equation for the *in vivo* LC_{50} data from Chapter 2 the 24, 48 and 96 hr exposure *in vivo* LC_{50} concentrations can be estimated to help compare the *in vivo* and *in vitro* LC_{50} data. This data is shown in Figure 5.3. The *in vivo* LC_{50} results for 24, 48 and 96 hours exposure to NP are between 2 to 5 times lower than the equivalent *in vitro* LC_{50} concentrations for the gill and digestive gland cells respectively.

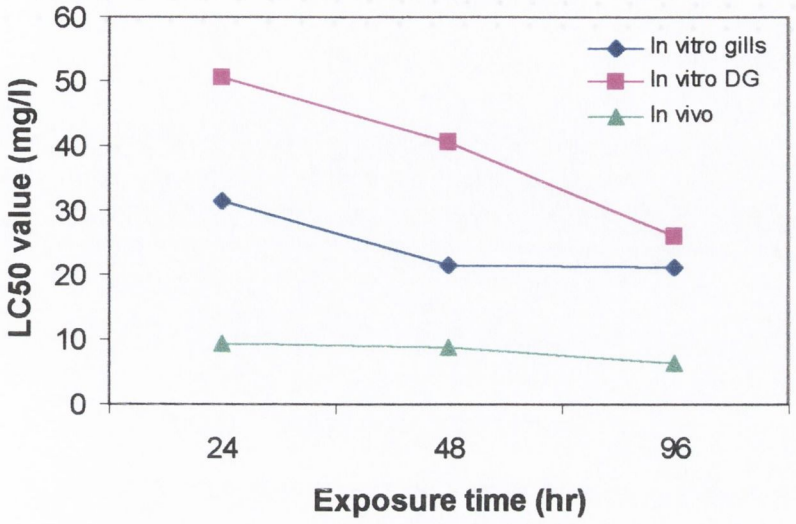


Figure 5.3. Graph showing the LC_{50} data obtained from the exposure of gill and digestive gland (DG) cells to NP *in vitro*. The *in vivo* data for 24, 48 and 96 hour exposure was calculated using the quadratic equation from the *in vivo* data presented in Chapter 2.

Table 5.7. LC_{50} values for gill and digestive gland (DG) cells for exposure to NP *in vitro* calculated using the Trimmed Spearman-Karber method with 95% confidence limits (CL). The lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC) were found from Tables 5.4(a&b) using the solvent control values. Toxicity threshold (TT) = (NOEC x LOEC)$^{1/2}$.

	LC_{50} mg l^{-1}	95% CL mg l^{-1}	LOEC mg l^{-1}	NOEC mg l^{-1}	TT mg l^{-1}
Gill cells					
24hr	31.62	5.6-179	5	1	2.2
48hr	21.45	11.7-39.2	0.5	0.1	0.22
96hr	21.19	9.8-45.6	0.5	0.1	0.22
DG cells					
24hr	50.65	29.2-87.9	5	1	2.2
48hr	40.84	22.2-75	5	1	2.2
96hr	26.00	2.2-312	10	5	7.1

MTT assay

For both the gill and digestive gland cells no decrease in absorbency was observed with increasing NP exposure using the MTT assay (Figure 5.4 & 5.5). These graphs show an increase in absorbency after exposure to higher NP concentrations (10 and 100 mg l^{-1}), indicating that the assay did not work. Very high p-values ($p=0.3 \geq 0.9$) were found for both gill and digestive gland cells at each NP concentration when compared with controls

after each exposure period indicating no reduction in cell viability. Therefore using the MTT assay no significant reduction in absorbency indicating cell death was found with increased exposure to NP.

