Assessment and Utilisation of Zequanox® for Zebra Mussel (*Dreissena polymorpha)* Control in Irish Waters

by

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Declaration

I declare that this thesis is my own work, and that it has not been previously submitted to any other institute or university.

I give permission to the Yeats library, Institute of Technology, Sligo to lend this thesis on request.

Sara Meehan

October, 2014

For Daniel

“And seek not the depths of your knowledge with staff or sounding line. For self is a sea boundless and measureless”

-Kahlil Gibran

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Abstract

Since the first arrival of the zebra mussel (*Dreissena polymorpha*) to Ireland in the early 1990's, there has been a rapid secondary spread of these highly invasive mussels within many Irish freshwater systems suitable for their establishment. This has resulted in direct invasive impacts to infested lakes, rivers and canals, both to the freshwater biota and to man-made infrastructure. The availability of an effective, yet environmentally benign, mussel control product to replace those currently used, such as chlorine and other biocides is particularly needed to mitigate mussel fouling impacts in drinking water plants and other infested facilities.

Zequanox® is a selective control product for dreissenids, i.e. zebra and quagga (*D.rostriformis bugensis*) mussels. It is a natural biocide, with the active ingredient being dead *Pseudomonas fluorescens* CL145A cells; a patented strain of a naturally occurring soil bacterium, which works by disrupting the mussels' digestive system. This product was developed in the USA, and has been tested and used in a number of North American trials, with the aim of commercialising a cross-continental, effective and regulatory compliant control product.

Research outputs from this thesis were utilised in the Zequanox regulatory application for commercial use within Europe. Ecotoxicology trials were carried out on nine species found commonly in Irish ecosystems. Results indicated that Zequanox does not negatively affect eight of these organisms at concentrations and treatment lengths required to get a >80% zebra mussel kill.

Field trials were carried out to demonstrate the effectiveness of Zequanox in industry and open water and to monitor water quality during and after treatment. These included a biobox trial at a drinking water treatment plant and an open water trial in a canal. Both trials achieved high levels of zebra mussel mortality (up to 81%) and provided insights into practical application techniques.

Additional laboratory assays were undertaken to determine the exact response relationship of juvenile zebra mussels to Zequanox. The results showed that juvenile mortality, on both experimental and control plates, are decreased with reduced handling,

with subsequent recommendations given for future juvenile treatment and counting procedures in the field.

After the two field trials (drinking water treatment plant and canal) were undertaken, it was apparent that mortality rates after treatment were lower in Ireland and also took longer to occur than similar trials carried out in North America. It was suspected that the higher water temperatures during North American trials (>25°C) meant that the zebra mussels were more metabolically active and therefore ingested more product. A number of comparative studies commenced to evaluate the effect of Zequanox on North American zebra mussels versus European zebra mussels. The results of this study showed that under the same temperature regimes mortality is similar. North American mussels were found to ingest more product in the initial eight hours, however by 24 hours, product concentration was similarly low for both mussel groups. The results of this trial allowed for industry recommendations to be made regarding the timing of treatments in Europe.

In conclusion this research has bridged the gap between the use of Zequanox in Europe and North America, showing there is potential for Zequanox to control zebra mussels in Europe not only in industry but also in open water. This study has also demonstrated Zequanox's potential to replace chlorine as the traditionally used control method, thereby reducing the environmental impact of mussel control on freshwater ecosystems.

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Chapter 1

Introduction

1.1 Overview of Chapter

This introduction provides background information to this thesis, by discussing invasive species and relevant legislation before focusing on the zebra mussel (*Dreissena polymorpha*), by outlining their life cycle, feeding habits and environmental requirements. The impact they have on their surrounding environment and industry will be reviewed including control needs. This will be followed by a review of control methods that currently exist including Zequanox. Finally I will present the aims and objectives of my research.

1.2 Introduction to Invasive Species

Invasive alien species can be defined as non-native species that are deliberately or unintentionally introduced by human action outside their natural habitats where they establish, proliferate and spread in ways that cause damage to biological diversity (European Commission, 2013). Invasive species have invaded Europe successfully due to its position as a centre for international trade, leading to a large and diverse population of these species, with the potential to reduce biodiversity (European Commission, 2013). In Europe it is estimated that there are 33 non-native established mammal species and 77 bird species; 5,789 alien plant species have been recorded in the wild, as well as 737 non-native multi-cellular marine species and 262 freshwater species (Keller et al., 2011). According to the European Commission (2013) there are over 12,000 alien species in the EU out of which 10 to 15% are causing damage. Keller et al. (2011) states that there has been recent growth in the field of invasion biology where scientists are now testing theories on how species enter and leave communities. Hulme et al. (2008) and Minchin and Gollasch (2002) provide theories on how invasive species enter Europe via a number of pathways: 1) via intentional release where species are released to improve the natural fauna or as game animals; 2) the escape of alien species from a managed environment such as a fish farm or unwanted pets released into the wild; 3) invasive species are often introduced attached to a host commodity; and 4) invasives may be transported out of their native range within the interior and exterior of

commercial ships, aircrafts, trains, canals, and other forms of transportation as well as via the pet trade (Minchin and Gollasch, 2002.; Minchin et al., 2002b; Minchin et al., 2003; Padilla and Williams, 2004; Minchin et al., 2005). According to the European Commission (2013) some species can also travel through transport infrastructure (for example the Danube-Main canal) (Commission of Europe, 2013).

Climate change, increasing vectors and pathways, changes in land use, and the absence of pathogens and predators have allowed for non-native species to invade new territories (Mooney and Cleland, 2001; Minchin and Gollasch, 2002; Broennimann et al., 2007). Invasive species possess a number of traits that make them so successful; firstly this includes feeding behaviours that allow them to exploit unused food resources, meaning they can inhabit new areas through both unused and vacant niches, niche displacement or from a lack of serious competition (Mooney and Cleland 2001; Edelist et al., 2012). The rapid reproduction abilities of invasive species and a short juvenile period also make for successful invaders (Rejmanek and Richardson, 1996). The continuous reproduction of successful invasive species during their expansion phase leads to unregulated exponential growth (Arim et al., 2006). In terms of aquatic invasive species this includes *Corbicula fluminalis* (Müller, 1774) the Asian clam, which can reproduce twice a year and *Hemimysis anomala* (Sars, 1907; Morton, 1980) the Ponto-Caspian mysid shrimp, which has been reported to have up to four broods a year (reviewed in Sousa et al., 2008, Minchin and Boelens, 2010, Rajagopal et al., 2000); prolonged reproduction also increases the success of becoming an established species, for example summer seasonal sequential spawning in the zebra mussel (Borcherding, 1991; Lucy, 2006).

1.2.1 European and Irish Legislation Relevant to Invasive Species

Invasive alien species are part of the assessment to determine ecological status in European countries, including Ireland as part of the Water Framework Directive (WFD) and are noted as a biological pressure (Water Framework Directive, 2005). Eight aquatic invasive species, including the zebra mussel, were listed in the Water Framework Directive (2005) as those posing the highest threat to a water body. Also included is an appendix of 13 other high impact species, 25 low impact species and 58 with the effect not fully known; all posing a threat to the achievement of good ecological status. In Ireland, EU legislation has helped to preserve biodiversity affected

by the introduction of invasive species. The European Communities (Birds and Natural Habitats) Regulations 2011 contains two sections (49 and 50) outlining the prohibition of the dealing and keeping of certain invasive species as well as prohibiting the introduction and dispersal of certain species. The European commission have now published a proposal for 'Regulation of the European parliament and of the council on the prevention and management of the introduction and spread of invasive alien species' (2013). This proposal aims to develop regulations to minimise the effect of invasive species on biodiversity, ecosystem services and the economy, through measures ensuring co-ordinate action and preventative action (European Commission, 2013).

1.3 The Zebra Mussel

1.3.1 Introduction to the Zebra Mussel

The zebra mussel *Dreissena polymorpha* (Pallas, 1771), is an invasive, exotic bivalve that has greatly impacted freshwater aquatic ecosystems in Europe including Ireland and many North American water bodies (Griffiths, 1993, Leach, 1993; Karatayev et al., 1997; McCarthy et al., 1997; McCarthy and Fitzgerald, 1997; Minchin and Moriarty, 1998; Karatayev et al., 2002; Minchin et al., 2003; Lucy et al., 2014). It has become a very successful invasive species due to its biological and ecological characteristics. The main known reasons for the rapid spread and colonisation of the zebra mussel are due to its high reproduction potential, its ability to attach to almost any hard surface and its high filter feeding capabilities. These aspects will be reviewed in following sections of this review.

The zebra mussel is native to the brackish and freshwaters of the Ponto Caspian Sea drainages (Black, Caspian, and Azov) (Karatayev et al., 1997). The construction of canals between the Eurasian river basins in the 18th century aided its distribution west to the North Sea and Baltic Sea, with shipping traffic further aiding in its distribution (as reviewed in May et al., 2006, Bij de Vaate et al., 2002). The zebra mussel was first recorded in North America in 1988 by Hebert et al. (1989), with the first individual collected in North America in Lake St. Clair (Hebert et al., 1989). Large populations were subsequently found in Lake St. Clair and Lake Erie (Mackie et al., 1989). By September 1989 the impacts of the zebra mussel came to the forefront. A water treatment plant in Monroe, Michigan (LePage, 1993) experienced a pumping outage.

Thereafter zebra mussels continued their spread around North East America eventually making their way west to Lake Mead in Nevada (Benson, 2013)

The zebra mussel first invaded Ireland in 1993 or 1994 (Minchin and Moriarty, 1998) in the River Shannon; it is most likely that they were attached to the hulls of boats or used leisure crafts imported from Britain and were introduced via this pathway (Minchin et al., 2005). *Dreissena* established British populations in the 19th century (reviewed in Karatayev et al., 1997). This late twentieth century colonisation was part of a second wave of zebra mussel invasions in Europe, which included Italy and Spain (Araujo et al., 2010). The zebra mussel spread rapidly using the Shannon-Erne waterway as a pathway for its movement (McCarthy et al., 1997; Minchin and Moriarty, 1998; Rosell et al., 1999; Minchin et al., 2002a; Minchin et al., 2003; Minchin et al., 2005). Inland waterways have allowed for movement of the zebra mussel in Ireland, both of its own accord and by boater movement (Minchin et al., 2003).

1.3.2 Zebra Mussel Reproduction

The zebra mussel life cycle consists of a sessile adult phase and a planktonic free swimming larval phase (Nichols, 1996) (Figure 1.1). Zebra mussels generally become sexually mature in their first year of life in North America and in the first or second year in Europe (Vailati et al., 2001; Lucy, 2006; Karatayev et al., 2007). The life cycle begins with external fertilisation of the gametes (Ackerman et al., 1994), after which there are three life cycle stages (Claudi and Mackie 1994); the veliger, post veliger and settling stage. Neumann et al. (1993) reviewing the data, concluded a large range in the duration of the larval stage from eight days to five weeks. After fertilisation the embryo develops into a free swimming trochophore (Claudi and Mackie, 1994), the veliger is transported by water currents where further developmental changes occur; including the secretion of a second shell, the development of a velum (organ for feeding and movement) and a foot (Akerman et al., 1994; Ludyanskiy et al., 1993). Here the inhalant and exhalent siphons develop and the mussel secretes byssal threads to firmly attach itself to its chosen substrate (Ludyanskiy et al., 1993). The final metamorphosis occurs after attachment whereby the mussel becomes a settled juvenile after the development of gills, the secretion of an adult shell and the loss of the velum. (Claudi and Mackie, 1994). The most commonly selected substrates by the juveniles are natural substrates such as rock, stone, wood and other plants as well as manmade substrates

such as concrete, plastic, vinyl and glass (Lucy et al., 2005). The amount of time it takes for a fertilised egg to reach a settled juvenile depends on the water temperature, with increased temperature promoting better growth and development (Nichols, 1996; Neuman et al., 1993). Marsden (1992) reports that it typically takes between eight and fifteen days to occur in American waters while Lucy and Sullivan (2001) report that in Irish waters it takes between two and three weeks in the July/August period. Settling stages have the highest mortality 20-100% according to Nichols (1996) and 90-99% mortality according to Mackie and Schloesser (1996).

The zebra mussel is rapidly able to colonise new areas, due to its high fecundity and the fact that it is a broadcast spawner, releasing up to one million eggs per season (Borcherding, 1991). The temperature of the water directly affects spawning: according to Borcherding (1991) waters must rise above the threshold temperature of 12°C for spawning to occur, although temperatures of 15°C have been widely cited (Karatayev et al., 1998). A relationship was found between temperature and peak spawning times, with the highest densities occurring during the highest temperatures (Garton and Haag, 1993; Borcherding, 1991; Karatayev et al., 2006). According to Lucy (2006), over a six year period in Lough Key, Ireland (1998-2003), spawning generally started in early July with peak spawning from the last week in July until the last week in August with larvae present in samples until mid-October. In most cases in Lough Key, temperatures were generally greater than 15°C during reproduction.

Growth experiments are often carried out on zebra mussels to determine age as there is a lack of distinguishing features to determine cohorts. Dorgelo (1993) and Neumann et al. (1993) carried out growth experiments and found that growth rates are affected by a number of external factors such as the eutrophic status of a lake, the flow and the algal species present. Dorgelo (1993) found that in eutrophic conditions shell lengths of the mussels increases between 0.54-0.59 mm a week under eutrophic conditions and 0.35mm a week under meso-oligotrophic conditions. Lucy et al. (2005) in Lough Key, Ireland found the zebra mussel population ranging in size from 1-34mm reflecting the presence of three year classes.

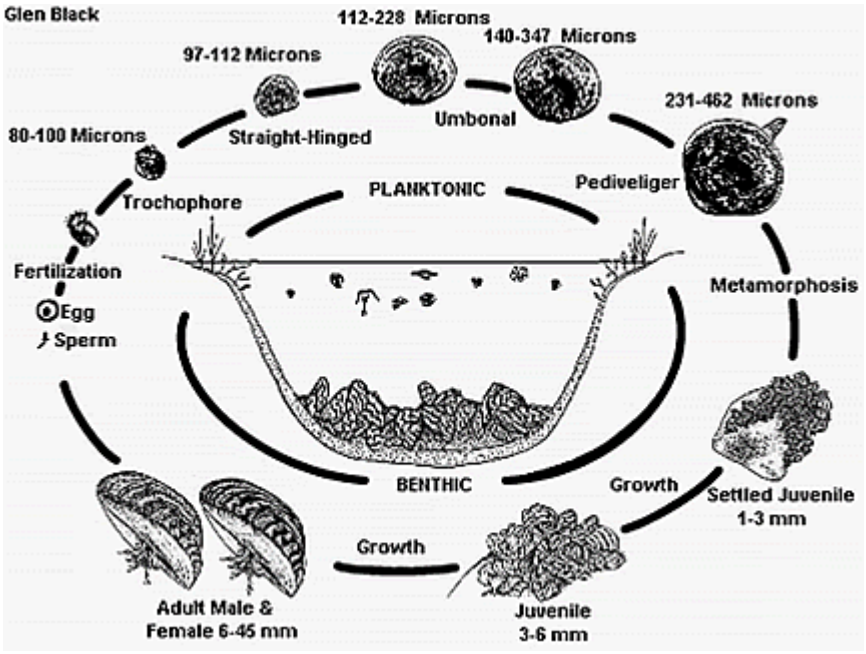

Figure 1.1 Zebra mussel life cycle. http://www.mass.gov/eea/agencies/dfg/fba/zebra-mussels.html [last accessed 4th November 2013]

1.3.3 Feeding in Zebra Mussels

The filter feeding process and ability to constantly filter when food is available are other attributes that make the zebra mussel such a successful invasive species. Kryger and Riisgård (1988) in lab trials found that zebra mussels can filter 5.0-7.0 millilitres (ml) of water an hour. Fanslow et al. (1995) found that in Saginaw Bay, Lake Huron, the average rate of mussel filtration was 16.2 millilitres/milligrams/hr (ml/mg/h) (ranging from 4.0 to 40.7 ml/mg/h) over the entire two year observation period and Yu and Culver (1999) found in Hargus Lake, Ohio, U.S.A that clearance rates of the zebra mussel ranged from 15.3 to 68.6 ml/hr.

Zebra mussels take in food particles via their inhalant siphon where food particles are sorted on the labial palps, unwanted particles are engulfed in mucus and exit through their exhalant siphon in the form of pseudofaeces (Stanczykowska and Planter, 1985; Horgan and Mills, 1997) (Figure 1.2). According to Ten Winkel and Davids (1982), zebra mussels can filter a broad range of particle sizes and also exhibit size selection having the ability to ingest very fine particles. As zebra mussels are selective feeders feeding on particles ranging in size from 15-50μm (Ten Winkel and Davids, 1982). MacIssac et al. (1995) found that clearance rates of zooplankton by the zebra mussels are relative to mussel size. The greater the mussel size the greater their capability to

suppress zooplankton in Lake St Clair. According to Horgan and Mills (1997), small zebra mussels (9-11mm shell length) readily ingest particles from 10µm - 150µm, whereas mussels (≤20.6 mm shell length) have been found to ingest particles up to 1.2mm; filtration rates did not differ among phytoplankton of different shapes, mussels were able to ingest uni-cells, filament and globular colonies. Figure 1.3 displays different filtration rates of the zebra mussel relative to temperature. Clearance rates do vary depending on size class of the mussel and according to Horgan and Mills (1997) do not vary between day and night. Due to their high filtration rates the zebra mussels have the potential to significantly reduce the concentrations of suspended solids, chlorophyll-*a* and phytoplankton bio volume (reviewed in Lucy et al., 2005, Higgins et al., 2008). Filtering can also cause the bioaccumulation of human waterborne pathogens, e.g. *Cryptosporidium*, *Giardia* and microsporidia, making them effective sentinels of water quality (Graczyk et al., 2004; Lucy et al., 2008). Moreover, filtration is particularly relevant to this research project as the control product Zequanox® depends on this process for success, as upon ingestion it destroys the mussel's digestive system (Molloy et al., 2013a, Molloy et al., 2013b). The active ingredient in Zequanox is *Pseudomonas fluorescens* and targets the zebra mussel only causing mortality through the disruption of the digestive system after ingestion (Molloy et al., 2013b).