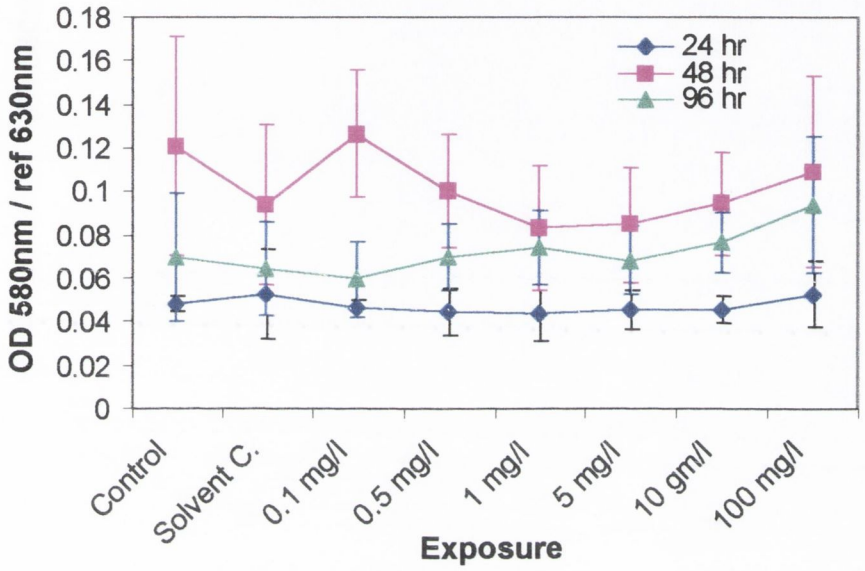


Figure 5.4. Percentage absorbency of zebra mussel gill cells in suspension exposed to various concentrations of NP for 24, 48 and 96 hr using the MTT assay. Error bars represent the standard error of the mean (SEM) for n=9 replicate cultures. Solvent C. = solvent control.

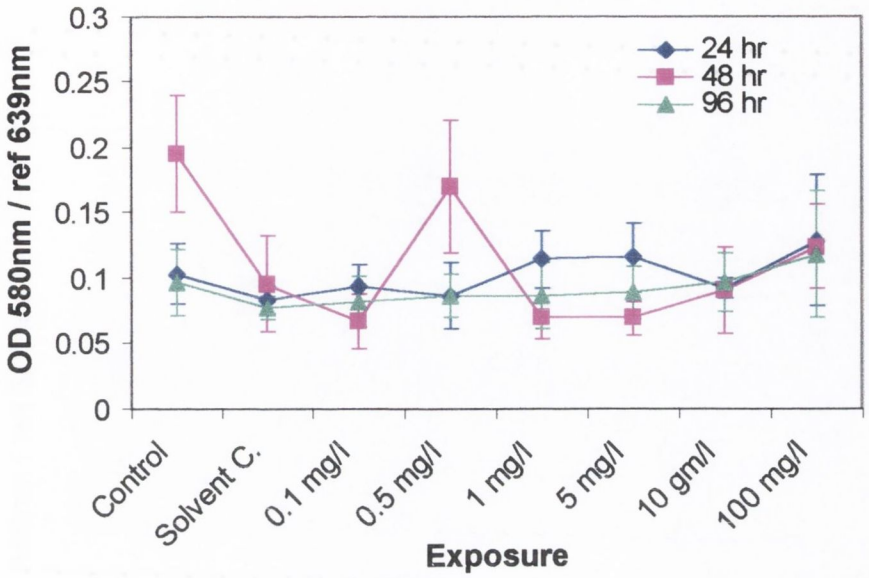


Figure 5.5. Percentage absorbency of zebra mussel digestive gland cells in suspension exposed to various concentrations of NP for 24, 48 and 96 hr using the MTT assay. Error bars represent the standard error of the mean (SEM) for n=9 replicate cultures. Solvent C. = solvent control.

An exposure of varying concentrations of gill cells to NP was undertaken (Figure 5.6). An increase in absorbency was found with higher cell concentrations, but as before no reduction in absorbency occurred with increased NP exposure.

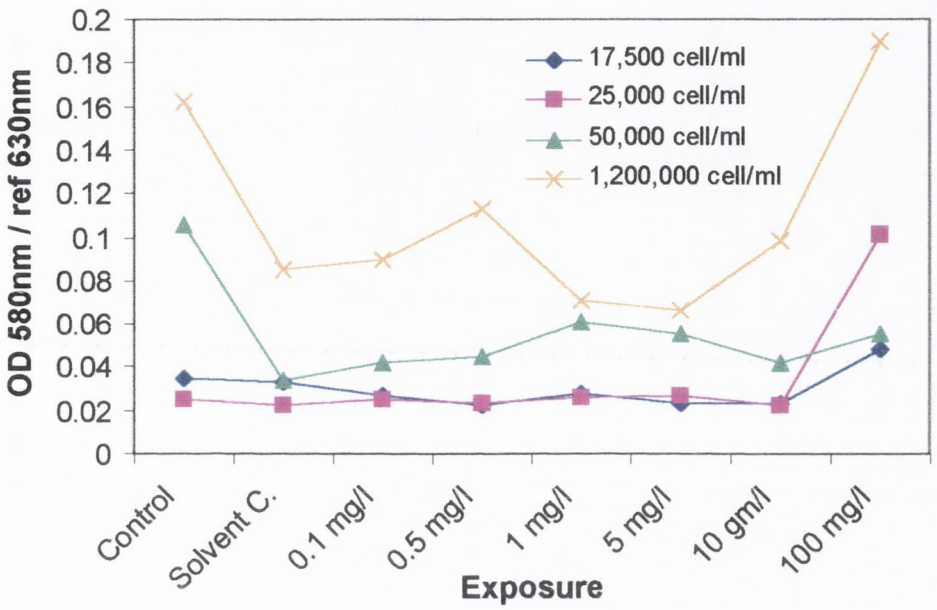


Figure 5.6. Effect of cell density on the viability of gill cells from the zebra mussel exposed to various concentrations of NP for 24 hr measured using the MTT assay. Solvent C. = solvent control.

The MTT reduction assay was also undertaken using the cell line CHSE cells. Absorption intensity at different cell densities is plotted in Figure 5.7. This graph shows a linear relationship (r= 0.975-0.937) with intensity increasing relative to cell density for the 3 time periods. Absorption also increases relative to culture time with the cells in culture for 96 hr producing a higher rate of absorption. After exposure to NP CHSE cells showed a decrease in intensity relative to the NP concentration (Figure 5.8). When compared to the

control cells, those exposed to each NP concentration for each exposure time (24, 48 and 96 hr) showed a highly significant ($p<0.005$) decrease in absorption (Table 5.8).

When compared to the solvent control only those cells exposed to 10 and 100 mg l^{-1} NP for 24 and 48 hr and 100 mg l^{-1} for 96 hr continued to show a significant ($p<0.05$) reduction in intensity. Exposure time seems to have little effect on cell viability as all 3 of the exposure times for each NP concentration have a similar intensity (Figure 5.8).

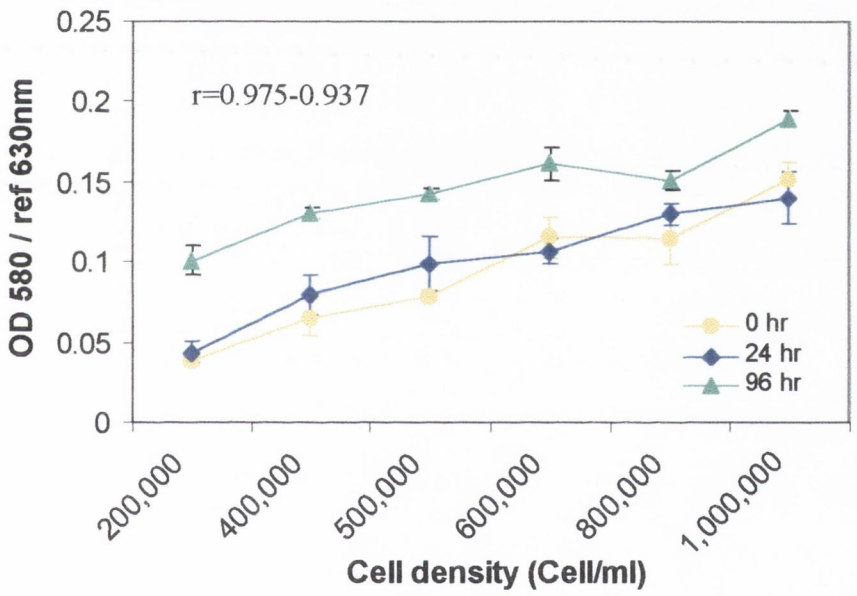


Figure 5.7. Absorption intensity for CHSE cells at different cell densities using the MTT assay after 0, 24 and 96 hr incubation. The r value represents the Pearson product moment correlation coefficient. Error bars represent the standard error of the mean (SEM) for n=9 replicate cultures. Solvent C. = solvent control.