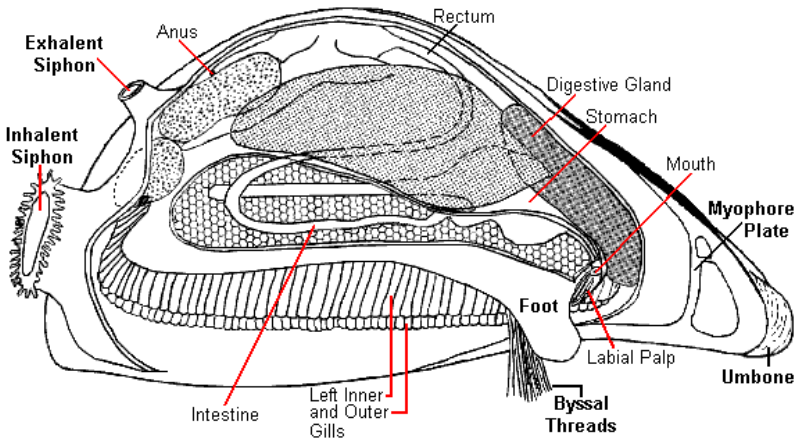

Figure 1.2 Digestive and respiratory structure of the zebra mussel. The zebra mussel resource http://zebramusselresource.weebly.com/physiology.html [last accessed 24th October 2013]

TABLE 1. A Review of Filtration Rates of *Dreissena polymorpha* (Shell Length 22 mm) from the Literature

Author	Food	Temperature (°C)	Filtration Rate (mL/mussel/hr)[a]
Kondratev (1963)	Clay suspension	16–17	(133)
Mikheev (1966)	*In situ*	20–22	(52)
Mikheev (1967)	*In situ*	—	2–50
Mikheev and Sorokin (1966)	C^{14}	—	45.5
Stanczykowska (1968)	*In situ*	—	35
Stanczykowska et al. (1975)	*In situ*	22–25	8–44
Alimov (1969)	Clay suspension	18–20	(159)
L'vova-Katchanova (1971)	—	—	43–56.3
Morton (1971)	Graphite	5–30	6.9
Hinz and Scheil (1972)	Suspension	5	13
Walz (1978)	*Nitzschia*	15	(63)
Benedens and Hinz (1980)	Graphite	17.7	4
Kryger and Riisgård (1988)	Suspension	20	286.8
Reeders et al. (1989)	*Chlorella*	13–17.7	40–75
Reeders and bij de Vaate (1990)	*In situ*	10–21	40–75
	In situ	3–5	18–51

[a] Numbers between parentheses have been recalculated for mussels with a shell length of 22 mm after Kryger and Riisgård (1988).

Figure 1.3 Filtration rates of the zebra mussel relative to temperature (Reeders et al., 1993)

1.3.4 Environmental Requirements

The zebra mussel is able to attach to any firm substrate such as rocks or stones or other hard surfaces, by their strong byssal threads (Karatayev et al., 1997). This gives them great flexibility in terms of where they can colonise and grow.

The zebra mussels preferred habitat is large freshwater lakes and rivers (Mackie, 1996) usually in water depths of between 0.5-7m. This preferred depth zone (1-5m) is where productivity is at its highest and has the optimum phytoplankton availability as a food source (Mackie et al., 1989; Marsden, 1992; Mackie and Schloesser, 1996; Karatayev et al., 1998; Lucy et al., 2005).

The zebra mussel inhabits clean silt free river or lake bottoms, with its preferred substrate being rocks and in some cases coarse sand; however in shallower water bodies their settlement even on suitable substrates can be inhibited by water currents which can dislodge them (Karatayev et al., 1998). Shelly sediment, silty sand and submerged macrophytes also provide suitable substrate for settlement (as reviewed in Karatayev et al., 1998). Macrophytes include stem fragments of *Schoenoplectus lacustris* (club rush) and *Phragmites australis* (common reed) and also leaves of *Nuphar lutea* (water lily) (Horvath et al., 1996; Lucy et al., 2005). In Lough Corrib, Ireland, zebra mussels are

found attached to another invader, *Lagarosiphon major*, the African curly pondweed (CAISIE project, 2008). It is possible that floating plant fragments aid the further dispersal of the mussel. The zebra mussel usually lives in dense aggregates, possible reasons for this are the preferential settlement of larvae on adults, the lack of other suitable hard substrate, and the preference of mussels for settlement on cracks or crevices (Kobak, 2013). Zebra mussels can be negatively affected by currents and are therefore larger at increased distances from the shore (Garton et al., 2013). Zebra mussels cannot survive freezing water temperature and require higher temperature to grow, develop and reproduce (Karatayev et al., 1998; Lucy, 2006). Temperatures greater than 30°C have been recorded by Claudi and Mackie (1994), Karatayev et al. (1998) and Boeckman and Bidwell (2013) as lethal to the zebra mussel. However if there is a gradual acclimation period the mussels may survive longer. For successful fertilisation the temperature must be greater than 10°C; at 12-24°C eggs can be fertilised between two and five hours after release (Sprung, 1993). The optimum temperature for larval development is 18°C (Sprung, 1993). Fisher et al. (1993) found that in adult zebra mussels the optimum temperature for increased clearance rates was between 15-23°C. Therefore a temperature of between 12-24°C allows for successful, feeding reproduction and survival of the zebra mussel.

pH can often be a limiting factor for the zebra mussel with a value of <6.5 effecting the metabolism of sodium, potassium and calcium (Vinogradov et al., 1993; Garton et al., 2013)). A pH of between 7.4 and 9.4 with an optimum pH of 8.5 is reported by Sprung (1993), and a pH tolerance range between 6.0 and 9.6 is reported by Garton et al. (2013), with a calcium value of > 25 mg l^{-1} is required for successful and healthy larval development (Karatayev et al., 2007).

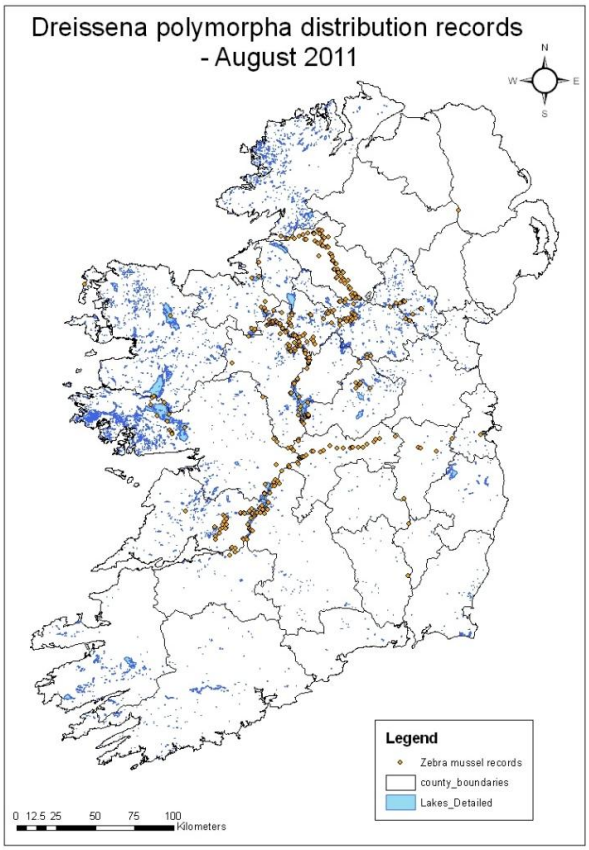

Figure 1.4 Distribution of the zebra mussel in Ireland 2011, National Biodiversity Data Centre [http://invasives.biodiversityireland.ie/zebra-mussel-continues-spread/ last accessed 20 March 2013]

In Ireland (Figure 1.4) the zebra is initially established widely within the River Shannon and Erne systems and is encroaching further into other waterways and lakes. Populations of zebra mussels now also exist in the Western and midland lakes in the Grand Canal (Minchin et al., 2003). In Ireland pleasure craft and angling gear are the most likely vectors for this spread (Minchin et al., 2003).

1.4 Impacts of Zebra Mussels

1.4.1 Introduction

The zebra mussel is an ecosystem engineer defined by Karatayev et al. (2002), i.e. as a species that 'directly or indirectly controls the availability of resources to other organisms by causing physiological state changes in biotic or abiotic materials'. Zebra mussels are very successful ecosystem engineers as they both directly and indirectly

control the availability of resources to other species. They provide habitat for some species and change existing ones for others. This will be further outlined below.

1.4.2 Impacts to the Benthic Environment

The zebra mussel removes material from the water to the benthic environment by filter feeding, and then releases faeces and pseudofaeces (Winkel and Davids 1982). This increases water clarity and allows the benthic environment to thrive through the provision of a new food source (Karatayev et al., 2002). However native unionid filter feeders like *Anodonta* are colonised by the zebra mussels and their numbers decrease (Maguire et al., 2006; Lucy et al., 2014).

Benthification is described by Mayer et al. (2014) as an impact of the invasion of zebra mussels where increased water clarity triggers a systematic change in the benthic ecosystem including structure, composition, distribution and function. The results of benthification described by Mayer et al. (2014) are similar to some of the interactive results of invasion, listed below by Karatayev et al. (1997), including the creation of habitat from mussel beds, an increased food source for benthic grazers from pseudofaeces, an increase in benthic primary production and an increase in benthic feeding by fish.

Zebra mussels and their associated species can form an interactive community all gaining from one another. These relationships have been divided by Karatayev et al. (1997) into the following; 1) formation of habitat, where zebra mussels provide habitat for other species through forming large masses, 2) trophic relationships, zebra mussel provide food for other species through faeces and pseudofaeces, 3) material relationships, zebra mussel provide materials such as shell fragments and small mussels used by other organisms for the construction of homes and finally, 4) dispersal relationships, zebra mussels can be transported by other plant or algal species like *Cladophora* as previously described.

1.4.3 Impacts to Fish

The introduction of the zebra mussel also marked a change in fish abundance, population structure and biomass. A study on fish populations in Lough Erne by Maguire et al. (2006) looked at six fish species making up the fish population in Lough Erne; roach *(Rutilus rutilus)*, common bream *(Abramis brama)*, European perch (*Perca*

fluviatilis), northern pike *(Esox lucius)*, European eel (*Anguilla anguilla)* and brown trout (*Salmo trutta)*.

A change in fish diets was noted trough gut dissections with roach, bream and roach bream hybrids now consuming zebra mussels. The reduction of zooplankton due to filtration by the zebra mussel in Lough Erne will also have a major impact on zooplanktivouros fish. Having their food source reduced may affect their growth, development and have an effect on species interactions (Maguire et al., 2006). In terms of fish population in Lough Erne the number of perch has increased and now compared with roach has a population of 2:1. This is most likely due to the reduction in competitive interactions between the roach and perch as the roach now has another food source. Millane (2008) carried out a study to assess predation of fish populations in Lough Sheelin, Ireland, on the zebra mussels by examining gut contents. Roach and bream hybrids presented the highest amount of mussel shells in the gut dissections and were found to consume mussels in each season sampled (Spring, summer and Autumn). Trout were found to consume zebra mussels only in the spring and only one perch out of 88 was found with a zebra mussel shell in its gut. This demonstrates that through increased food source available to the roach it reduces competition to the perch therefore allowing the population to increase.

1.4.4 Impacts to Water Clarity

Through extensive zebra mussel filtration the depth of the photic zone of lakes and rivers may be extended, total lake volume available for photosynthesis by phytoplankton is enlarged, and macrophytes whose depth distribution is limited by light can thrive at greater depths than pre-invasion. The mussel's high filtration rates reduce phytoplankton abundance allowing for further light penetration which in turn causes excessive macrophyte growth (Karatayev et al., 2002; Lucy et al., 2005). In both Lough Key (Lucy et al., 2005) and Lough Erne (Maguire et al., 2006) research showed that water clarity has increased significantly following zebra mussel invasion; invasion here was directly related to reductions of the peak summer chlorophyll *a* concentrations. In terms of the phytoplankton community, monospecific blooms of *Microcystis* species have been recorded after zebra mussel invasion. It is thought that the occurrence of these blooms may be due to selective rejection by zebra mussels (Winkel and Davids, 1982; Maguire et al., 2006; Vanderploeg et al., 2001; Vanderploeg et al., 2013). The

filtering activity of the zebra mussels means water transparency is increased and macrophyte abundance increases (Leach, 1993; Griffiths, 1993). Extensive macrophyte growth can cause problems for boat users angling activities and other leisure activities as well as increasing costs for local authorities with regards to waterways maintenance (Zhu et al., 2006). The zebra mussels themselves can cause problems for leisure crafts, blockages may occur from zebra mussel settlement in the water intake slots of engines leading to engine damage (Minchin et al., 2002b). Fouling can also increase fuel consumption on leisure crafts due to increased drag, and can sink mooring buoys and zebra mussels settled in shallower areas of the Shannon navigation may also cause problem for bathers and paddlers (Minchin et al., 2002b). Zebra mussels are also known to carry human waterborne parasites such as *Cryptosporidium parvum* oocysts, *Giardia lamblia* cysts and *Encephalitozoon intestinalis* spores (Graczyk et al., 2004). The shells of dead and live mussel can be quite sharp, cutting feet and leaving them open to infection.

1.4.5 Impacts to Elements and Compounds

The zebra mussels' physiological activities can have a large impact on cycling elements in the ecosystem. Through respiration, oxygen levels can be reduced particularly in systems where photosynthesis is light limited. Also because of the abundance of the zebra mussel in some areas, their high rates of consumption and excretion can affect nitrogen (N) and phosphorus (P) levels (Karatayev et al., 2002). Stańczykowska and Planter (1985) found that zebra mussels in lakes of central and Northern Poland play a huge role in N and P cycles, by removing large amounts and accumulating it in their soft bodies and shells for up to and over a dozen years. The amount of N accumulated by the zebra mussels varied between 11%-12.8 and the percentage P accumulated by the mussels ranged from 0.84-0.92% (Stańczykowska and Planter, 1985). Also Effler et al. (1997) demonstrated the mobilisation of ammonia (NH_3) and phosphorus (P) by the zebra mussel. Their study focused on an area of the Seneca River, Baldwinsville, New York. An abrupt oxygen depletion in the river originally prompted surveys where they found that zebra mussels occupied all available cobble substrate on the river bottom. Zebra mussel populations, water flow and water quality parameters (including P and NH_3) were monitored over July-September 1994. This study found that P and NH_3 were increased during low flow. There was a reduction in fluxes of P and NH_3 after late August were there was also a reduction in zebra mussel biomass proving that the

increase in P and NH_3 is directly related to zebra mussel population. This mobilisation of P and NH_3 can have many impacts. In this case it led to an increase in macrophyte growth further downstream.

Bootsma and Liao (2013) presented the results of mass specific excretion of P by Dreissenid mussels in North America, with values for P excretion ranging from 0.2 to 3.22 μmol $gDW^{-1}h^{-1}$ and 1.2-26.1 μmol $gDW^{-1}h^{-1}$ for Nitrogen. Similar to the study by Stańczykowska and Planter (1985), Bootsma and Liao (2013) found that levels of excretion were affected by outside factors such as food quantity, quality and temperature.

1.4.6 Impacts to Unionids

The zebra mussel is also directly responsible for the depletion in numbers of Ireland's native unionid clams (*Anodonta*) that can be found to co-occur with zebra mussels (Minchin et al., 2005). Evidence suggests that zebra mussels often preferentially colonise unionids (Karatayev et al., 1997 and Schloesser et al., 1996). Ricciardi et al. (1995a) presented data from a Lake St. Clair study where virtually all unionids become infested in two to three years in habitats that support zebra mussel densities of at least $1000/m^2$. This depletion in unionids from the zebra mussel is due to several factors; through attachment to native unionids it impairs their movement and burrowing, also via attachment it impairs valve opening and closing which affects, feeding, respiration, reproduction and excretion. They reduce food intake by stripping the inhalant water of phytoplankton (Maguire et al., 2006); the large numbers that attach to unionid shells impairs growth, causing shell deformities and leading to breakages of the thin unionid shells (Figure 1.5). Lucy et al. (2014) suggest that native unionids are most severely impacted by zebra mussel colonisation during the initial stages of invasion, that impacts to the unionids may decrease after ten years and in fact it is possible for zebra mussel and unionids to coexist.

Figure 1.5 Damage to unionid shells from zebra mussel attachment (photo by Sara Meehan).

1.4.7 Impacts to Industry

Systems that are most vulnerable to zebra mussel infestation include those associated with inland waterways management (navigation structures, water level control structures, pumping stations and drainage structures), raw water systems used in potable water treatment, agricultural systems, industry and power generation (McMahon et al., 1994).

Unattached juveniles and veligers are often small enough to pass through mesh screens present at water intake points, the presence of a hard shell allows for their immediate attachment if suitable conditions exist such as slow flow and appropriate surface for settlement e.g. pipes or concrete chambers (Claudi and Mackie, 1994). They will attach, grow and reproduce quickly, causing an infestation. Water intake structures especially those of small diameter (60 -180 centimetres [cm]) according to Claudi and Mackie (1994) are particularly vulnerable to fouling by zebra mussels; therefore dams, reservoirs, aqueducts, drinking water plants, power plants and fish passes are all commonly infested by adult zebra mussels (Mackie and Claudi, 2010). This causes reduced flow through pipes which, in the case of drinking water treatment plants, can lead to drinking water shortages, deposition of mussel shells at pipe outlets, clogging of machinery and may impair the taste of drinking water (LePage, 1993; Mackie and Claudi, 2010).

1.5 Control of Zebra Mussels

1.5.1 Introduction

As a rule, facilities that abstract water from infested sources are required to develop and apply a control method. In many cases facilities have been constructed prior to the arrival of the zebra mussel, which means that control options are not part of the initial plant processes and instead evolve as part of an environmental management plan. As well as this due to the effect of the zebra mussel on native unionids and navigational structures, research is ongoing into the possibility of control in open water. The most effective control method will successfully kill all life stages of the zebra mussel and have no negative effects on the environment. Claudi and Mackie (1994) outline five different chemical treatment strategies; (1) once at end of the reproductive season; (2) periodically, by tackling adults; (3) intermittently, every few hours to prevent initial infestation; (4) semi-continuously, to allow for the mussels to reopen their shells after initial exposure to a chemical; and (5) continuously, to discourage any settlement. Here, consideration must be given to the particular population of mussels and the discharge limitations of the chemical method used. Periodic applications often occur in industrial facilities. An initial high treatment concentration is generally applied to first tackle an existing mussel population followed by regular maintenance or treatment to tackle the more vulnerable life stage the veligers (McMahon et al., 1994). Here, low dose intermittent treatment suffices. Many control methods have been explored but the debate is ongoing as to which is best utilised in specific settings.

Control of the zebra mussel is never 100% effective i.e., it is not eradication, and the results of treatment are often hard to measure as will be described below, it is often useful to combine treatment with sidestream monitoring to measure the effects (Claudi and Mackie, 1994). Mitigation of the zebra mussel must be carried out yearly at a minimum otherwise infestation is likely to continue to re occur (due to new settlement) and reach previous infestation levels.

1.5.2 Temperature

Heat treatment is a possible and often successful control method for zebra mussels. According to Claudi and Mackie (1994) the amount of heat applied and the duration needs to be established with several factors such as the ambient water temperature and

the rate of temperature increase requiring consideration. The lower the ambient temperature the more susceptible the mussels are and if the temperature increase is gradual the mussels may acclimatise making them harder to kill. A study by Rajagopal et al. (1997) involved numerous experiments in the lab to determine the lethal and sub lethal response of zebra mussels to a variety of temperatures. These sub lethal responses included filtration rate, foot activity and byssal thread production. The mortality observed was temperature dependant with 100% mortality reached in 114 minutes at the lowest temperature of 34°C and three minutes at the highest temperature of 38°C. The filtration rate of the mussels showed a decline as the temperatures increased and for foot activity a dramatic decline was noted as temperatures increased past 27.5°C. For byssal thread production the rate of production decreased as the temperatures increased: for example byssal thread production at 20°C for the 5mm group was 13 threads per mussel per day whereas this number decreased to 1 thread per mussel per day at 30°C. It should be noted that the effect of lethal temperatures are subject to the temperature that the mussels are living in and the rate at which the temperature rises. McMahon et al. (1995) noted that the greater the acclimation temperature the lower the lethal temperature and vice versa the lower the acclimation temperature the greater the lethal temperature. Similar to this Matthews and McMahon (1999) looked at the effect of temperature acclimation on zebra mussels during extreme hypoxia. The results of this study were as expected, the greater the acclimation temperature the lower the tolerance of hypoxia. In power stations the types of heat treatment as mentioned above could be applied by circulating the heated effluents through cooling pipes instead of automatic discharge this would be a more economical way to implement this kind of heat treatment (Claudi and Mackie, 1994).