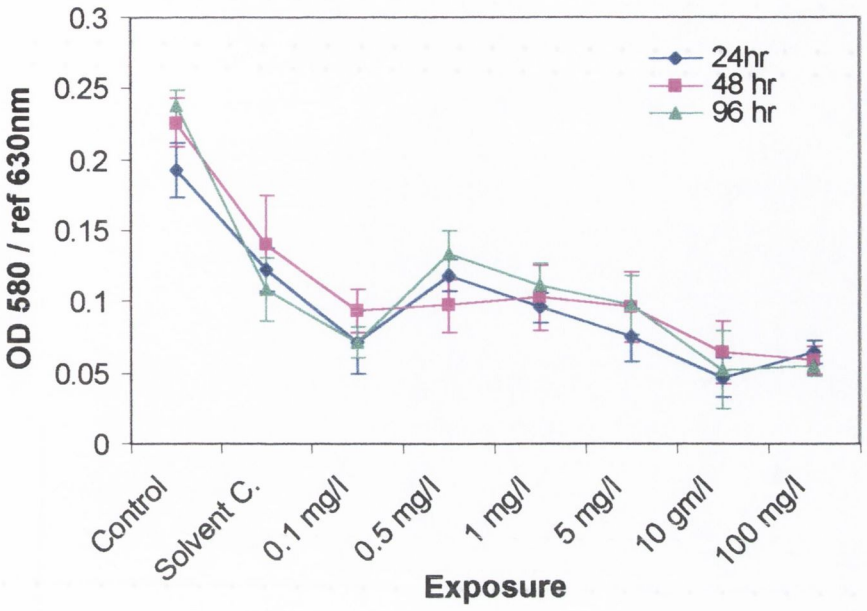


Figure 5.8. Percentage absorbency of CHSE cells in suspension exposed to various concentrations of NP for 24, 48 and 96 hr using the MTT assay. Error bars represent the standard error of the mean (SEM) for n=9 replicate cultures.

Table 5.8. Comparison of CHSE viability using the MTT assay after NP exposure with control and solvent control cells. Significance (p=0.05) tested using Dunnet's test (ANOVA). Actual p-values calculated using Student's t-test. # indicates not significant; * p=<0.05 significant; ** p=<0.01 highly significant; *** p=0.001 very highly significant. SC = solvent control.

	SC.	0.1 mg l^{-1}	0.5 mg l^{-1}	1 mg l^{-1}	5 mg l^{-1}	10 mg l^{-1}	100 mg l^{-1}
Control 24hr	*	***	**	***	*	***	***
SC. 24 hr		#	#	#	#	***	***
Control 48hr	#	***	***	***	***	***	***
SC. 48 hr		#	#	#	#	*	*
Control 96hr	***	***	***	***	***	***	***
SC. 96 hr		#	#	#	#	#	*

DISCUSSION

The *in vitro* method described in Chapter 4 for maintaining cells from the zebra mussel in culture proved suitable for use in a toxicity study of cells from the gill and digestive gland in suspension. Using this method viable cells were dissociated from the gill and digestive gland and maintained in suspension for use in obtaining the *in vitro* LC_{50} for exposure to NP. Bacterial contamination was maintained under control and was not a serious problem. DMSO was the solvent of choice as acetone may react with the plastic of the

culture vessel. Although suitable in other experiments (Kilemade & Mothersill, 2001) the concentration of solvent used (2.5%) was perhaps too high for cells from the zebra mussel as in one or two instances there was a significant reduction in the solvent control. Exposures were therefore compared with both the control and solvent control. Digestive gland cells seemed to be better maintained in culture with no significant decrease in cell viability occurring in the control cells between 0 hour before seeding and after 10 days in culture, corresponding to the 96 hr exposure control, whereas gill cell viability was significantly reduced after 8 days in culture (48 hr exposure control). Most samples from both the digestive gland and gill showed a significant reduction in cell viability in the 7 day period between day 0 and 24 hr exposure to NP. It therefore seems more realistic to compare the 48 and 96 hr exposed samples with the 24 hr samples for each NP concentration instead of 0 hr.

Digestive gland cells appeared more resistant to the toxic effect of NP than the gill cells. The toxicity threshold (TT) calculated was an order of magnitude higher for the digestive gland cells than for the gill cells after the 48 and 96 hour exposure respectively. Increased exposure time to NP reduced the TT value for gill cells indicating that longer-term exposures to smaller quantities of NP reduce gill cell viability. In contrast to this the TT for digestive gland cells increased with exposure time. This would indicate that once the initial cells have been killed off after 24 hr exposure to NP the cells that were left have increased resistance to the toxic effect of the chemical. This is consistent with other reports of reduced sensitivity to toxins of trout hepatocyte cells in culture when compared to gill epithelial cells (Lilius, 1997; Lilius et al., 1994). This is thought to be due to the

high bio transformation capacity of the hepatocytes making them more tolerant to chemicals than whole organisms and other cells. Rainbow trout hepatocyte primary cultures retain their central metabolic pathways and maintain stable levels of several enzymes important in xenobiotic metabolism such as the cytochrome P450 system for up to 2 weeks (Rabergh et al., 1997). Isolated digestive gland cells from *Mytilus edulis* showed similar specific activities of superoxide dismutase (SOD) and catalase as the whole tissue, indicating that the enzymes are stable in culture and have a wide distribution in different cell types in culture (Bermelin et al., 1998). These cells were also indicated to have a capacity for cytochrome P450-catalysed xenobiotic metabolism in culture (Michelmore et al., 1997). The digestive gland is the major site of detoxification in the zebra mussel and has been proven to be highly effective in reducing the toxicity of numerous heavy metals and pesticides (Busch & Schuchardt, 1991; Camusso et al., 1994; Comba et al., 1996; Dauberschmidt et al., 1997). This could help to explain the high LOEC value found for the digestive gland cells of the zebra mussel. It is possible that the live cells remaining in culture may be metabolising the toxin as they do *in vivo* reducing its toxicity and increasing resistance. However as it was not proven that these cells were metabolising *in vitro* this theory is just speculation.

The LC_{50} calculated for the digestive gland cells was also considerably higher than that for the gill cells. The 24 hr LC_{50} value of 31.62 mg l^{-1} NP for the gill cells reduced to around 21 mg l^{-1} after 48 and 96 hr exposure. There was a larger drop from 50.65 to 26 mg l^{-1} for the digestive gland cells after 24 and 96 hr exposure. A similar trend was seen in fish where the LC_{50} data for gill epithelial cells from rainbow trout showed better concordance

concordance with the literature data for fish LC_{50} values than for hepatocyte EC_{50} values (Lilius et al., 1994). Lilius (1997) concluded that of the two cell types the gill epithelial cells were superior for the detection of general (basal) toxicity using trout, possibly for the reasons explained above.

These *in vitro* LC_{50} levels are considerably higher than the *in vivo* values previously calculated for zebra mussels exposed to NP and reported in Chapter 2. Direct comparison of the LC_{50} values calculated using the Trimmed Spearman-Karber method is difficult resulting from the inability to calculate the *in vivo* LC_{50} for below 8 days due to the necessary trim being too high for the calculation. The *in vivo* LC_{50} for 8 days exposure of zebra mussels was 7.67 mg l^{-1}, reduced down to 3.71 mg l^{-1} after 15 days and 1.76 mg l^{-1} after 50 days exposure. However using the quadratic equation for the *in vivo* data the *in vivo* 24, 48 and 96 hour LC_{50} data can be estimated. From this data *in vivo* exposures are 2 to 5 times more sensitive than gill and digestive gland cells (respectively) in culture. It has been previously observed that *in vivo* exposures were more sensitive, in some cases 2 orders of magnitude more sensitive than *in vitro* exposures (Kilemade & Mothersill, 2001) as appears to be the case here. Another *in vitro* toxicity study using nonoxynol (α-(4-nonylphenol)-ω-hydroxypoly-(oxy-1-ethanediyl) formed by reacting nonylphenol with ethylene oxide on rainbow trout primary epidermal cell cultures found an LC_{50} value of 10.24 and 7.96 mg l^{-1} for 24 and 48 hours respectively (Dowling & Mothersill, 1999). This is also considerably higher than the *in vivo* 96 hr LC_{50} values of 0.9 and 3 mg l^{-1} for juvenile salmon (*Salmo salar*) and cod (*Gadus morrhua*) exposed to NP and nonylphenol ethoxylate (NPE) respectively (Swedmark et al., 1971; McLeese et al., 1980).