1.5.3 Chlorine

Chlorine is one of the most widely used methods of zebra mussel control worldwide, it can have direct toxic effects on adults, inhibit settlement and larval growth and can weaken the mussels ability to remain attached to substrate. (Claudi and Mackie, 1994; Mackie and Claudi, 2010). Continuous chlorine treatment can be used to eliminate nearly all mussel settlement from a treatment area, and intermittent chlorine treatment can be used as a proactive measure in preventing zebra mussel settlement.

Chlorine treatments can often take up to ten days minimum where operations must shut down for the treatment period (Meehan et al., 2013) adding to the treatment time after tanks/pipes are dosed with chlorine they must be then drained in order to physically remove all the dead mussel shells. Although the use of chlorine meets all regulatory discharge limits more recently studies have shown that the use of chlorine can be potentially dangerous when it combines with organic compounds in the water forming carcinogenic substances (United States Environmental Protection agency, 1999). The formation of chlorinated organic compounds is also enhanced when dead mussels are present (Wright et al., 1997). The veligers are much more sensitive to chlorine then adults and low chlorine levels in pipes can be used to prevent settlement. Much higher doses are required to tackle adult mussels however as it is highly toxic to other aquatic life its use must be minimised and thus chlorine could never be applied in open water (Payne and Lowther, 1992).

Mackie and Claudi (2010) uses Ontario Hydro, Canada as an example of successful intermittent treatment where high levels of chlorine were applied for 1.5 hours every 12 hours at the end of the breeding season this will help prevent new settlement, but will not kill off adult mussels.

1.5.4 Other Chemicals

As a control method chemical hypoxia has been utilised by industries such as those with water intake pipes (Matthews and McMahon, 1999), application of this kind of chemical treatment does however require sufficient mixing in the water to evenly distribute the product which can often be hard to achieve.

Waller and Fisher (1996) looked at 14 different chemicals serving various purposes for aquaculture operations for their potential in the control of zebra mussels. Such chemicals included disinfectants (for equipment and ponds) and therapeutants (fish quarantine and transport). Static toxicity tests were carried out on three different size classes of zebra mussels. Most of the chemicals tested such as hydrogen peroxide and potassium proved effective against veligers but not adult zebra mussels. In order for these chemical tested to be effective against adult zebra mussels they must be applied at higher concentrations then recommended for fish (Waller and Fisher, 1996). Similar to the above study, Cope et al. (1997) identified and tested 47 chemicals with the potential for preventing the attachment of zebra mussels. These chemicals were selected based on

their antioxidant properties or their properties that inhibit catalytic enzymes involved in byssal development. It must be noted however that these chemical were chosen based on the assumption that similarities exist in byssal thread development of the blue mussel, *Mytilus edulis* and the zebra mussel. Eleven chemicals inhibited zebra mussel re attachment after 48 hours exposure. Based on this together with analysis of chemical cost, and hazards to humans in its application and to the environment, three were selected and tested on non-target fish the bluegill (*Lepomis macrochirus*), catfish (order Siluriformes) and rainbow trout (*Oncorhynchus mykiss*). Overall these chemicals proved not to be selective to the zebra mussels only.

Non fouling coatings have also been the focus of extensive studies researching their ability to prevent macro fouling growth focusing on the blue mussels and the zebra mussels (Becker van Slooten and Tarradellas, 1994; Gross, 2007). This control method has its limitations as it can be expensive and is non selective, therefore toxic to all aquatic organisms due to the presence of tributyltin.

1.5.5 UV Light

Wright et al. (1997) looked at UV light as a way of controlling larvae. In this study the larvae were exposed to broad band UV and narrow band UV wave lengths. Larvae were exposed for periods of up to four minutes to narrow band UV wavelength of 297nm, 280nm and 254nm, for significant mortality to occur exposure needed to exceed 30 seconds. The higher the wavelength, the greater is the mortality. Also mortality was noted for up to 12 days after exposure. From this study UV light proves to be an effective way of killing larvae, however this can take up to 12 days and does not include adult mussels.

1.5.6 Microbial Biofilm

As the focus of zebra mussel control is now turning away from chemical eradication due to stringent laws regarding water quality, other non-chemical methods are being explored.

One area of natural control explored by Gu et al. (1997) examined microbial biofilms and how they inhibit or induce invertebrate settlement. Most surfaces that are submerged in fresh and marine water over time become coated with a microbial biofilm. This thin layer of organic matter may have specific chemicals that can inhibit or induce

invertebrate attachment, in particular the zebra mussel. This study isolated a large number of microorganisms from live and dying tissue of the zebra mussel that were determined to be lethal to the zebra mussel and impair their attachment to surfaces. Between 95 and 100% of mussels attached to clean polystyrene surfaces compared with less than 50% attaching to surfaces coated with biofilms. Swabs of an aquarium environment were also tested for their repulsion properties for the zebra mussel this study found that more than 60% of the bacteria strains tested were *Pseudomonas* one of which was *Pseudomonas fluorescens* which is incidentally the active ingredient in Zequanox.

1.5.7 Natural Antifouling Agents

Taylor and Zheng (1997) looked at natural antifouling agents to include 23 species of algae and invertebrates including chlorophytes, phaeophytes, sponges and echinoderms. Of the species tested above, lab trials and field deployments were carried out for *Fucus* and *Ulva* using frosted slides and settling plates. For zebra mussels one valve of each adult mussel tested was glued to the clean section of the microscope slide, from here the mussels had the choice to attach to the clean or coated part of the slide. These results were compared with positive controls (CuO_2 antifouling paint) and negative controls (clean slides). For field deployment the same principle was applied with each settlement plate divided into sections. For the lab trial zebra mussels in all but one instance attached outside the extract coated zones with the same results for the field deployments. The coated sections of the plates were completely free from zebra mussels and the non-coated sections had significant numbers. As the *Fucus* and *Ulva* extracts were avoided by the adult zebra mussels it proved to be an effective inhibitor of larval settlement. Overall this study demonstrated the potential in naturally occurring products like seaweed as inhibitors of zebra mussel attachment.

1.5.8 Combinations

Sometimes effective control of the zebra mussel requires a combination of different methods as demonstrated by Mead and Adams (1997) in the Ohio-American water company plant in Ashtabula. Here a mix of physical and chemical control methods were explored beginning with the redesign of the intake screens. Due to the small gaps between the previous intake screens (24 and 30 inches) the mussels were readily able to cluster and attach restricting the flow of water. New intake screens were designed

leaving a gap of six inches on the flat. Thirty inch screens and the 24 inch screens were remoulded to form a dome shape which also increased the distance between the bars. Twice a year a diver physically removes mussels from the water intake screens. Chemical control methods could not be applied at the intake screens due to restrictions on water quality however this application was possible on the intake pipes which also had a severe mussel problem. A high molecular weight polymer, dimethyl-diallyl-ammonium chloride (DMDAAC) was applied to the intake pipes via permanent feed lines (at 1.5 to 2.0mg/l) as lab trials showed this substance to have a 96 hour LC_{50} of between 1.5 and 3mg/l. The lab trial also showed that the zebra mussel did not attach to the test container as well as the controls. This is a good example of a zebra mussel control method being implemented into a working plant using numerous techniques put together to suit this particular operation. Although the zebra mussels here were not eradicated completely these techniques help reduce their numbers and keep the plant running. The use of increased temperature with a chemical control method has been observed to have greater efficiency on causing higher mortality (Harrington et al., 1997; Claudi and Mackie, 1994)

1.5.9 BioBullets

BioBullets are another possible treatment for zebra mussels. These microencapsulated bullets consist of potassium chloride covered in a slurry premix so as to disguise the potential danger of ingestion to the zebra mussel. The potential effects on water quality and non-target organisms other than *A. anatina* has not yet been shown (Aldridge et al., 2006; Costa et al., 2012) and therefore it is not possible to say if BioBullets are suitable for use in open water. BioBullets are essentially a form of chemical control and therefore non-target studies on different functional feeding groups are required before this product can be considered for use in industry and open water.

1.5.10 Zequanox

Zequanox is the commercial name for an environmentally friendly zebra mussel control product. It is made up of dead *Pseudomonas fluorescens* cells *pf-* CL145A. *Pseudomonas fluorescens* is worldwide in its distribution and is found in all kinds of environments such as plants, dead animals, water and soil (Peix et al., 2009). It is a North American isolate strain of a bacterial species found in all North American water bodies protecting the roots of plants from plant disease that is used in Zequanox

(Marrone Bio Innovations, 2012a). In an effort to control zebra mussels using a more environmentally friendly method, Dr Dan Molloy of the New York State Museum investigated the use of bacteria and their natural metabolic properties as selective control agents. Screening trials resulted in the discovery of the lethality of CL145A a strain of *Pseudomonas fluorescens* to zebra mussels (Molloy et al., 2013a).

The zebra mussel does not recognise Zequanox as a potentially harmful substance and readily ingests it which is unlike chlorine treatment which often causes the zebra mussels to shut their shells and cease feeding leading to prolonged chlorination (Rajagopal et al., 2003; Molloy et al., 2013a). Dead *Pseudomonas fluorescens* cells are just as effective against the zebra mussels as live cells as intoxication and not infection is the mode of action (Molloy et al., 2013a). Using dead *Pseudomonas fluorescens* cells a study by Molloy et al. (2013b) revealed that mortality occurs from lysis and necrosis of the digestive gland and sloughing of the stomach epithelial cells.

Non target ecotoxicology trials carried out by Meehan et al. (2014a) and Molloy et al. (2013c) shows that Zequanox and the active ingredient in Zequanox dead *Pseudomonas fluorescens* cells are not harmful to a range of organisms tested at concentrations required to gain a >80% zebra mussel kill. Molloy et al. (2013c) tested the active ingredient *Pseudomonas fluorescens* strain CL145A on a number of non target organisms at 100-200ppm to include ciliate *Colpidium colpoda*, the cladoceran *Daphnia magna*, three fish species (*Pimephales promelas, Salmo trutta,* and *Lepomis macrochirus*), and seven bivalve species (*Mytilus edulis, Pyganodon grandis, Pyganodon cataracta, Lasmigona compressa, Strophitus undulatus, Lampsilis radiata,* and *Elliptio complanata*). No mortality was recorded, however low mortality was recorded (3-27%) in the amphipod *Hyalella azteca*, but additional trials suggested that the mortality could be attributed to some other unidentified factor.

In the USA a license has been granted by the US EPA for the use of Zequanox in defined enclosures and therefore trials are ongoing. Meehan et al. (2014b) also demonstrated the efficacy of Zequanox in reducing the zebra mussel population at the Grand Canal, Tullamore harbour using an impermeable curtain and treating settled juveniles, seeded adults and naturally settled adults, resulting in a reduction in adults and juveniles. In the USA the Great Lakes restoration programme is leading the way forward for the use of Zequanox to help restore the native unionid population which was

reduced due to zebra mussels (Great Lakes Restoration). To do this they are first evaluating the toxicity of Zequanox to freshwater fish and unionids from the Great Lakes and Mississippi River. They are developing treatment protocols to use Zequanox to kill planktonic larvae in contained systems such as fish transport passes in hatcheries, and finally developing application methods for using Zequanox to reduce zebra mussel populations within unionid beds and restoration structures (Great Lakes Restoration).

1.6 Zebra Mussel Monitoring

1.6.1 Introduction

Zebra mussel monitoring is required for industries that abstract water from zebra mussel infested sources, such as drinking water treatment plants, power plants and fish hatcheries. Figure 1.6 displays the treatment process of a drinking water treatment plant in Co. Sligo, Ireland. Here the raw water is taken from an infested source (Lough Gill), therefore the raw water chambers (pre ozone chambers) have a severe mussel infestation. Zebra mussel monitoring in industry and in the wild is important for a number of reasons; in natural water bodies it is important to monitor for detection of a new population or for those already established, alternatively monitoring programmes are carried out in industrial or natural settings to determine the management and effectiveness of a control method(s). The monitoring of veligers in an industrial water source is important to determine when to begin and when to end a treatment, as well as this, monitoring naturally occurring mussel populations helps to determine where treatment is necessary and helps to inform if and when natural population declines occur (Marsden, 1992). In industries like power plants or water treatment facilities early detection of zebra mussels is key to mussel reduction. Monitoring for veligers or settled juveniles is the best process and treatment of these life stages is easier. Lower doses of treatment methods will kill early life stages and due to their microscopic size, physical removal is not necessary after. As the distribution of veligers is often patchy the monitoring of juvenile settlement using settlement plates is more reliable (Lucy, 2006).

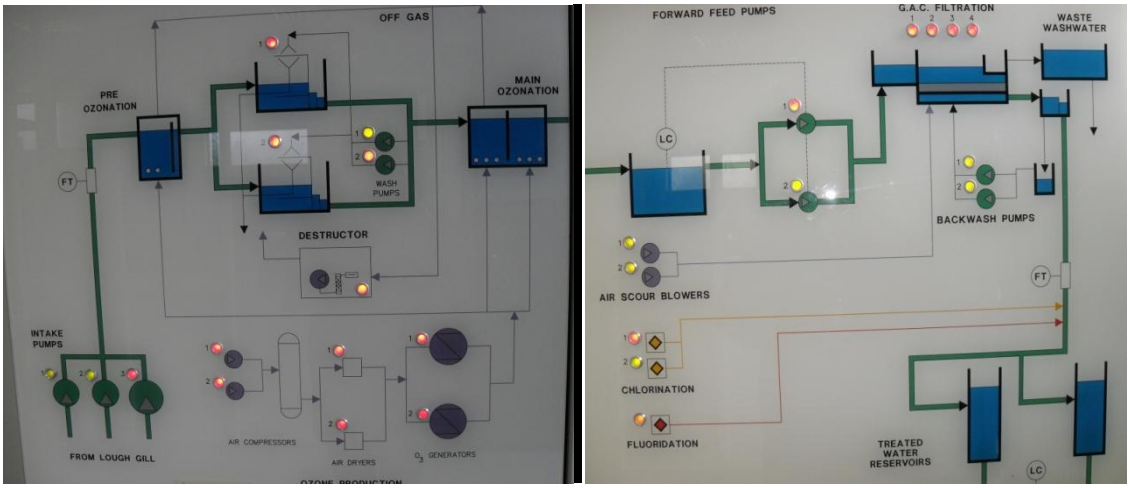

Figure 1.6 Infrastructure of a water treatment plant Ireland (photo by Sara Meehan).

Standard sampling procedures such as those set out by Marsden (1992) allow for comparison between studies and between years. These standard procedures make recommendations, such as considering substrate type for settlement as well as the recording of ancillary data such as temperature and depth, as mussel populations can be affected by these parameters.

For sampling settled juveniles in order to make a study comparable there are a number of factors that require consideration; substrate type, texture, depth where settlement is to be collected, light and water currents. As well as this juvenile settlement can be affected if there is bio film on the settlement substrates with preference given to surfaces with bio film as opposed to clean ones. Areas of higher turbulence causes a decline in zebra mussel settlement, as well as the plate orientation, whether it is horizontal or vertical with shaded horizontal surfaces being favoured by the mussels. Roughened PVC surfaces are also favoured (Marsden 1992; Karatayev et al., 1998; Lucy et al., 2005).

PVC plates can be deployed horizontally on a rope or using multi plate samplers (a plate holder that can house a number of plates) to gather natural settlement which can then be counted. Adult zebra mussels can be counted *in situ* (Marsden, 1992) on natural substrates using divers, where quadrats are placed randomly over a population of mussels and a razor blade is used to remove all mussels within the quadrat. Alternatively a long handled scraper with a net attached can be used to scrape away an adult mussel population and catch them in the net (Minchin et al., 2002a). Again the collection of ancillary data is important here to make population studies comparable.

1.6.2 Settlement Plates

Settlement plates can be used to calculate the number of settled juveniles per square meter. According to Mackie and Claudi (2010) they are made up of almost any solid non toxic substrate such as, carbon steel, stainless steel, polyvinyl chloride (PVC), bricks clay or cement tiles (Figure 1.7). They are usually attached to a rope and submerged in the water body to be monitored (Mackie and Claudi, 2010). The area of the settlement plate must be known in order to calculate the number of juveniles per square meter. Settlement density can then be determined from these plates once initial settlement is observed. Sampling may be carried out weekly, bi weekly or monthly where plate scrapes can be taken or settlement can be directly counted under the microscope. Mackie and Claudi (2010) also recommend the recording of temperature as this can help interpret settlement patterns. An example of the working use of settlement plates comes from Lucy et al. (2005) adapted from Marsden (1992) where settlement plates were deployed in order to estimate juvenile settlement density and size. In this instance, three plates per site were deployed. At each site the bottom two of the three plates were changed on a two week rotation where the mean of thirty 1cm x 1cm quadrats was calculated to determine the estimated density per meter squared (Figure 1.7). The top plate was removed and counted once in July and once in October to estimate seasonal density of mussel settlement, these seasonal plate counts were then compared with the two week cumulative plate counts. In the 'Standard Protocol for Monitoring and Sampling Zebra Mussels' (Marsden, 1992) settlement plates are again referred to as the best option for monitoring juveniles. Slide racks are also discussed for monitoring settlement where a periphyton slide rack that can hold a number of glass slides is used to gather natural settlement. The same principals for monitoring are applied here where the slides are removed at regular intervals and replaced by clean ones and all settlement is counted. Also in this instance it is recommended that once a month two slides are removed that have been in place since the rack was deployed to count overall settlement. For counting using glass slides, graph paper is placed under the slide with five 1cm squares marked out and juveniles within these squares are counted (Marsden, 1992).

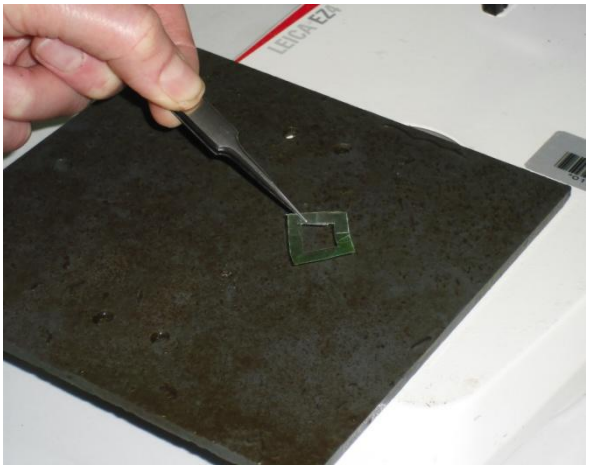

Figure 1.7 Settlement plate with counting quadrat (1cm^2) (photo by Sara Meehan).

1.6.3 Sidestream Monitoring

Sidestream monitoring using bioboxes according to Mackie and Claudi (2010) is recommended to monitor zebra mussel settlement in power plants or other such facilities. Bioboxes can be a box of any size or shape that is connected to a facilities main inflow of raw water, to demonstrate the possible effect of zebra mussel fouling in the piping of a facility. The outflow of this water goes to the drain and helps to balance the inflow maintaining a continuous circulation. Plates are set up inside bioboxes to monitor juvenile settlement. This type of biobox can also be used to demonstrate if treatment of a piping system in a plant is effective, by placing a second biobox further down the piping system that is receiving treated water. If this biobox is not colonised by zebra mussels the plants treatment measures are working. These bioboxes according to Mackie and Claudi (2010) are also very useful in determining when treatment commences and finishes. It is best to start treatment as soon as settlement is observed and cease when no more settlement is observed on the plates. Seeding bioboxes with a number of adults from a wild population will give insight into whether the treatment is as effective against adults as well as juveniles and be a representation of the state of the pipes in the plant after treatment (Figure 1.8).