In another study the EC_{50} values for fish gill and hepatocytes *in vitro* were 1 or 2 orders of magnitude higher than for the whole fish *in vivo*, indicating that cellular tests with the endpoints used are less sensitive than whole organisms (Lilius et al., 1995). Most cytotoxicity tests only measure one endpoint, usually reflecting cell viability and do not provide any information about the possible toxic mechanisms involved. As a cell may be severely disturbed at a concentration far below that resulting in cell death, the viability endpoint may well underestimate the toxicity of a compound (Lilius, 1997) resulting in higher than expected *in vitro* LC_{50} values.

The results obtained from the MTT reduction assay indicate that this test is not suitable for use with the cell suspension method developed for the zebra mussel. No trend of decreased MTT activity was found with exposure to increased concentrations of NP for 24, 48 and 96 hours in either the gill or digestive gland cells. This would suggest that NP is not toxic to the cells at the concentrations tested. However as the trypan blue results indicate this is not the case. The assay was tested using the fish cell line CHSE cells exposed to the same NP concentrations, with a reduction in MTT activity correlated with increased NP exposure, proving that the assay worked as expected. The most likely reason why the assay didn't work with the zebra mussel cells in suspension is due to a difference in cell density pippetted into each vial. This could be due to the tissues not being fully dissociated with clumps of tissue concentration in some wells, resulting in an uneven cell distribution. There is also a possibility that due to the cells not adhering to the culture vessel properly uneven numbers of cells were removed from the wells during the media change, resulting in an uneven cell distribution. This could explain the results as a

direct correlation was observed between CHSE cell density and the absorbency measured. This direct correlation has also be reported in other studies (Mosmann, 1983; Domart-Coulon et al., 1994). This problem of uneven cell distribution was encountered by other authors who noticed high levels of variability between assays using the MTT assay on bivalve primary cell cultures (Domart-Coulon et al., 2000). Increased MTT activity was correlated with an increase in gill cell densities indicating that this assay could indeed be used to measure zebra mussel cell viability *in vitro*. However care should be taken to ensure an even cell distribution and the media should not be changed when using the MTT assay.

The initial aim of this work was to establish a technique of investigating methods of ED in the zebra mussel at a cellular level *in vitro*. Several techniques have been developed to allow the study of EDC *in vitro*. Many of these rely on vertebrate cells particularly fish cell culture, have not yet been standardised and are only in the testing stage. Vitellogenin synthesis has been stimulated in cultured hapatocytes of various fish species exposed to 17β-estradiol (Maitre et al., 1985; Flouriot et al., 1993; Smeets et al., 1999). Work has also been carried out to study *in vitro* the effect of endocrine disrupting chemicals (EDCs) on invertebrates. Some *in vitro* methods established for invertebrates have the potential to be used to investigate ED. These include the study of vitellin synthesis in ovaries of the shrimp *Penaeus semisulcatus* incubated *in vitro* (Browdy et al., 1990), vitellogenisis in the isolated hepatopancreas and ovary of the shrimp *Penaeus vannamei* (Quackenbush & Keeley, 1986), and the biosynthesis of steroids in the gonad of the mussel (*Mytilus edulis*) (De Longcamp et al., 1974).

Primary cultures that have hormonal responses would be the most useful for detecting EDCs and for studying their mechanisms of action (eg. Hepatopancreas and ovarian cultures). Primary cultures of the digestive gland have been highlighted as particularly useful to study the effects of xenobiotics on the production and distribution of lipovitellin. Ideally tissue and cell cultures of the zebra mussel would be established and used to measure the effect of endocrine disrupting chemicals on steroids or vitellin as described in Chapter 3. Antibodies developed against lipovitellin of several species of insects, crabs, shrimp and crayfish (Hasegawa et al., 1993; Lee & Watson, 1994; Oberdorster et al., 2000) could also be tested for cross reactivity in cultured tissues of the zebra mussel.