In a study by Lyons et al. (1990) bioboxes were used to assess if the treatment of the J.R Whiting plant on the Lake Eire shoreline in the USA was successful. This plant had a very severe zebra mussel infestation so a molluscicides treatment programme was designed to eradicate the mussels. Separate molluscicide treatments were applied to the main condenser, circulating water systems and the service water system. For each

molluscicide application bioboxes were used to assess the success of the treatment. Bioboxes seeded with adult mussels were placed at the inlet and outlet ends of the treated cooling water systems. Water flowed through these bioboxes for each of the 12 hour applications and for several weeks after allowing the J.R Whiting power plant to determine the success of their treatment.

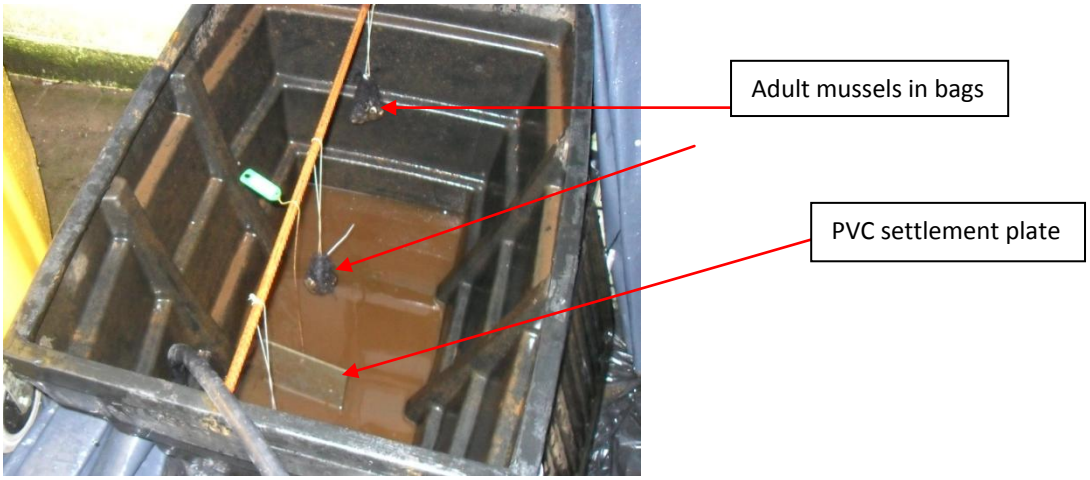

Figure 1.8 Biobox showing seeded adult mussels and PVC plate (photo by Sara Meehan).

1.6.4 Mesocosm

A mesocosm provides a link between observational field studies and controlled laboratory experiments. Mesocosm experiments are generally used to determine an ecosystem response to an added nutrient or a change in environmental conditions. A mesocosm can be open water or land based where a large body of water is enclosed (Watts and Bigg, 2001). They are used to assess the effect an environmental change can have on organisms or ecosystems (Taucher et al., 2012; Moss et al., 2004). Mesocosm experiments have previously been used to assess the effect that zebra mussels have on the surrounding ecosystem (Wilson, 2001).

1.7 Ecotoxicology Testing

Acute toxicity testing is essential in determining the potential effect of a substance on organisms as well as in determining the potential ecological effects. It is necessary to use organisms from different trophic levels to determine the potential impact on an ecosystem as a whole (Kumar et al., 2010). To compare the toxicity of one substance to another, it is important to measure the same effect. The median lethal concentration

(LC_{50}) is used to determine the dose that kills 50% of the test organisms and the median effective concentration (EC_{50}) is used to determine the dose that has an effect on 50% organisms (namely immobilisation). Waller et al. (1993) carried out toxicity testing on the effect of molluscicides to the zebra mussel and other non target organisms. This study used the LC_{50} values to compare the effect of molluscicides amongst organisms. Using a universal method to describe effect makes the response from different species comparable. The taxa chosen for the non target testing as part of this research have widespread freshwater distribution, and are within various functional feeding groups, namely filter feeders, benthic grazers, decomposers and carnivores. Their response to Zequanox treatment will be discussed further in Chapter 2. Non target trials have previously been undertaken in America by Molloy et al. (2013c) using the active ingredient dead *P. fluorescens* CL145A and Marrone Bio Innovations using Zequanox however the results have not yet been published: further information can be viewed on the Marrone Bio Innovations website [http://www.marronebioinnovations.com/products/brand/zequanox/]. As discussed in section 1.5.10 numerous non target trials have been conducted by Molloy et al. (2013c) demonstrating the target specificity of the active ingredient *Pseudomonas fluorescens* CL145A however it is necessary to determine the effect of the commercialised product Zequanox to non target species native to Ireland and Europe.

1.7.1 Rational for Selecting Organisms for Ecotoxicology Trials

Chironomus (non-biting midges) Order Diptera: Freshwater non-biting midges (Chironimidae), often account for over half of the biomass of macroinvertebrates in many aquatic habitats. Chironomids are a very diverse and tolerable group of species (396 known species in Ireland) so they can live in most climates and are tolerant to a wide range of water qualities (Ristola, 2000). They have four life stages: egg, larva, pupa and adult. Chironomid midges make up the diet of predatory aquatic insects like dragon flies and beetle larva as well as fish particularly bottom feeding fish like carp as larva and surface feeding fish like trout as adults (Apperson et al., 2006). *Chironomus*, a common genus of this family were chosen for this study.

According to OECD test guidelines 219 and 235, chironomids are abundant and have a well described endocrine system, they are easy to handle and culture as they are very durable. These reasons make chironomids a suitable candidate to represent insects in

conducting ecotoxicology trials using Zequanox. Testing on *Chironomus* took place using the larva, pupae or both. The pupae stage would be more sensitive than the larval stage as the larval stage can survive in low oxygen conditions.

Asellus aquaticus (waterlouse), Order Isopoda: This species is found in rivers, streams and standing water, is omnivorous, although largely known as a decomposer (Bundschuh et al., 2012). It breathes through lamellar gills on the ventral side of the body. As water passes through its gills at a constant rate testing the effect of Zequanox is important as they would constantly be exposed to the treatment. This species can reproduce in large numbers and are often found in association with zebra mussels. *Asellus aquaticus* also serves as a preferential food item for fish such as perch (*Perca fluviatilis*) and brown trout (*Salmo trutta*) so if the waterlouse is affected by Zequanox it may be important to look at the fish that feed on this species (Bundschuh et al., 2012; Aigbavbiere, 2010; Gargan and O'Grady 1992). There are no standards set out for toxicity testing in *Asellus aquaticus* however ASTM guidelines set out for 'Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians' (ASTM, 2007) can apply.

Ephemerella ignita (mayfly), Order Ephemeroptera. This genus of mayfly is generally found in well oxygenated water. These aquatic insects go through many nymph stages and two flying stages (subimago and imago). The nymph stage looks completely different from the adult and lives in freshwater (Salles, 2000). Nymphs after they hatch from eggs are less then 1mm in length and have no gills. The *Ephemerella* nymph lives mainly in rivers and streams or at the edge of large lakes with wind-swept shores. They are usually found clinging to, or crawling amongst submerged plants and stones, although they may swim in short bursts if disturbed [Olsen et al., 2001; The Ephemeroptera Recording Scheme last accessed 14 March 2013].

In older nymphs, gills are found in pairs on each segment of the abdomen. These gills beat to control the flow of water through the body which in turn controls the amount of salt and oxygen in the body (Salles, 2000). *Ephemerella* nymphs are easily recognised by their three caudal filaments at the tip of the abdomen where the middle one is much shorter than the other two (Salles, 2000). Mayfly nymphs are generally grazers, they feed on algae and bacteria from stones and weeds. Mayflies also are an important food source for other organisms. For example mayfly eggs are eaten by snails and caddis fly

larvae. The nymphs may be eaten by fish, frogs, birds, flies or water beetles and the subimagos are eaten by fish, birds, dragon flies, water beetles and other predatory insects (Salles, 2000).

Mayfly nymphs are very sensitive to water pollution and are often used to determine pollution in a water body (Hering et al., 2003). Due to their sensitive nature and the abundance of *Ephemerella* spp. in Ireland it is important to carry out ecotox trials on them for the use of Zequanox.

Mytilus edulis (Blue mussel), Order Mytiloida. The blue mussel is a saltwater bivalve mussel, they have similar properties to the zebra mussel such as their modes of feeding and reproduction. Zebra mussels reside in freshwaters in Ireland, while *Mytilus edulis* are found in marine environments. It is important to consider the discharge of MBI-401 from freshwater systems to transitional or coastal areas, where aquaculture and shellfisheries with *Mytilus edulis* may be. The blue mussel is an ideal species to test the effect of Zequanox as they are filter feeders taking in a large amount of water daily. Also they are bio accumulators and can accumulate toxins in the water even when present at low concentrations, therefore being sensitive to any changes in their environment (Mubiana et al., 2006). Blue mussels are also an important commercial fishery and are produced by aquaculture in Ireland.

ASTM International (American Society for Testing and Materials) has toxicity testing guidelines for a wide range of freshwater and marine organisms. *Mytilus edulis* is covered under 'ASTM E724 - 98(2004) Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs'. This describes procedures for conducting acute toxicity tests with embryos or larvae of saltwater bivalve molluscs. Endpoints measured in Guide E 724 include survival or shell deposition.

Anodonta (swan/duck/pearl mussel), Order Unionoida. In Ireland difficulty has been noted in distinguishing between the two common species of *Anodonta; Margaritifera margaritifera* (L., 1758) (pearl mussel) *Anodonta anatina* (L., 1758) (duck mussel), and *Anodonta cygnea* (L., 1758) (swan mussel). Until molecular phylogenetic investigations are carried out it is not possible to confirm which species are present in Ireland, therefore in this thesis will not be distinguished and will be referred to as *Anodonta* (Lucy et al., 2014). This mussel is the largest freshwater mussel in Ireland and Britain.

This mussel mainly inhibits standing water bodies like ponds and lakes, where here they have the ability to survive extreme conditions. The *Anodonta* mussel resides in muddy, silty and organic rich substrates among tall plants like the common reed and club rush. *Anodonta* are considered to play an important role in the food chain as primary consumers and are located at the first link of the food web due to their role as filter feeders (Başçınar et al., 2009). Like the zebra mussel *Anodonta* are filter feeders and they often co-occur with the zebra mussel. It is important to test the effects of Zequanox to ensure it does not cause similar disruptions to their digestive system.

Anodonta are widespread throughout much of lowland Europe however they are becoming scarcer in southern Europe and Scandinavia. In Ireland they can be found in most freshwater systems, but have been largely extirpated in waters where zebra mussels have invaded (Byrne et al., 2009; Killeen and Aldridge, 2011; Lopes-Lima, 2014; Lucy et al., 2014). In addition to widespread mortality caused by zebra mussels, some of the causes for their scarcity in numbers include anthropogenic factors like eutrophication and waste inflow leading to a decrease of oxygen in the water which can in turn hinder fertility (reviewed in Lucy et al., 2014). Within Ireland, river or canal modification cause serious damage to the *Anodonta* mussel habitat leading to their decline. As *Anodonta* are known to co-exist with the zebra mussel is imperative to test for effect from Zequanox.

ASTM also has a standard guide 'for conducting laboratory toxicity tests with freshwater mussels (E2455-05)'. This particular standard describes methods for conducting lab toxicity tests with early life stages of the freshwater mussel, (glochidia and juvenile mussels) in water-only exposures (ASTM, 2013).

Daphnia pulex (water fleas), Order Cladocera. *Daphnia pulex* are small planktonic crustaceans measuring between 3-5 mm in females and approximately 2 mm in length for males. *Daphnia* species live in freshwater rivers, streams, lakes and ponds all over Ireland and are commonly known as water fleas. The optimum temperature range for *Daphnia pulex*, is 18-22°C (Clare, 2002).

Daphnia play an important role in the food web, providing food for young bream, roach and perch (*Perca fluviatilis*) as well as invertebrate predators like the phantom midge (GeoChemBio). *Daphnia*, like the zebra mussel are filter feeders so it is important to carry out ecotoxicology trials on them, as although they are selective filter feeders they

still maintain a steady stream of water through the body and so may be susceptible to any changes in the water (Ryther, 1957; Porter et al., 1983).

For *Daphnia*, toxicity testing guidelines have again been laid out by ASTM under guideline 'E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macro invertebrates, and Amphibians', and OECD guideline 202 '*Daphnia sp.*, Acute Immobilisation Test'. This guide describes procedures for obtaining laboratory results of the adverse effect of a test material added to dilution water, using three basic exposure techniques; static, renewal, and flow-through. With these guidelines the results are reported as LC_{50} (median lethal concentration) or EC_{50} (median effective concentration) at the end of the test (ASTM, 2007).

Austropotatamobius pallipes (white clawed crayfish), Order Decapoda. This species of crayfish was introduced to Ireland many hundreds of years ago, and is also widely distributed around Europe (Füreder, 2010). It is listed under the IUCN red list as endangered and as an Annex 2 species under the EU Habitats Directive. Crayfish are quite vulnerable and are susceptible to a range of threats. The most aggressive threat affecting the crayfish is the introduction of invasive alien crayfish species (e.g. signal crayfish) and disease. (Füreder et al., 2010). In Ireland surveys have indicated the loss of stocks from several midland lakes (Stokes et al., 2004).

Austropotatamobius pallipes can be found in lakes, streams, rivers or canals, submerged under cobbles, rocks, logs, tree roots and fallen leaves (Füreder et al., 2010). Crayfish generally occur in hard mineral rich waters with a pH value between 7 and 9. They often co-occur in lakes with zebra mussel. They require good water quality and moderate summer temperatures below 20°C. They are very sensitive to acidity and heavy metals (Reynolds, 1998). Crayfish can live for more than 10 years and feed on animal and plant food. Their diet will depend on whether they are living in streams or in lakes. In Irish lakes adult white-clawed crayfish prey on a wide variety of benthic invertebrates including snails, crustaceans and insect larvae and in turn are controlling the abundance of some species (Reynolds, 1998).

Due to the fact that they are a protected and endangered species in Ireland it is very important to carry out ecotoxicology trials on the white-clawed crayfish to make sure that there are no adverse effects to the use of Zequanox at all. If there were even any minor side effects it would seriously hinder Zequanox use in Ireland and Europe. Also

as can be seen from the list of species consumed by the crayfish in Ireland many of the species listed for non target trials are included. This increases the importance of testing some of the other non target species as any changes to the crayfishes food source would seriously affect *Austropotatamobius pallipes* numbers.

Lymnaea peregra (common pond snail), Order Basommatophora. The wandering pond snail lives primarily in slow moving to still water. It has a spiral, brownish shell with a large opening and a grey-brown translucent body. The head has a single pair of tentacles with eyes at the base. This snail usually reaches between 15-20mm (Islam et al., 2001). *Lymnaea peregra* usually colonises weedy garden ponds where it can be seen on emergent vegetation or on mud banks however it does not travel far from the water (Lance et al., 2006). It is widely distributed and this species is the most common freshwater snail in Europe. Its widespread distribution is down to a high tolerance of a wide range of water qualities (Islam et al., 2001).

This species is very useful to have as it can feed on rotting vegetation which otherwise would de-oxygenate the water. *Lymnaea* provides food for other freshwater organisms such as crayfish, leeches and fish which in turn are preyed upon by water birds and rats (Lance et al., 2006). As *Lymnaea peregra* thrive in the type of environment that the zebra mussel creates it would be important to test the effects of Zequanox on this snail as they are very likely to co-occur with the zebra mussel and so may be exposed to Zequanox.

Salmo trutta (brown trout), Order Salmoniformes. Brown trout are olive green to brown in colour, covered with black spots along their side, back and dorsal fin and have a slightly forked tail (Staley, 2007). Male *Salmo trutta* can reach up to 1.5 m in length and weigh 36 kg, females can reach a maximum of 1.2 m in length and 20 kg in weight (Hendry and Cragg-Hine, 2003). In Ireland trout can be found in nearly all lakes and rivers. The natural range of the brown trout was originally Europe but now they can be found all around the world, this is due to their introduction for sport (Fishlock, 2011). Although not always possible in today's environment in order for the trout to be successful it needs a narrow range of water temperatures (an average of less than 20° C in the summer). It needs to have medium to strong current speeds with good water quality and finally it needs to have access to a clean bottom with coarse gravel for reproduction (Baglinière and Maisse, 1991). In Ireland trout fishing is a major sport

with the river Nore, Suir and their tributaries both containing a good stock of brown trout and good fly hatches (Inland Fisheries Ireland).

The brown trout feeds on a large number of insects both aquatic and terrestrial such as caddis flies (Trichoptera), stoneflies (Plecoptera), mayflies (Ephemeroptera), beetles (Coleoptera), grass hoppers (Orthoptera) and grubs (Diptera) that fall into the water (Fishlock, 2011). Larger brown trout also feed on other fish such as young brown and rainbow trout (*Onchorhynchus mykiss*).

Apart from their conservation value as a native salmonid species, *Salmo trutta* contributes to an important part of Ireland's economy, i.e. brown trout angling. In Ireland the overall impact of recreational angling is estimated at approximately €755 million to the economy (Inland Fisheries Ireland, 2013). It is very important to ensure that they are not affected by the use of Zequanox because if brown trout numbers are affected it will lead to a drop in the tourism industry. For the ecotoxicology trials it would be best to carry out on the juvenile stages of the brown trout either fry or parr as they are most sensitive at these stages. ASTM has guidelines laid out for 'Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians' (ASTM 2008). This guide outlines the procedures for obtaining laboratory data on the adverse effects of aqueous ambient samples and effluents on certain species of freshwater fish using static or flow through techniques. In conjunction with this ASTM have a 'Standard Guide for Measurement of Behaviour During Fish Toxicity Tests ASTM E1711 - 95(2008)'. This guide gives general information on methods for qualitative and quantitative assessment of behavioural response of fish to toxicity testing and can be used in conjunction with standard toxicity tests.

1.8 Aims and Objectives of Thesis

This research has two overarching aims; to examine in depth the potential effects Zequanox could have on the environment including water quality and organisms found in the Irish aquatic ecosystem and to explore Zequanox use in industry and open water. To meet these aims this research can be divided into the following objectives:

- Carry out non target trials to assess the target specificity of Zequanox to zebra mussels only

- Perform a biobox trial to test the efficacy of Zequanox in controlling zebra mussels at a commercial facility
- Execute a field trial using a mesocosm set up, to test the ability of Zequanox to control wild mussel populations
- Compare the efficacy of Zequanox in controlling North American V European zebra mussels
- Utilise lab trials to further explore effects and mortality rates of Zequanox on settled juvenile zebra mussels

1.9 Tasks and Methods

This research can be divided into five sequential sections:

- Chapter 2 explores the effect of Zequanox on non target organisms to first ensure there were no negative impacts from the use of Zequanox. A paper composed of *Anodonta*, *Austropotamobius pallipes* and *Chironomous plumosus* was published in the journal of Ecotoxicology and Environmental Safety, where co-contributors were Dr. Adam Shannon, Ms. Bridget Gruber, Dr. Sarahann Rackl and Dr. Frances Lucy.
- Once Zequanox was deemed safe, its use in controlling zebra mussels at a drinking water plant was explored as in Chapter 3. A biobox study was carried out to mimic the effect Zequanox would have on the infested water chambers of an Irish drinking water treatment plant. This work was published in the journal Management of Biological Invasions, where co-contributors were Dr. Frances Lucy, Ms. Bridget Gruber and Dr. Sarahann Rackl.
- Chapter 4 demonstrates an *in situ* application carried out in Tullamore Harbour. This study was published in 'International Conference on Aquatic Invasive Species' (ICAIS) edition of Management of Biological Invasions where co-contributors were Bridget Gruber and Dr. Frances Lucy. In Tullamore Harbour, infested canal walls currently have no control procedures in place. This trial involved the containment of canal sections in a mesocosm type experiment in order to treat the infestation.

- Following on from the two *in situ* trials it was apparent that mortality was lower after treatment in Ireland when compared to North America. Research was carried out to further explore this and assess the possible reasons (Chapter 5).

- Also following on from the two *in situ* trials the effect of Zequanox on juvenile mussels was unclear. Further lab research was carried out to get exact survival after treatment and determine how long it takes for survival to reach zero (Chapter 6).

- A trouble shooting chapter was composed (Chapter 7) to allow for knowledge contribution and expansion. Here recommendations were made for future research taking into consideration lessons learned from this thesis.

Chapter 2

The impact of Zequanox® on Selected Non Target Irish Aquatic Organisms

2.1 Introduction to paper

This chapter will examine the impact, if any, of Zequanox to nine non target organisms. The results of ecotoxicology trials on the non target organisms will be presented and discussed for each individual species. Ecotoxicology trials on three of these taxa were compiled as a paper and published in the Journal of Ecotoxicology and Environmental Safety (volume 107): *Anodonta* and *Austropotamobius pallipes* were chosen for inclusion in the paper as their depleting numbers and conservation status means stakeholder interest is high (Byrne et al., 2009; Füreder et al., 2010; Killeen and Aldridge, 2011; Lopes-Lima, 2014; Lucy et al., 2014); *Chironomus plumosus* were chosen for inclusion as they are one of Irelands most commonly occurring invertebrates and are quite tolerant of poorer water quality and would therefore serve as a good baseline study (Ristola, 2000). The published paper in its entirety is presented in Appendix A. [http://www.sciencedirect.com/science/article/pii/S0147651314002267].

2.2 Introduction

Ecotoxicology is defined by Forbes and Forbes (1994) as 'the field of study that integrates the ecological and toxicological effects of chemical pollutants on populations, communities and ecosystems with the fate (transport, transformation and breakdown) of such pollutants in the environment'. Determining the potential effects of Zequanox (although it is a natural biocide) on aquatic organisms with which it may come into contact is imperative in progressing the licensing of this product in Europe. Testing has been carried out in the USA on a number of fish, molluscs, plants and algae (Marrone Bio Innovations, 2012a; Molloy et al., 2013c). Prior to *in situ* trials with Zequanox in Ireland it was important to test its effect on organisms (fish and macroinvertebrates) found in an Irish ecosystem. These non target studies consisted of testing developmental formulations of Zequanox called MBI-401 FDP (Freeze dried powder) and MBI-401 SDP (spray dried powder) on nine different organisms. MBI-401 FDP was an earlier developmental formulation of Zequanox (2011-2012); high unionised ammonia levels

(NH_3) were documented with its use in the early stage non target bio assays. The formulation was further developed to MBI-401 SDP (2012-present), which had lower unionised ammonia levels and was therefore used for further non target testing. Due to the change in product formulation, bridging studies were carried out where two organisms previously tested with the FDP formulation (*Chironomus, Ephemerella ignita)* were also tested using the SDP formulation to check for any differences in potential effects. If the SDP bioassays gave a higher LC_{50} value with lower unionised ammonia levels, the other organisms tested with FDP did not require re testing. The difference between the two formulations lies with the percentage active ingredient; MBI-401 FDP contains 100% active ingredient or active substance (a.i., a.s.) whereas MBI-401 SDP contains 50% active ingredient. Concentrations of SDP were adjusted accordingly for testing therefore all concentrations in all tests are referred to as active substance in mg/L (a.i. mg/L).

The results of this study are important for gaining regulatory approval for the use of MBI-401 in European waters. Non target studies have previously been carried out in North America, however in order to successfully use Zequanox in Europe, testing on European species was necessary and also required by the Irish National Parks and Wildlife Service (NPWS) prior to the Cairns Hill and Tullamore Harbour treatment in Ireland (Chapters 3 and 4). Another important aspect of these non target tests were to assess the effect on the endangered and protected crayfish species *Austropotamobius pallipes* and native molluscs (genus *Anodonta*) whose numbers have been directly reduced by the zebra mussel. In the case of *Anodonta* and other Unionidae, Zequanox has the potential to restore populations following treatment, which results in the removal of attached zebra mussels, thereby preventing mortality (Great Lakes Restoration Initiatives, 2013).

2.2.1 Guidelines for Ecotoxicology Testing

Guidelines for the testing of chemicals and biocides include those by the Organisation for Economic Cooperation and Development (OECD). The OECD guidelines relating to the non target organisms tested in this research are numbers 219 ‘Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment; 202 *Daphnia* sp.*,* ‘Acute Immobilisation Test, 203 ‘Fish, Acute Toxicity Test, and 235 *Chironomus* sp., ‘Acute Immobilisation Test’ (OECD). These guidelines layout the test

set up and recommended procedures, including the preparation of the test water and test organism, observations, data and reporting. In non target studies carried out by Molloy et al. (2013c) where *Pseudomonas fluorescens* CL145a (the active ingredient in Zequanox) was tested for effect, US EPA guidelines for 'Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms', were used (United States Environmental Protection Agency, 2002). This guideline document is a comprehensive document outlining all aspects of ecotoxicology testing on freshwater and marine organisms including health and safety, types of test, equipment and facilities and data handling. A technical manual for monitoring white-clawed crayfish in Irish lakes published in Irish Wildlife Manual 45 outlines the best survey methods for the crayfish including; preparation and location, capture methods and handling (Reynolds et al., 2010). All the guidelines listed here were consulted prior to testing, however as OECD guidelines were more species specific they are referenced throughout.

2.3 Objectives

The objectives of the non-target ecotoxicology studies were to:

- Further evaluate the target specificity of Zequanox (assessed previously in North America) with respect to brown trout (*Salmo trutta*) and macroinvertebrate species.
- Evaluate the effect of Zequanox on organisms protected in EU aquatic ecosystems.
- Assess the effect of Zequanox to water quality.
- Compare treatment with MBI- 401 FDP and MBI- 401 SDP and examine the difference in effect.

2.4 Materials and Methods

This chapter lays out general methods followed by species specific ones. The results and discussion section first comprises of a general presentation and discussion of the raw water quality recordings followed by a discussion of the statistical analysis of water quality. The individual mortalities for each species followed by statistical analysis of mortality is then presented and finally the conclusions. These non target studies consisted of 72 hour static renewal ecotoxicology testing, with treatments ranging from low to high concentrations, 50 mg/L - 900 mg a.i./L.

Nine organisms [eight macroinvertebrate and one vertebrate (*Salmo trutta*, brown trout)] were selected for non-target testing (Table 2.1).

Table 2.1 List of organisms for ecotoxicology testing

Chironomus sp.	Non-biting midge
Asellus aquaticus	Waterlouse
Ephemerella ignita	Mayfly
Mytilus edulis	Blue mussel
Anodonta sp.	Swan/duck mussel
Daphnia pulex	Water flea
Austropotatamobius pallipes	Crayfish
Lymnaea peregra	Common pond snail
Salmo trutta (parr)	Brown trout

2.4.1 General Procedures

Six out nine organisms were collected in the wild, with the exception of *Daphnia pulex*, *Salmo trutta* and the *Lymnaea peregra*. All except *Salmo trutta* were tested in the research lab at IT Sligo. All tests followed the same standard methodology, developed as part of this thesis research (a compilation of all relevant OECD guidelines);

- All organisms were allowed at least 24 hours to acclimatise in the lab before treatment began.
- All test chambers were exposed to test concentrations ranging from 50 mg a.i. /L. – 900 mg a.i./L. for 72 hours (Figure 2.1).
- Water and product was renewed every 12-24 hours depending on whether MBI-401 FDP or MBI-401 SDP was used. As with the use of MBI-401-FDP it was anticipated that 12 hour renewals would reduce unionised ammonia levels. In some instances, depending on the taxa size, only 90% of the water could be removed, as care was taken not to accidentally pour out the smaller taxa.
- Water quality parameters pH, temperature (°C), dissolved oxygen (DO), unionised ammonia (NH_3), and turbidity (NTU) were measured every 12-24 hours.

- For all taxa only one replicate per concentration was used to measure water quality.

- All water quality parameters measured were compared to three different guidelines and regulations. First of these was the European Communities (Quality of Salmonid Waters) Regulations, 1988 (Irish Statute Book, 1988 S.I. No. 293/1988). Salmonid waters are referenced here because they demand the highest quality of water to support game fish (freshwater). These stringent water quality regulations are considered a good proxy for water quality results observed in this study; results with levels close to Salmonid waters were considered as having no negative effect on test organisms in terms of water quality. Water quality parameters were also compared to the less stringent levels acceptable for Cyprinid waters (Freshwater Fish Directive (78/659/EEC)). The Freshwater Fish Directive (78/659/EEC) also contains regulations for Salmonid waters many of which overlap. 'Parameters of water quality: interpretation and standards' (EPA, 2001) provided both the European Communities (Quality of Salmonid Waters) Regulations and the Freshwater Fish Directive in a summarised form. Finally parameters recorded were compared to the OECD water quality guidelines; 219 'Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment', 202 *Daphnia* s*p.,* 'Acute Immobilisation Test' and 203 'Fish, Acute Toxicity Test'.

- The same validity criterion was applied to all tests taken from OECD guidelines.

- The 72 hour LC_{10}, LC_{50}, LC_{100} (lethal concentration, concentration at which 50% of the organisms are killed) or EC_{50} EC_{10} EC_{100} (effective concentration, concentration at which 50% of the organisms are immobilised) was calculated depending on the organism. Mortality can often be difficult to determine in certain organisms, therefore the effective concentration is used (determined by lack of response after gentle agitation).

- All data was recorded in the same way, on three separate sheets individually labelled; ITS/DC/000 (treatment concentration), ITS/OBS/000 (mortality

observations), ITS/WQ/000 (water quality parameters). Any additional observations were made in a lab notebook where the relevant data sheets were referenced

- After testing was completed statistical analysis took place using Excel and Minitab 16; the same analysis was applied to all tests where possible. Where mortality was observed a general linear model was applied to raw untransformed data to determine if concentration, exposure time, effected the organism's survival. Replication was also analysed to determine if there was a difference in mortality between replicates: if no difference was found it could it be eliminated, and the respective means used for post other analysis including graphs and ANOVAs. Throughout the text the reference of increasing treatment duration is referred to as "time". Raw untransformed data was used in separate two-way ANOVAs to determine whether the treatment concentration and treatment exposure or time had an effect (caused fluctuations) on water quality. Measurements taken every 12-24 hours (depending on water renewals) were used in order to examine the effect to water quality over time (Fowler et al., 1998). A *t*-test was used to examine if there was a statistically significant difference in the means of NH_3 recorded between treatment with FDP and SDP.

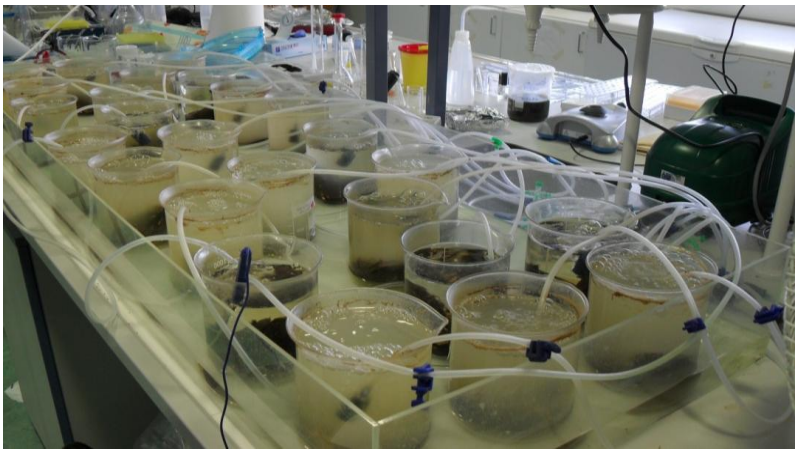

Figure 2.1 Test chambers containing *Anodonta* and zebra mussels during Zequanox treatment, in the lab at IT Sligo (photo by Sara Meehan).

2.4.2 Test Organism Origin

***Chironomus,* Meigen, 1803**

For FDP testing *Chironomus w*ere collected from Drumcliff River, Co. Sligo (54º32'71.36"N, 8º49'38.05"W) and for SDP testing *Chironomus plumosus* were collected from Lough Gill, Co. Sligo (54°15'16.91"N, 8°19'22.81"W) via kick sampling. After collection the samples were returned to the lab and sorted. The organisms selected were all swimming when taken from the sample container and placed in sample beakers. The sizes ranged between approximately 2 and 10mm.

***Asellus aquaticus,* (Linnaeus, 1758)**

The waterlouse used in this study were collected from Lough Gill, Co. Sligo (54°15'16.91"N, 8°19'22.81"W) via kick sampling. The samples collected were returned to the lab and sorted. The organisms selected were all swimming when taken from the sample container and placed in test chambers. The sizes ranged between approximately 2 and 10 mm.

***Ephemerella ignita*, (Poda, 1761)**

The mayfly used in this study for both FDP and SDP tests were collected from Drumcliff River, Co. Sligo (54º32'71.36"N, 8º49'38.05"W) via kick sampling. The samples collected were returned to the lab and sorted. The organisms selected were swimming and had all appendages attached when taken from sample container and placed in test chambers. The sizes ranged between approximately 2 and 8mm.

***Mytilus edulis*, Linnaeus, 1758**

Mytilus edulis were collected by hand from rocks on Ross beach, Killala, Co. Mayo (54º22'06.61"N, -9º22'07.48"W). The organisms selected all had either their shells closed or were filtering when placed in test chambers. The sizes ranged between approximately 6 and 45mm**.**

***Anodonta* sp., Lamarck, 1799**

Anodonta for testing were collected from Trinity Lake, Co. Cavan (53°59'45.6"N 7°27'57.3"W) via snorkelling. The organisms selected all either had their shells closed

or were filtering when placed in test chambers. The sizes ranged between approximately 51 and 162 mm**.**

***Daphnia pulex*, Leydig, 1860**

Daphnia pulex were purchased from Blades Biological Ltd in the UK. They were delivered in a knotted plastic bag and placed in a box with soft packaging. *Daphnia* were cultured in beakers where the young were separated from the adults daily using a pipette. The *Daphnia* were fed yeast and the water was changed three times a week. Organisms < 24 hours old were used for testing. Five organisms were used per test chamber.

***Austropotamobius pallipes*, (Lereboullet, 1858)**

Removal of this species for experimental purposes requires a licence, which was obtained by National Parks and Wildlife Services (NPWS). Crayfish were collected from Lough Owel, Co. Westmeath (53°55'52"N, -7°36'36"W) *via* trap use and hand picking (Figure 2.2). Two crayfish were placed into each of the test chambers, which were eight litre plastic tanks, filled with two litres of water. The crayfish were separated in each tank for the acclimatisation period and for the duration of testing by a piece of thin plastic with holes cut in the bottom to allow for water circulation (Figure 2.3). The organisms selected all had their appendages intact and were reactive to touch when placed in the test chambers. The sizes ranged between approximately 15 mm and 41 mm carapace length.

Figure 2.2 Crayfish in traps (photos by Sara Meehan).

Figure 2.3 Crayfish in test chambers

***Lymnaea peregra*, (Müller, 1774)**

Lymnaea peregra, used in this study were purchased from Blades Biological Ltd in the UK. They were delivered in a knotted plastic bag and placed in a box with soft packaging; the organisms were selected at random. However, when they were placed in the test chamber, each individual was monitored to make sure it was alive. When the snail's head and tentacles came out of the shell it was considered alive. The sizes ranged between approximately 7mm and 18mm shell length.

***Salmo trutta*, Linnaeus, 1758**

Salmo trutta, used in this study were purchased from Inland Fisheries Ireland's Roscrea fish farm and delivered to the Shannon Aquatic Toxicity Laboratory on May 31, 2012. Approximately 150 juvenile (0+) fish were placed in a 1000L tank of carbon filtered Shannon municipal supply water and acclimated for eleven days prior to testing (Figure 2.4).

Figure 2.4 *Salmo trutta* in 1000L tank for acclimatisation (photo by Sara Meehan).

2.4.3 Dilution water

Chironomus (FDP), *Asellus aquaticus* and *Ephemerella ignita* were kept in Drumcliff River water. The *Mytilus edulis* were kept in sea water from Rosses Point beach, Co. Sligo. *Chironomus* (SDP), *Anodonta, Daphnia pulex, Lymnaea peregra* were kept in Lough Gill water. *Austropotamobius pallipes* were kept in Lough Owel water and *Salmo trutta* were kept in carbon filtered municipal water. Test water was either collected fresh at the beginning of treatment or daily. Water quality parameters (pH, DO, NTU, NH_3 and °C) are presented in (Appendix B: Tables1-10).

The water in the test chambers was changed every day prior to treatment. For 24 hour renewals, the water was changed at approximately 8.30 am, water quality parameters were recorded prior to water change and after at approximately 4 pm. For 12 hour renewals the water was changed at approximately 7.30 am and 7.30 pm and water quality parameters were then recorded at 7.00 am and 7.00 pm (Appendix C: Tables1-11). Temperature, dissolved oxygen, pH and NH_3 were measured using an Orion 5 star plus meter and turbidity was measured using 2100N Hach Turbidity meter. For *Chironomus* (FDP), *Asellus aquaticus,* and *Ephemerella ignita* (FDP), unionized ammonia (NH_3) was measured using an aquarium ammonia test kit (API ^{TM} Aquarium pharmaceutical ammonia aquarium test strips). *Chironomus* (SDP), *Ephemerella ignita* (SDP), *Mytilus edulis, Anodonta, Daphnia pulex, Lymnaea peregra,* and *Austropotamobius pallipes* were measured using the Orion 5 star plus meter. For *Daphnia pulex,* water quality parameters were only measured as the water was being changed (every 24 hours) due to the small amount of water used in the test chambers. The water was poured into a separate beaker and parameters were recorded so that the *Daphnia pulex* would not be disturbed.

2.4.4 Determination of Test Concentrations

Small range finders were carried out on a number of species, to help determine the range of concentrations to be tested in the definitive test. For other species due to lack of samples range finders were not carried out and concentrations were based on previous studies or available information.

Chironomus

A range finder was carried out prior to testing with MBI-401 FDP using 200 mg a.i./L and 300 mg a.i./L so to better understand the tolerance of *Chironomus*. All organisms in the 200 mg a.i./L test survived, but only one individual survived in 300 mg a.i./L. 100, 200, 300, 400, 500 mg a.i./L and controls were analysed in the main test.

Asellus aquaticus

A range finder was not carried out for the waterlouse. The test concentrations were decided based on the results obtained from the *Chironomus* study as they inhabit similar conditions. Concentrations of 100, 200, 300, 400, 500 mg a.i./L and controls were analysed in the main test.