The need for the development of *in vitro* assays such as receptor-binding assays that are applicable to invertebrate endocrine process has been highlighted (De Fur et al., 1999). These assays are needed to better understand invertebrate endocrine processes and to understand the mechanism of action of EDCs. By mixing the *in vitro* tissue culture method described here with the gill estrogen receptor (ER) assay described in Chapter 3 it may be possible to develop a new EDC screening test capable of screening large quantities of xenoestrogenic compounds in a relatively short period. This would be able to quantify the ER binding of a chemical by the gill cells after *in vitro* exposure to various compounds, eliminating the need for time consuming and costly *in vivo* exposures and could provide important information on the mechanism of action.

CONCLUSION

The *in vitro* LC_{50} measured for the digestive gland and gill of the zebra mussel exposed to NP was considerably higher than the previously measured *in vivo* values. This is in keeping with the results from other studies indicating that cells *in vitro* are less sensitive to the toxic effects of chemicals than whole animals *in vivo*. The very high value obtained for digestive gland cells may indicate that the cells are capable of metabolising the toxin and reducing its toxicity *in vitro*. Although the ED effect of the chemical could not be measured *in vitro*, there is the potential of developing an exciting new *in vitro* EDC screening assay using the ER assay described in Chapter 3.

Chapter 6.

GENERAL CONCLUSIONS

- Endocrine distruption (ED) was observed in the zebra mussel *Dreissena polymorpha* after exposure to tertiary treated effluent *in situ* and nonylphenol (NP) *in vivo*. The effects of ED were measured in both male and female mussels by a significant increase in expression of vitellin-like proteins measured using both the alkali labile phosphate (ALP) assay and gel electrophoresis (GE).

- Elevated levels of the sterol cholesterol, the precursor for sexual steroids, were measured in mussels exposed to tertiary treated effluent and NP using HPTLC. The measurement of cholesterol levels has the potential to become a very useful biomarker of ED in other aquatic organisms.

- Effects of ED were also seen at a histological level, with an increase in the interstitial tissue in the gonads of male mussels exposed to tertiary treated effluent, reducing the area of the sperm-producing seminiferous tubules. Electron microscopy (EM) also revealed possible effects on the sperm producing ability of the male gonad. These effects could have a negative effect at the population level.

- The LC_{50} value of NP on the zebra mussel was higher than for other bivalves with values of 7.67, 2.13 and 1.76 mg l^{-1} calculated for 8, 35 and 50 days respectively. Effects on sub-lethal parameters were observed at concentrations as low as 0.1 mg l^{-1}.

- Tissues from the zebra mussel were maintained *in vitro* as both viable cell suspensions and explants for up to two weeks using the tissue culture techniques developed. This technique is suitable for use in *in vitro* cytotoxicity tests.

- The *in vitro* LC_{50} of NP calculated for cell suspensions from both the gill and digestive gland was 2 to 5 times higher than the *in vivo* LC_{50}, indicating that cells maintained *in vitro* are less sensitive to the chemicals toxic effects than they are *in vivo*. Proper understanding of this *in vitro* / *in vivo* relationship could lead to the replacement of costly *in vivo* toxicity exposures.

- Using the estrogen receptor assay, gill cell suspensions from the zebra mussel exposed to xenoestrogens *in vitro* have the potential for development into a novel method for screening endocrine disrupting chemicals. This has the advantage of replacing costly *in vivo* exposures and is specific to xenoestrogens.

- Immunohistochemistry using techniques and antibodies developed for mammalian species was not suitable for zebra mussel samples preserved in paraffin wax. Although immunoreactivity was achieved, results were not reliable with quantification proving very difficult.

- Zebra mussels are suitable animals for use in *in situ* exposures as they can survive very harsh environments. However they are not well suited for long-term *in vivo* laboratory exposures when studying gonad development.

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