Ephemerella ignita

A range finder was carried out prior to FDP testing using 300 mg a.i./L, 400 mg a.i./L and 500 mg a.i./L. No mayfly survived in 300 mg a.i./L, two survived in 400 mg a.i./L and three survived in 500 mg a.i./L. As survival was zero in the lowest test concentration used it was assumed that outside influences caused such high mortality and not Zequanox. Concentrations of 100, 200, 300, 400 and 500 mg a.i./L and controls were analysed in the main test.

Mytilus edulis

A small 24 hour static renewal range finder was carried out prior to this test using 100 mg a.i./L, 200 mg a.i./L, 300 mg a.i./L and 400 mg a.i./L and a control all containing three organisms each. Mussels were treated at zero and 12 hours. All organisms in all concentrations survived. Concentrations of 200, 300, 400, 500, 600 mg a.i./L and controls were analysed in the main test.

Anodonta

In Ireland as a decline in *Anodonta* spp. has been noted due to zebra mussel invasion a range finder was not carried out (Byrne et al., 2009). The test concentrations were based on a previous test carried out by Marrone Bio Innovations on different species of unionids, where mussels were exposed to live cells of *Pseudomonas fluorescens* strain CL145A (bacterium making up MBI-401 FDP) at concentrations of 100 and 200 mg a.i./L (Molloy et al., 2013c). Concentrations of 100, 200, 300, 400, 500 mg a.i./L and controls were analysed in this test.

Daphnia pulex

A small 24 hour range finder was carried out prior to this test using 100 mg a.i./L, 150 mg a.i./L and 200 mg a.i./L with 5 *Daphnia pulex* per concentration. All organisms survived the range finder. Acute toxicity tests have previously been carried out by MBI on *Daphnia magna* so concentrations were also based on these results (Marrone Bio Innovations Ecotoxicology Studies, 2012). Concentrations of 50, 100, 150, 200, 250 mg a.i./L and controls were analysed in the main test.

Austropotamobius pallipes

A. pallipes is listed on the IUCN Red List as an endangered species (Füreder, 2010) and is also protected in Ireland under the Irish Wildlife Act 2000, therefore a range finder was not carried out for *A. pallipes* due to the small sample size gathered. The concentrations were determined on the basis that crayfish are known burrowers and can therefore withstand high turbidity levels (Holdich et al., 2006). Concentrations of 350, 450, 550, 650, 750 mg a.i./L and controls were analysed in the main test.

Lymnaea peregra

A small 24 hour range finder was carried out prior to this test using 600 mg a.i./L, 700 mg a.i./L and 800 mg a.i./L with four snails per concentration. All organisms survived the range finder. Concentrations of 500, 600, 700, 800 and 900 mg/L and controls were analysed in the main test.

Salmo trutta

The concentrations tested were based on previous tests carried out by Still Meadow labs for Marrone Bio Innovations using rainbow trout. Concentrations of 180, 100, 56, 32 and 18 mg a.i./L and controls were analysed in the main test (Figure 2.5).

Figure 2.5 *Salmo trutta* in test chambers at Shannon aquatic lab (photo by Sara Meehan).

2.4.5 Preparation of Test Concentrations

An 8 g/L concentrated product solution was made up for testing: this was done by weighing out the appropriate amount of Zequanox depending on the treatment concentration and mixing with sample water. This was stirred on a magnetic stir plate until the product was thoroughly and entirely mixed. It is important to note that MBI-401 SDP is a 50% active substance (a.i.) unlike MBI-401 FDP, which is a 100% active substance therefore concentrations of Zequanox used for MBI-401 SDP testing was in effect double that used for MBI-401 FDP testing.

2.4.6 Test Conditions

Table 2.2 Test conditions for each organism (all test conditions are representative of readings taken every 24 hours before product renewal)

	***Chironomus* FDP**	***Chironomus plumosus* SDP**	***Asellus aquaticus* FDP**	***Ephemerella ignita* FDP**
Test Procedure	semi-static 24 hour renewal test	semi-static 24 hour renewal test	semi-static 24 hour renewal test	semi-static 12 hour renewal test
Duration (Hours)	72	72	72	72
Temperature (°C)	18.1-19.9	14.7-16.8	18.6-20.8	17.8-20.0
Dissolved Oxygen (mg/L)	5.87-8.56	7.93-9.85	3.27-9.20	4.56-9.96
pH Value	7.88-8.37	7.81-9.64	7.34-8.60	6.94-8.74
Turbidity (NTU)	6.47-129	16.9-102	5.34-244	28.2-173
Ammonia (NH_3) (mg/L):	0-3.0	0.003-5.682	0-2.4	0.03-7.54
Exposure to light	8 light/16 dark hours	8 light/16 dark hours	8 light/16 dark hours	8 light/16 dark hours
Feeding	None	None	None	None
Test vessels	1000ml beakers - 500ml water	1000ml beakers - 500ml water	1000ml beakers - 500ml water	1000ml beakers - 500ml water
Aeration	Koi air pump with bubblers	Koi air pump with bubblers	Koi air pump with bubblers	Koi air pump with bubblers
Replication	3 replicates - 5 controls	3 replicates - 5 controls	3 replicates - 5 controls	3 replicates - 5 controls

Table 2.2 Test conditions for each organism (all test conditions are representative of readings taken every 24 hours before product renewal) (contd.)

	***Ephemerella ignita* SDP**	***Mytilus edulis* FDP**	***Anodonta* FDP**	***Daphnia pulex* SDP**
Test Procedure	semi-static 24 hour renewal test	semi-static 12 hour renewal test	semi-static 12 hour renewal test	semi-static 24 hour renewal test
Duration (Hours)	72	72	72	72
Temperature (°C)	15.6-17.2	15.1-17.6	16.3-18.1°C	18.4-19.7
Dissolved Oxygen (mg/L)	4.69-9.54	6.68-8.96	7.63-9.4	2.12-8.39
pH Value	7.55-8.4	7.63-8.43	8.0-8.66	6.63-7.93
Turbidity (NTU)	19.2-187	47.6-770	17.8-148	28.9-632
Ammonia (NH_3) (mg/L):	0.0-0.719	1.38-16.2	0.23-7.25	0.0022-0.138
Exposure to light	8 light/16 dark hours	8 light/16 dark hours	8 light/16 dark hours	8 light/16 dark hours
Feeding	None	None	None	None
Test vessels	1000ml beakers - 500ml water	1000ml beakers - 800ml water	1000ml beakers - 800ml water	1000ml beakers -200ml water
Aeration	Koi air pump with bubblers	Koi air pump with bubblers	Koi air pump with bubblers	Koi air pump with bubblers
Replication	3 replicates - 5 controls	3 replicates - 5 controls	3 replicates - 5 controls	3 replicates - 5 controls

Table 2.2 Test conditions for each organism (all test conditions are representative of readings taken every 24 hours before product renewal) (contd.)

	***Austropotamobius pallipes* SDP**	***Lymnaea peregra* SDP**	***Salmo trutta* SDP**
Test Procedure	semi-static 24 hour renewal test	semi-static 24 hour renewal test	semi-static 24 hour renewal test
Duration (Hours)	72	72	72
Temperature (°C)	14.7-17.4	18.3 - 23.2	14.2±14.9
Dissolved Oxygen (mg/L)	4.01-9.92	6.28 - 9.05	7.8-10.3
pH Value	7.15-8.0	7.17 - 8.02	7.6 - 8.1
Turbidity (NTU)	79.8-231	53.9 – 185	6.0 – 68
Ammonia (NH_3) (mg/L):	0.168-3.06	0.033 - 0.107	0.03 - 0.027 (NH_4)*
Exposure to light	8 light/16 dark hours	8 light/16 dark hours	16 light/8 dark hours
Feeding	None	None	None
Test vessels	8L tanks - 1.5L water	1000 mL - 500 mL water	25L glass aquaria - 20L water
Aeration	Koi air pump with bubblers	Koi air pump with bubblers	air pump with bubblers
Replication	3 replicates - 5 controls	3 replicates - 5 controls	2 replicates - 2 controls

* NH_4 was measured at Shannon Aquatic Toxicity Labs instead of NH_3

2.4.7 Test Procedures

After organisms were collected, on the same day they were moved from sample buckets and trays to test chambers using either a pasteur pipette, a paint brush or by hand. The amount of organisms varied between two and eight (Table 2.3). Each taxa except *Salmo trutta* and *Austropotamobius pallipes* involved the use of five control chambers (Table 2.2). In most cases species were acclimatised in test chambers and any dead or compromised individuals were replaced prior to the first round of treatment. According to OECD guidelines all organisms were acclimatised prior to treatment. *Chironomus, Ephemerella ignita, Mytilus edulis, Lymnaea peregra* and *Asellus aquaticus* were acclimatised overnight*; Salmo trutta* were acclimatised for eleven days in an 1000 L flow through tank; *Anodonta* were acclimatised for 13 days in two litre tanks (Figure 2.6), *Austropotamobius pallipes* were acclimatised for 48 hours and the *Daphnia* were cultured in beakers prior to testing and neonates < 24 hours old were used.

The water was changed daily before treatment. This was executed by pouring off water into the laboratory sink, very slowly, to stop organisms from falling out. For smaller organisms like *Ephemerella ignita* and *Chironomus* a spoon was placed at the top of the beaker to prevent them from falling out and for *Daphnia* due to their size they were removed for water renewal. Some of the discarded water was collected in a separate beaker and used to measure turbidity. Fresh river/lake/sea water was then slowly poured down the side of the beaker in order to cause minimal disturbance to the organisms. Any individuals that were removed were put on a petri dish for further inspection to confirm mortality/immobility and make any additional observations. When necessary, ice packs and fans were used to reduce and hence control the temperature of the test beakers. Temperatures were tailored to the individual organism depending on OECD guidelines and the optimum temperature recommended for their survival. It must be noted that *Ephemerella ignita* were continuously moulting during the trial and all exuviae were removed and checked.

Figure 2.6 *Anodonta* in test chamber, (photo by Sara Meehan).

Table 2.3 Number of organisms per taxa, per test chamber

Taxa	Number per test chamber
Chironomus	6
Asellus aquaticus	5
Ephemerella ignita	5
Mytilus edulis	8
Anodonta	2
Daphnia pulex	8
Austropotamobius pallipes	2
Lymnaea peregra	5
Salmo trutta	5

2.4.8 Validity of the Test

In accordance with OECD guidelines 219 'Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment', 202 *Daphnia* sp., 'Acute Immobilisation Test' and 203 'Fish, Acute Toxicity Test' for the test to be valid there should be;

— No more than 10% mortality in the control

— The dissolved oxygen should be ≥6 mg/L in the control and test vessels

— The pH should be between 6-9 in all test vessels

— The water temperature should not differ more than ±1.0°C

For *Daphnia* all the validity criteria bar the DO levels were applied. According to OECD guidelines 202 the *Daphnia* test should not being aerated and therefore DO must be >3 mg/L. The validity criteria were based on water quality parameters recorded every 24 hours, after treatment and before water refreshment.

2.5 Results and Discussion

2.5.1 Water Quality

2.5.1.1 Water Quality Parameters (Raw Data Comparisons)

Table 2.4 displays the upper and lower limits of water quality parameters recorded in treated chambers and compares this to the limits set out by the European Communities (Quality of Salmonid Waters) Regulations, 1988, the Freshwater Fish Directive (78/659/EEC) (Cyprinid and Salmonid waters) and OECD guidelines 202, 203, 219. The water quality parameters reported here are indicative of every 12 or 24 hours after treatment and includes all treatment concentrations (50-900 mg/L), all other parameters recorded including the controls and parameters recorded after treatment are reported in Appendix C. It is very important to note, that the water quality limits set out for Salmonid and Cyprinid waters are intended for the purpose of lakes and rivers and not saltwater or small test chambers. As Zequanox is also intended for use in open water these regulations serve as an interesting proxy, however in most cases the NH_3 recorded will fall outside of these limits as testing was over a 72 hour period and test chambers mostly contained only 500 ml of water. OECD guidelines are designed for ecotoxicology testing and give a better idea of appropriate water quality parameters in order to keep organisms healthy.

There are no OECD guidelines for NH_3 levels during testing, there are however, Salmonid and Cyprinid limits (European Communities, Quality of Salmonid Waters, Regulations, 1988 and the Freshwater Fish Directive, 78/659/EEC), which state that NH_3 must stay below 0.02 mg/L and 0.025 mg/L respectively. As testing was carried out over a 72 hour period unionised ammonia levels were often >0.025 mg/L. The two way ANOVAs discussed below will examine the relationship between NH_3 and test

concentration and the effect to NH_3 due to the passing of time. For all species tested, NH_3 levels exceeded 0.025 mg/l (Table 2.4). For many organisms NH_3 in the control jars was also above acceptable limits for Salmonid waters; NH_3 is a product of excretion and when keeping organisms in small static environments it becomes elevated (Burrows, 1964; Eddy, 2005). No great mortalities were noted at low concentrations to any of the organisms other than *S. trutta;* this species is susceptible to ammonia especially when under stress (Eddy, 2005). Therefore, due to the low mortality (0-1 death) at low concentrations (100-200 mg/L), with the exception of *S. trutta,* it can be said that NH_3 did not contribute to any mortalities at concentrations required for >80% zebra mussel kill.

pH recorded for all test organisms was between 6-9 and therefore fell within all three guidelines/ regulations. Dissolved oxygen concentrations for *Anodonta, S. trutta* and *Chironomus plumosus* SDP was above Salmonid water quality regulations (European Communities, Quality of Salmonid Waters, Regulations, 1988, Freshwater Fish Directive 78/659/EEC), which state that DO must exceed 7 mg/l. *Chironomus* FDP, *M. edulis, and L. peregra* DO levels were above those required for Cyprinid waters (Freshwater Fish Directive, 78/659/EEC) (>5 mg/L) and OECD guidelines (>6 mg/L). Those that fell below the recommended guidelines were for tests on *A. aquaticus, E. ignita* FDP, *E. ignita* SDP, *Daphnia* and *A. pallipes.* For *A. aquaticus* DO ranged between 4.30 and 8.61 mg/L this is close to the cyprinid regulations of 5 mg/L. On average, DO was 7.19 mg/L which is greater than the Salmonid water quality parameters. For *Ephemerella ignita* FDP/SDP the drop in oxygen concentration during testing occurred only in the higher treatment concentrations of 400 mg/L and 500 mg/L, which is not representative of a real time application. Oxygen concentration for the *Daphnia* test dropped below that of the Salmonid water quality limits; this occurred in the higher concentrations treated as there was no aeration; therefore this was expected due to the high turbidity and the natural degradation of the product. For *A. pallipes*, the oxygen concentration drop occurred only on two occasions once at 650 mg a.i./L at 48 hours, no mortalities occurred here so it is considered insignificant. The other drop in DO was at 48 hours at 450 mg a.i./L, with one small crayfish mortality (18mm carapace length), this low DO level may have only partly contributed to this; as mortality did not occur in higher concentrations it is unlikely Zequanox was the sole cause.

According to Salmonid and Cyprinid water quality regulations (European Communities, Quality of Salmonid Waters, Regulations, 1988 and the Freshwater Fish Directive, 78/659/EEC) temperature must stay below 21.5 °C, downstream of a thermal discharge point or must not exceed the unaffected temperature by 1.5°C. As this was a lab trial it was not possible to make an exact comparison, but all temperatures were <21.5°C. Temperature fluctuations were within OECD guidelines (±1°C) for *Chironomus* FDP, *Ephemerella ignita* SDP, *Anodonta, Daphnia*, and *S. trutta*, those that fluctuated more are addressed individually under the validation heading for each species and for the most part fluctuations were minor.

There are no Salmonid or Cyprinid water quality regulations for turbidity. The turbidity increases with the concentration of treatment, therefore the high turbidity seen would not be present in a real time application as only a 200 mg a.i./L or less treatment is required to get a >80% zebra mussel kill (Meehan et al., 2013; Meehan et al., 2014b). The only organism affected by high mortality at low treatment concentrations was *S. trutta* therefore it is necessary to examine turbidity as a potential cause. *Salmo salar* is another common fish species in Ireland, which like *S. trutta* plays a premier role in Irelands angling tourism. High turbidity has been noted by Bash et al. (2001) to be fatal to *S. salar* at high concentrations; whilst at lower levels it can affect their foraging capabilities, resistance to disease and can increase stress. According to Sweka and Hartman (2001) increased turbidity does decrease growth rates of brook trout (*Salvelinus fontinalis*) however it does not affect their ability to find and consume prey. This demonstrates that these trout do have the ability to move away from their highly turbid environments in search of more favourable conditions. Even though the mortality rates here were higher than the other organisms tested it must be remembered that in a real application to target zebra mussel control, only an eight hour treatment is required.

2.5.1.2 Statistical Analysis of Water Quality (Two-Way ANOVA)

Prior to ANOVA and GLM testing, the data was first checked for normality and a Bartletts test was applied to determine if variances were equal. If data was not normal or variances were not equal data was log transformed. Raw untransformed data was used for all ANOVA and GLM tests. Table 2.5 displays a summary of all the Anova results. The two way ANOVA tests applied show that NH_3 measurements were affected and fluctuated with the passing of time. NH_3 was not often affected by concentration (Table

2.5). As Zequanox degrades over each 12-24 hour period it can cause elevated levels of ammonia as evident from past ecotoxicology tests carried out by MBI (MBI pers. comm.). The organism most affected by Zequanox treatment was *S. trutta*; trout species along with most fish species are known to be sensitive to NH_3 fluctuations (Randall and Tsui, 2002). Coupled with this, fish themselves produce NH_3 from metabolic waste and urea excretion across the gills. For this test the fish were starved prior to treatment therefore it is likely that the majority of ammonia came from the fish as a by product of protein metabolism (which is excreted via the gills) and not faecal ammonia (Van Waarde, 1983; Wright et al., 1993; Randall and Tsui, 2002; Eddy, 2005; Ogbonna and Chinomso, 2010; United States Environmental Protection Agency, 2013). Where pH and temperature levels rise, ammonia production is also increased. Furthermore when fish are stressed NH_3 levels are amplified. In this non target testing the fish would have been stressed due to the high turbidity present and their removal from a large acclimatising tank to a smaller test tank followed by their immediate treatment (Wright et al., 1993; Bash et al., 2001; Randall and Tsui, 2002; Eddy, 2005; Ogbonna and Chinomso, 2010; United States Environmental Protection Agency, 2013). In addition to the fish causing an increase to NH_3 levels it is suspected that residual Zequanox build up in chambers (in some instances, depending on the organism it was not possible to remove all the water) caused NH_3 fluctuations. Even though levels were lower with the use of the SDP formulation, NH_3 fluctuations still occurred.

The temperature of the test chambers was not affected by concentration, in all but one case (*Salmo trutta),* but was affected by the passing of time. As the chambers were in ambient lab temperatures it was expected that temperature would be subject to a certain amount of fluctuation over time. These fluctuations were small and did not affect mortality of the organisms. In the case of *S. trutta*, the temperature fluctuated between 14.2-14.8 ºC, a minor fluctuation and within the normal range for distribution of this fish (Elliot, 1975).

Dissolved oxygen, in most cases was affected by concentration and the passing of time. The degradation of Zequanox over time causes the biochemical oxygen demand (BOD) to rise and the dissolved oxygen levels to drop (Meehan et al., 2014b). Therefore, the higher the test concentration, the greater the BOD, and consequently the lower the DO. As Zequanox is made up of primarily particulate organic matter its breakdown in water is inevitable, however in test chambers the effects to BOD and DO are elevated due to

the small amount of test water, this is not representative of what would happen in a real time treatment. In Chapter 4 (Tullamore Harbour trial) after the treatment period was over the curtains were removed and Zequanox dissipated to almost undetectable levels, meaning that the BOD would be lower and DO levels would remain high.

Turbidity is directly correlated with Zequanox concentration, this is evident in Figure 2.1 where the test chambers are 'milky' in appearance, the varying degree of concentrated 'milkyness' shows different product concentrations. Therefore turbidity is affected by test concentration i.e. the higher the test concentration the higher the turbidity. Over each 24 hour test period (which includes the time from treatment to the time prior to water and product renewal (24 hours)) degradation of Zequanox occurs, this is evident from the turbidity data (Appendix: C, 24 hour renewals). Therefore the two way ANOVA tests applied examines only the fluctuation in the measurements taken every 12-24 hours (before the product was renewed) and assesses if, over time, there is a fluctuation in these turbidity measurements or if the different treatment concentrations causes a fluctuation.

Turbidity fluctuated with the passing of time in all taxa except *Chironomus* FDP, *Daphnia, Anodonta* and *Mytilus edulis.* These results show that for *Chironomus plumosus* SDP, *A. aquaticus*, *Emphemeralla ignita* (FDP/SDP), *A. pallipes, L. peregra,* and *S. trutta* that there was a statistically significant fluctuation in turbidity between measurements. For *Daphnia, Anodonta* and *Mytilus edulis* it was possible to fully remove all the water during product renewal either by pouring off or removing the organism, thus removing most of the Zequanox present in the test chambers and reducing residual build up over the course of the experiment. This may account for the turbidity fluctuation in those organisms where it was not possible to remove all the water.

Finally pH was affected evenly for the most part, by time and concentration, as temperature was affected mainly due to the passing of time it was expected that pH would be subject to fluctuations due to the direct relationship between pH and temperature. The individual results are stated under each organism heading.

The comparison of water quality across MBI-401 FDP and SDP shows that temperature and pH were within acceptable levels according to Salmonid water quality limits. Reported NH_3 was higher for MBI-401 FDP than SDP except for two measurements

after 24 hours for *Chironomus*, here NH_3 at 200 and 300 mg/L reached 4.21 and 5.68 mg/L, these were two isolated recordings and NH_3 remained <1.7 mg/L for all other MBI-401 SDP measurements. The *t*-test test applied took into consideration NH_3 readings at all treatment concentrations. It was found that there is a statistically significant difference in NH_3 readings between *Ephemerella ignita* FDP and SDP and no statistically significant difference in NH_3 readings between *Chironomus* FDP and SDP ($p > 0.05$ for *Chironomus* and $p < 0.05$ for *E. ignita*). It is suspected that the reduction in NH_3 with MBI-401 SDP played a role in reducing mortality in the SDP studies (MBI pers. comm.).

Table 2.4 Upper and lower water quality parameters recorded before treatment every 12-24 hours dependent on species. Limits for Salmonid Water Quality and Cyprinid water quality are also given.

		DO (mg/L)	pH	Temperature °C	Unionized ammonia (NH_3)(mg/L)	Turbidity (NTU)
Salmonid Water Quality Limits (S.I. No. 293/1988)		**>7**	**6-9**	**<21.5**	**<0.02**	**N/A**
Freshwater Fish Directive (78/659/EEC)	**Cyprinid Water Quality Limits**	**>5**	**6-9**	**<21.5**	**<0.025**	**N/A**
	Salmonid water Quality Limits	**>7**	**6-9**	**<21.5**	**<0.025**	**N/A**
OECD Validity Criteria (202, 203, 219)		**>6**	**6-9**	**±1°C**	**N/A**	**N/A**
Chironomus FDP		5.87 - 8.56	7.4 - 8.4	18.1-19.9	0-3	6.47 - 129
Chironomus plumosus SDP		7.93-9.85	7.81-9.64	14.7-16.8	0.003-5.682	16.9 - 102
Asellus aquaticus FDP		4.30 - 8.61	7.34-8.60	18.6-20.8	0-2.4	5.34-244
Ephemerella ignita FDP		4.56-9.96	6.94-8.74	17.8-20.0	0.03-7.54	28.2-173
Ephemerella ignita SDP		4.69-9.54	7.55-8.4	15.6-17.2	0.0-0.719	19.2-187
Mytilus edulis FDP		6.68-8.96	7.63-8.43	15.1-17.6	1.38-16.2	47.6-770
Anodonta FDP		7.63-9.4	8.0-8.66	16.3-18.1	0.23-7.25	17.8-148
Daphnia pulex SDP*		2.12-8.39	6.63-7.93	18.4-19.7	0.0022-0.138	28.9-232
Austropotamobius pallipes SDP		4.01-9.92	7.15-8.0	14.7-17.4	0.168-3.06	79.8-231
Lymnaea peregra SDP		6.28 - 9.05	7.17-8.02	18.3-23.2	0.033-0.107	53.9 – 185
Salmo trutta SDP**		7.8-10.3	7.6 - 8.1	14.2-14.9	0.03-0.027 (NH_4)	6.0 – 68

*not aerated

** parameters recorded differently by Shannon Aquatic Toxicity Lab

Table 2.5 Summary of Anova's on water quality parameters for non target studies*

Water Quality	**Temperature**		**Dissolved Oxygen**		**pH**		**Turbidity**		**NH_3**	
Species Tested	Time	Concentration	Time	Concentration	Time	Concentration	Time	Concentration	Time	Concentration
Chironomus FDP	X	X	X	X	X	X	X	X	✓	X
Chironomus SDP	✓	X	✓	X	✓	X	✓	✓	X	X
Asellus aquaticus	X	X	✓	✓	✓	X	✓	✓	✓	X
Ephemerella ignita FDP	✓	X	✓	✓	✓	✓	✓	✓	✓	✓
Ephemerella ignita SDP	✓	X	X	X	X	X	✓	✓	X	X
Mytilus edulis	✓	X	X	✓	✓	X	X	X	✓	✓
Anodonta	✓	X	✓	✓	X	X	X	✓	✓	✓
Daphnia pulex	✓	X	X	X	X	✓	X	X	✓	X
Austropotamobius pallipes	✓	X	X	X	X	X	X	X	✓	X
Lymnaea peregra	✓	X	X	X	X	X	✓	✓	✓	X
Salmo trutta	X	✓	✓	X	✓	X	✓	✓	N/A	N/A

*✓ = effect; X = no effect

2.5.2 *Ecotoxicology*

2.5.2.1 *Chironomus*

Chironomus were exposed to both MBI-401 FDP and MBI-401 SDP in a 72 hour static renewal toxicity test at concentrations of 100, 200, 300, 400, 500 mg a.i./L. MBI-401 FDP testing was carried out on July 5, 2011 and MBI-401 SDP testing was carried out on February 21, 2012 as part of a bridging study in the transition from the use of MBI-401 FDP to MBI-401 SDP. Immobilisation of *Chironomus* was determined by the lack of response after gentle agitation. Endpoints are reported as $LC_{50,}$ $LC_{10,}$ and LC_{100} after 72 hours.

Mortalities

Using averages, one mortality occurred in the control (3%) after 72 hours exposure to MBI-401 FDP. No mortalities were observed in 100 mg a.i./L, two mortalities were observed in 200 mg a.i./L (11%), three mortalities were observed in 300 mg a.i./L (17%), 15 mortalities were observed in 400 mg a.i./L (83%) and 17 mortalities were observed in 500 mg a.i./L (94%) (Figure 2.7 and Table 2.6).

Table 2.6 Mean number of live individuals (3 treated replicates, 5 control replicates) and % mortality of *Chironomous* after 72 hours exposure to MBI-401 FDP.

Time	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L	Control
24	6.0	6.0	6.0	6.0	5.0	6.0
48	6.0	6.0	5.0	5.0	5.0	6.0
72	6.0	5.3	5.0	1.0	0.3	5.8
% Mortality	0	11	17	83	94	3

Using averages, two mortalities occurred in the control (7%) after 72 hours exposure to MBI-401 SDP. One mortality was observed in 100 mg a.i./L (12%), one mortality was observed in 200 mg a.i./L (12%), three mortalities were observed in 300 mg a.i./L (17%), four mortalities were observed in 400 mg a.i./L (22%) and five mortalities were observed in 500 mg a.i./L (28%) (Figure 2.8 and Table 2.7).

Percentage mortality was significantly higher with the use of FDP over SDP. With SDP there was no significant increase in mortality between the concentrations with 100 mg

a.i./L having 12% mortality and 500 mg a.i./L having 28% mortality. When this is compared with FDP there is a high difference with a jump of 0% to 94% mortality between 100 mg a.i./L and 500 mg a.i./L showing that FDP is more harmful to *Chironomus* than SDP.

Table 2.7 Mean number of live individuals (3 treated replicates, 5 control replicates) and % mortality of *Chironomus plumosus* after 72 hours exposure to MBI-401 SDP.

Time	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L	Control
24	6.0	6.0	5.3	6.0	6.0	6.0
48	6.0	5.3	5.0	5.3	5.0	5.8
72	5.3	5.3	5.0	4.7	4.3	5.6
% Mortality	12	12	17	22	28	7

Statistical Evaluation

FDP

The GLM results (Appendix D Table 1) indicate that the five concentrations were confirmed to have an effect on organism survival. Time was also shown to effect organism survival. There was no significant difference in mortality between replicates.

The results from separate two-way ANOVAs showed time had no effect on temperature, dissolved oxygen, pH and turbidity. There was an effect to ammonia due to time. Concentration had no effect to temperature, dissolved oxygen, pH and NH_3 (Appendix D Table 2).

SDP

GLM results confirm that concentration did not effect on organism survival, time however did effect organism survival. There was no significant difference in mortality between replicates. The lower mortality in this test compared to the FDP test accounts for concentration not affecting mortality. The mortality was not deemed statistically significant (Appendix D Table 3).

The results from separate two-way ANOVA tests (Appendix D Table 4) indicate that concentration had no effect on temperature, DO, pH, and NH_3. Temperature, DO, pH and turbidity were affected by time, but did not affect NH_3.

Validity of the Results

According to OECD 219 guidelines 'Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment' this study can be regarded as valid;

— In the control not more than 10% of the *Chironomus* died

— The dissolved oxygen did drop below 6 mg/L for the FDP test however only by 0.13 mg/L and therefore is considered only a minor deviation and did not affect the scientific validity of the test.

— The pH was between 6-9 in all test vessels

— The water temperature did differ by more than ±1.0°C however only by an extra 0.8°C for the FDP and 0.1°C for the SDP. This is considered only a minor deviation and did not affect the scientific validity of the test.

Conclusion

This test indicated that based on nominal concentrations the LC_{50} of MB1-401 FDP to *Chironomus* was 325 mg a.i./L and the estimated LC_{50} of MB1-401 SDP was 1074.86 mg a.i./L. Based on these results MBI-401 SDP is less toxic than MBI-401 FDP. Results from toxicity testing with MBI-401 FDP can be assumed "worst case." MBI-401 SDP is more suitable for zebra mussel control as it has less of an effect on *Chironomus* than MBI-401 FDP. These results coupled with zebra mussel control trials (Meehan et al., 2013 and Meehan et al., 2014b) show that the optimal concentration for controlling zebra mussels (approximately 100 mg a.i./L, with a maximum concentration of 200 mg a.i./L) would have minimal to no impact on *Chironomus*.

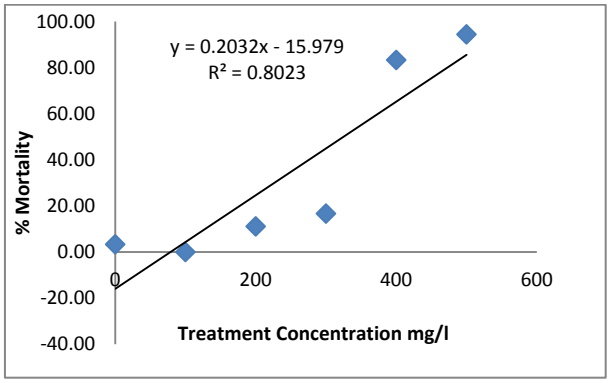

Figure 2.7 Concentration response graph for *Chironomus* after 72 hours exposure to MBI-401 FDP.

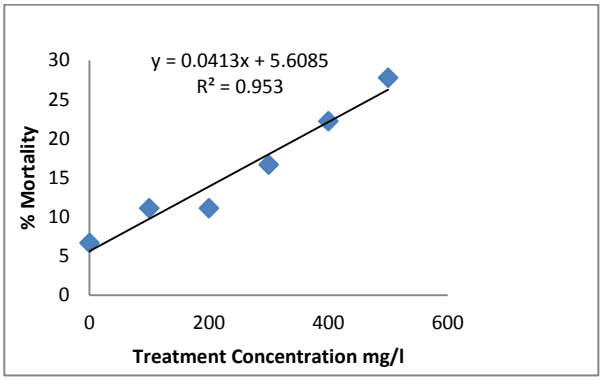

Figure 2.8 Concentration response graph for *Chironomus plumosus* after 72 hours exposure to MBI-401 SDP.

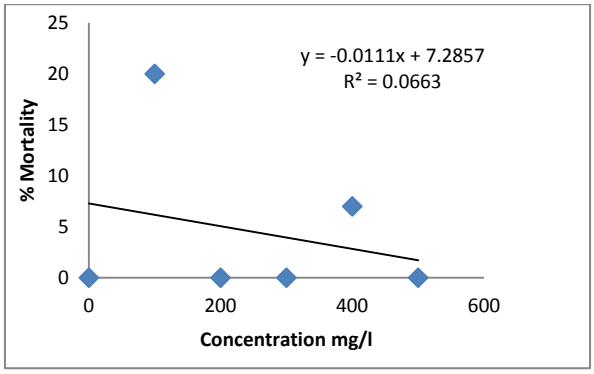

Figure 2.9 Concentration response graph for *Asellus aquaticus* after 72 hours exposure to MBI-401 FDP.

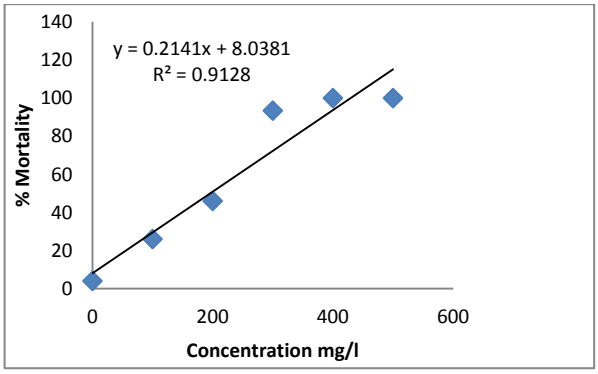

Figure 2.10 Concentration response graph for *Ephemerella ignita* after 72 hours exposure to MBI-401 FDP.

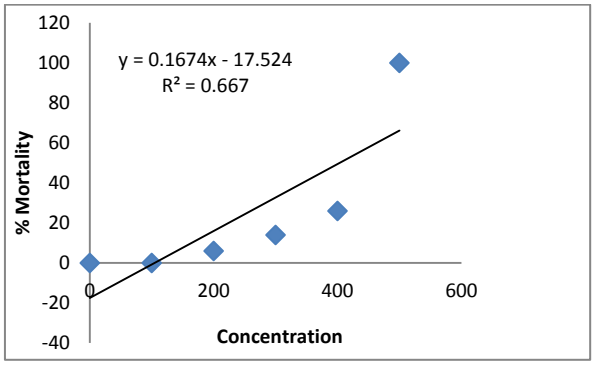

Figure 2.11 Concentration response graph for *Ephemerella ignita* after 72 hours exposure to MBI-401 FDP

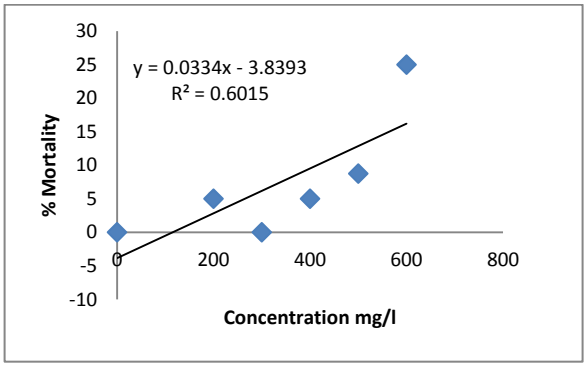

Figure 2.12 Concentration response graph for *Mytilus edulis* after 72 hours exposure to MBI-401 FDP.

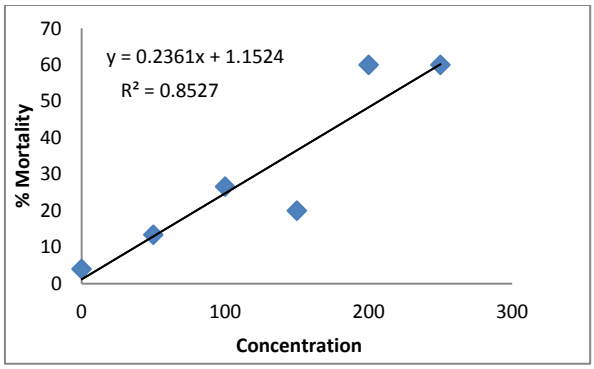

Figure 2.13 Concentration response graph for *Daphnia* after 72 hours exposure to MBI-401 SDP.

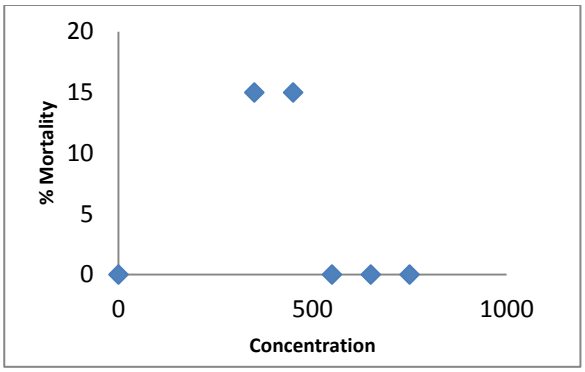

Figure 2.14 Concentration response graph for *Austropotamobius pallipes* after 72 hours exposure to MBI-401 SDP.

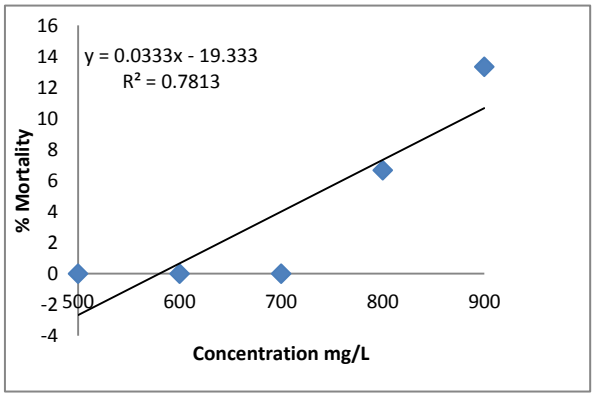

Figure 2.15 Concentration response graph for *Lymnaea peregra* after 72 hours exposure to MBI-401 SDP.

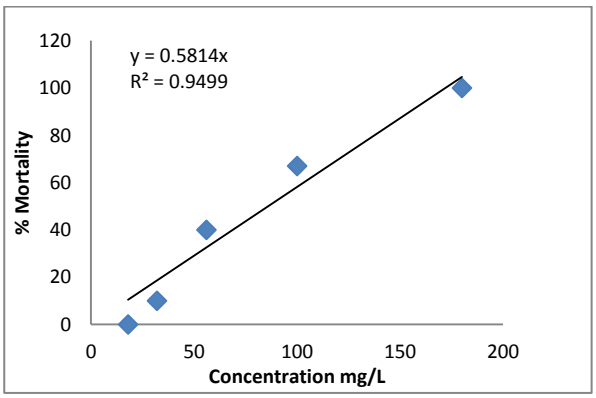

Figure 2.16 Concentration response graph for *Salmo trutta* after 72 hours exposure to MBI-401 SDP.

2.5.2.2 *Asellus aquaticus*

The test substance, MBI-401 FDP, was assessed on *Asellus aquaticus*, (July 26, 2011) exposed in a 72 hour static renewal toxicity test at concentrations of 100, 200, 300, 400 and 500 mg a.i./L. Immobilisation was determined by the lack of response from the waterlouse after gentle agitation. Endpoints are reported as EC_{50}, EC_{10} and EC_{100} after 72 hours.

Mortalities

Using averages, no mortality occurred in the control after 72 hours exposure. Three mortalities were observed in 100 mg a.i./L (20%), no mortalities were observed in 200 mg a.i./L, no mortalities were observed in 300 mg a.i./L, one mortality was observed in 400 mg a.i./L (8%) and no mortalities were observed in 500 mg a.i./L (Figure 2.9 and Table 2.8).

As the largest amount of mortality occurred in the lowest test concentration 100 mg a.i./L and no mortalities occurred in the highest concentration of 500 mg a.i./L it is evident that mortalities in 100 mg a.i./L were not due to the product concentration. For 100 mg/L all the mortalities occurred in one test chamber at the 72 hour mark. All water quality parameters at this point were within the limits required for Salmonid waters. Possibly there was an underlying disease/pathogen among the waterlouse in this particular test chamber or the result may simply be down to natural variation (United States Environmental Protection Agency, 2001).

Table 2.8 Mean number of live individuals (3 treated replicates, 5 control replicates) and % mortality of *Asellus aquaticus* after 72 hours exposure to MBI-401 FDP.

Time	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L	Control
24	5.0	5.0	5.0	5.0	5.0	5.0
48	4.0	5.0	5.0	5.0	5.0	5.0
72	4.0	5.0	5.0	4.6	5.0	5.0
% Mortality	20	0	0	8	0	0

Statistical Evaluation

GLM results confirmed that concentration did have an effect on organism survival. Time was shown to have no effect on organism survival (Appendix D Table 5). The mortality in this test was random as only one mortality occurred in all replicates at 400 mg/L and three occurred in one test chamber at 100 mg/L. It was expected that the general linear model would show no effect to mortality due to concentration and time because of the low mortality. It is recommended that a repeat test is carried out. There was no significant difference in mortality between replicates.

The results from separate two-way ANOVAs indicate that time and concentration had no effect on temperature. There was an effect on dissolved oxygen due to time and concentration. pH and ammonia fluctuated due to time but not concentration. Here turbidity fluctuated over time (Appendix D Table 6).

Validity of the Results

According to OECD 219 guidelines 'Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment', 202 *Daphnia* sp., 'Acute Immobilisation Test' and 203 'Fish, Acute Toxicity Test' this study can be regarded as valid because;

- In the control not more than 10% of the waterlouse died
- The dissolved oxygen was >6 mg/L in the control and test vessels
- The pH was between 6-9 in all test vessels
- The water temperature did differ by more than ±1.0°C however only by an extra 0.2°C. This is considered only a minor deviation and did not affect the scientific validity of the test.

Conclusion

This study indicated that based on nominal concentrations the estimated EC_{50} of MBI-401 FDP to *Asellus aquaticus* was 3,883 mg a.i./L, the estimated EC_{10} was 247.34 mg a.i./L and the estimated EC_{100} was 8,352 mg a.i./L. This study showed that at 200, 300 and 500 mg/l there were no negative effects to waterlouse, as mortality occurred at 100 mg/l and 400 mg/l it is recommended that these tests are repeated to further confirm that Zequanox is non toxic to *Asellus aquatics* at low concentrations.

2.5.2.3 *Ephemerella ignita*

Ephemerella ignita were exposed to MBI-401 FDP and MBI-401 SDP in a 72 hour static renewal toxicity test at concentrations of 100, 200, 300, 400 and 500 mg a.i./L. MBI-401 FDP testing was carried out on September 27, 2011 and MBI-401 SDP testing was carried out on June 13, 2012 as part of a bridging study in the transition from the use of MBI-401 FDP to MBI-401 SDP. The product was renewed every 12 hours for FDP and every 24 hours for SDP. The reason the product was renewed every 12 hours for the FDP testing was due to the elevated ammonia levels; by changing the test water and product every 12 hours it was hoped the ammonia levels in the test beakers would drop below 1 mg/L. Endpoints are reported as $LC_{50,}$ $LC_{10,}$ and LC_{100} after 72 hours.

Test Results

Mortalities

Using averages, one mortality occurred in the control (4%) after 72 hours exposure to MBI-401 FDP. Five mortalities were observed in 100 mg a.i./L (33.4%), seven mortalities were observed in 200 mg a.i./L (46%), 14 mortalities were observed in 300 mg a.i./L (93.4%), 100% mortality was observed in 400 mg a.i./L and 100% mortality was observed in 500 mg a.i./L. The results are presented in Figure 2.10 and Table 2.9.

Table 2.9 Mean number of live individuals (3 treated replicates, 5 control replicates) and % mortality of *Ephemerella ignita* after 72 hours exposure to MBI-401 FDP.

Time	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L	Control
12	4.6	5.0	5.0	5.0	5.0	5.0
24	4.6	5.0	4.0	4.3	3.3	4.8
36	4.6	4.6	1.3	0.0	0.0	4.8
48	4.6	3.0	0.7	0.0	0.0	4.8
60	4.6	3.0	0.7	0.0	0.0	4.8
72	3.3	2.7	0.3	0.0	0.0	4.8
% Mortality	33.4	46	93.4	100	100	4

Using averages, no mortality occurred in the control after 72 hours exposure to MBI-401 SDP. Zero mortalities were observed in 100 mg a.i./L (0%), one mortality was

observed in 200 mg a.i./L (6%), two mortalities were observed in 300 mg a.i./L (14%), four mortalities were observed in 400 mg a.i./L (26%) and 100% mortality was observed in 500 mg a.i./L (Figure 2.11 and Table 2.10).

One hundred percent mortality was reached in both the FDP and SDP tests however with the SDP this was only reached at the highest concentration of 500 mg a.i./L with 400 mg a.i./L reaching only 26% mortality. For FDP 100% mortality was reached at 500 and 400 mg a.i./L with 300 mg a.i./L at 93.4% mortality showing that the FDP has a much greater effect on the mortality of the mayfly than the SDP.

Table 2.10 Mean number of live individuals (3 treated replicates, 5 control replicates) and % mortality of *Ephemerella ignita* after 72 hours exposure to MBI-401 SDP.

Time	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L	Control
24	5.0	5.0	5.0	5.0	4.7	5.0
48	5.0	5.0	5.0	4.0	1.7	5.0
72	5.0	4.7	4.3	3.7	0.0	5.0
% Mortality	0	6	14	26	100	0

Statistical Evaluation

FDP

GLM results confirm that the five concentrations did have an effect on organism survival. Time had no effect on organism survival. Replication There was no significant difference in mortality between replicates (Appendix D Table 7).

The results from separate two-way ANOVAs indicate that concentration had no effect on temperature but time did affect temperature. For dissolved oxygen, pH, and ammonia both concentration and time had an effect on these parameters. Turbidity was affected by the passing of time (Appendix D Table 8).

SDP

GLM results confirm that time and concentration did effect organism survival. There was no significant difference in mortality between replicates (Appendix D Table 9).

The results from separate two-way ANOVAs indicate that temperature, DO, pH and NH_3 were not affected by concentration. Temperature and turbidity were affected by time whereas pH, DO, and NH_3 were not (Appendix D Table 10).

Validity of the Results

According to OECD 219 guidelines 'Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment', 202 *Daphnia* sp., 'Acute Immobilisation Test' and 203 'Fish, Acute Toxicity Test' this study can be regarded as valid;

— In the control not more than 10% of the mayflies died.

— The dissolved oxygen did drop below 6 mg/L, however, this occurred only in a small number of test chambers and therefore this is considered only a minor deviation and did not affect the scientific validity of the test

— The pH was between 6-9 in all test vessels.

— The water temperature did differ by more than ±1.0°C but only by an extra 0.2°C for the FDP testing. This is considered only a minor deviation and did not affect the scientific validity of the test.

Conclusion

This study indicated that based on nominal concentrations the LC_{50} of MB1-401 FDP was 195.99 mg a.i./L and the LC_{50} of MB1-401 SDP was 403.37 mg a.i./L. Based on these results, MBI-401 SDP is less toxic than MBI-401 FDP. Thus, results from toxicity testing with MBI-401 FDP can be assumed "worst case." MBI-401 SDP is more suitable for zebra mussel control as it has less of an effect on *Ephemerella ignita* than MBI-401 FDP. These results coupled with zebra mussel control trials (Meehan et al., 2013 and Meehan et al., 2014b) show that the optimal concentration for controlling zebra mussels (approximately 100 mg a.i./L, with a maximum concentration of 200 mg a.i./L) would have minimal to no impact on *Ephemerella ignita* species.

2.5.2.4 *Mytilus edulis*

The test substance, MBI-401 FDP, was assessed on *Mytilus edulis*, blue mussel, exposed in a 72 hour static renewal toxicity test at concentrations of 200, 300, 400, 500 and 600 mg a.i./L on October 25, 2011. Endpoints are reported as $LC_{50,}$ $LC_{10,}$ and LC_{100} after 72 hours.

Mortalities

Using averages, no mortality occurred in the control after 72 hours exposure. One mortality was observed in 200 mg a.i./L (3.75%), no mortalities were observed in 300 mg a.i./L, 1 mortality was observed in 400 mg a.i./L (3.75%), two mortalities were observed in 500 mg a.i./L (8.75%) and six mortalities were observed in 600 mg a.i./L (25%). (Figure 2.12 and Table 2.11).

The highest mortality reached was 25% in 600 mg a.i./L and these mortalities occurred after 36 hours. A small amount of mortalities were also observed in 200 mg a.i./L, 400 mg a.i./L and 500 mg a.i./L all of which were observed in the final count made at 72 hours. This demonstrates that the blue mussel can withstand high concentrations of MBI-401 FDP. Mortality in 600 mg a.i./L was expected as it is a very high concentration that would never be used in a real time application.

Table 2.11 Mean number of live individuals (3 treated replicates, 5 control replicates) and % mortality of *Mytilus edulis* after 72 hours exposure to MBI-401 FDP

Time	200 mg/L	300 mg/L	400 mg/L	500 mg/L	600 mg/L	Control
12	8.0	8.0	8.0	8.0	8.0	8.0
24	8.0	8.0	8.0	8.0	8.0	8.0
36	8.0	8.0	8.0	8.0	8.0	8.0
48	8.0	8.0	8.0	8.0	7.7	8.0
60	8.0	8.0	8.0	8.0	7.7	8.0
72	7.7	8.0	7.7	7.3	6	8.0
% Mortality	3.75	0	3.75	8.75	25	0

Statistical Evaluation

GLM results confirm that the five concentrations did have an effect on organism survival. Time was also shown to effect organism survival. There was no significant difference in mortality between replicates (Appendix D Table 11).

The results from separate two-way ANOVAs indicate that concentration did not affect temperature and pH but did affect ammonia and dissolved oxygen. Time did affect the temperature, pH and ammonia and had no effect on DO and turbidity (Appendix D Table 12).

Validity of the Results

According to OECD 219 guidelines 'Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment', 202 *Daphnia* sp.*,* 'Acute Immobilisation Test' and 203 'Fish, Acute Toxicity Test' this study can be regarded as valid;

— In the control not more than 10% of the *Mytilus edulis* died.

— The dissolved oxygen did drop not below 6 mg/L (however, only in a small number of the higher treatment test chambers). This is considered only a minor deviation and did not affect the scientific validity of the test.

— The pH was between 6-9 in all test vessels

— The water temperature did differ by more than ±1.0°C; however, only by an extra 0.5°C. This is considered only a minor deviation and did not affect the scientific validity of the test.

Conclusion

This study indicated that based on nominal concentrations the estimated LC50 of MB1-401 FDP to *Mytilus edulis* was 1,612 mg a.i./L, the estimated LC_{10} was 414.35 mg a.i./L and the estimated LC_{100} was 3,110 mg a.i./L. This study showed that that only a small number of mortalities occurred at all concentrations tested. These results coupled with zebra mussel control trials (Meehan et al., 2013 and Meehan et al., 2014b) show that the optimal concentration for controlling zebra mussels (approximately 100 mg a.i./L, with

a maximum concentration of 200 mg a.i./L) would have minimal to no impact on *Mytilus edulis*.

2.5.2.5 *Anodonta*

The test substance, MBI-401 FDP, was assessed on *Anodonta*, duck mussels exposed in a 72 hour static renewal toxicity test at concentrations of 100, 200, 300, 400 and 500 mg a.i./L on September 18, 2011. The product was renewed every 12 hours. Endpoints are reported as $LC_{50,}$ $LC_{10,}$ and LC_{100} after 72 hours.

Mortalities

No mortalities occurred after 72 hours exposure, in any of the concentrations tested, (100 mg a.i./L, 200 mg a.i./L, 300 mg a.i./L, 400 mg a.i./L and 500 mg a.i./L) or in any of the controls indicated MBI-401 FDP is not toxic to *Anodonta*.

Statistical Evaluation

As there were no mortalities a GLM was not applied. The results from separate two-way ANOVAs indicate that concentration had no effect on temperature, and time did affect temperature. For dissolved oxygen and ammonia time and concentration affected both. For pH and turbidity time showed no effect on both whereas concentration did affect pH (Appendix D Table 13).

Validity of the Results

According to OECD 219 guidelines 'Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment', 202 *Daphnia* sp.*,* 'Acute Immobilisation Test' and 203 'Fish, Acute Toxicity Test' this study can be regarded as valid;

— In the control not more than 10% of the *Anodonta* mussels died.

— The dissolved oxygen did not drop below 6 mg/L.

— The pH was between 6-9 in all test vessels.

— The water temperature did differ by more than ±1.0°C but only by an extra 0.2°C. This is considered only a minor deviation and did not affect the scientific validity of the test.

Conclusion

This study showed that based on nominal concentrations no negative effects to *Anodonta* occurred at any concentrations tested. These results coupled with zebra mussel control trials (Meehan et al., 2013 and Meehan et al., 2014b) show that the optimal concentration for controlling zebra mussels (approximately 100 mg a.i./L, with a maximum concentration of 200 mg a.i./L) would have minimal to no impact on *Anodonta.*

2.5.2.6 *Daphnia pulex*

The test substance, MBI-401 SDP, was assessed on *Daphnia pulex* exposed in a 72 hour static renewal toxicity test at concentrations of 50, 100, 150, 200, and 250 mg a.i./L on April 4, 2012. Endpoints are reported as EC_{50}, EC_{10} and EC_{100} after 72 hours.

Mortalities

Using averages, one mortality occurred in the control (4%) after 72 hours exposure. Two mortalities were observed in 50 mg a.i./L (13%), four mortalities were observed in 100 mg a.i./L (27%), three mortalities were observed in 150 mg a.i./L (20%), nine mortalities were observed in 200 mg a.i./L (60%) and nine mortalities were observed in 250 mg a.i./L (60%). (Figure 2.13 and Table 2.12).

It is important to note that the EC_{50} reported in this test is after 72 hours. OECD guidelines for *Daphnia* testing recommends carrying out 48 hour testing as the 72 hours can prove to stressful to the *Daphnia*. The 72 hour EC_{50} was used in order to keep all the testing uniformed and to present the very worst case scenario. When in reality exposure would not be this long.

Toxicity tests were also carried out by Shannon Aquatic Toxicity Lab on *Daphnia magna* to validate the testing carried out in IT Sligo. Concentrations of 56, 100, 180, 320, 560, 1000, 1800 mg a.i./L were tested with a total of ten replicates. The average 48 hour EC_{50} was 364.6 mg a.i./L. This is close to the 48 hour EC 50 of 308 mg a.i./L, which was obtained in IT Sligo.

Table 2.12 Mean number of live individuals (3 treated replicates, 5 control replicates) and % mortality of *Daphnia* after 72 hours exposure to MBI-401 SDP

Time	50 mg/L	100 mg/L	150 mg/L	200 mg/L	250 mg/L	Control
24	5.0	5.0	5.0	4.7	2.0	5.0
48	5.0	4.6	5.0	4.0	2.0	4.8
72	4.3	3.67	4.0	2.0	2.0	4.8
% Mortality	14	27	20	60	60	4

Statistical Evaluation

GLM results confirm that the different test concentrations did have an effect on organism survival. Time was shown to also have an effect on organism survival. There was no significant difference in mortality between replicates (Appendix D Table 14).

The results from separate two-way ANOVAs indicate that concentration did not affect temperature, DO and NH_3, There was an effect to pH due to concentration. With regards to time there was an effect to NH_3 and temperature over time, and there was no effect to pH, DO and turbidity over to time (Appendix D Table 15).

Validity of the Results

According to OECD 202 guidelines '*Daphnia* sp., Acute Immobilisation Test and Reproduction Test' this study can be regarded as valid since;

— In the control not more than 10% of the *Daphnia* were immobilised

— The dissolved oxygen did drop below 3 mg/L, however, this occurred only in once test chamber (250 mg a.i./L) at 72 hours and therefore is considered only a minor deviation and did not affect the scientific validity of the test

— The pH was between 6-9 in all test vessels

— The water temperature did not differ by more than ±1.0°C

Conclusion

This study indicated that based on nominal concentrations the EC_{50} of MB1-401 SDP to *Daphnia pulex* was 206.89 mg a.i./L, the EC_{10} was 37.47 mg a.i./L and the EC_{100} was 418.66 mg a.i./L. These results coupled with zebra mussel control trials (Meehan et al., 2013 and Meehan et al., 2014b) show that the optimal concentration for controlling zebra mussels (approximately 100 mg a.i./L, with a maximum concentration of 200 mg a.i./L) would have minimal to no impact on *Dapnia pulex*.

2.5.2.7 *Austropotamobius pallipes*

The test substance, MBI-401 SDP, was assessed on *Austropotamobius pallipes,* the white clawed crayfish, exposed in a 72 hour static renewal toxicity test at concentrations of 350, 450, 550, 650 and 750 mg a.i./L on May 17, 2012. Endpoints are reported as $LC_{50,}$ $LC_{10,}$ and LC_{100} after 72 hours.

Test Results

Mortalities

Using averages, no mortality occurred in the control after 72 hours exposure. One mortality was observed in 350 mg a.i./L (15%), one mortality was observed in 450 mg a.i./L (15%), no mortalities were observed in 550 mg a.i./L, no mortalities were observed in 650 mg a.i./L, and no mortalities were observed in 750 mg a.i./L.(Figure 2.14 and Table 2.13). Water quality has previously been discussed in section 2.4.1.1 in relation to the low DO at 450 mg/L possibly causing this mortality.

Table 2.13 Mean number of live individuals (3 treated replicates, 5 control replicates) and % mortality of *Austropotamobius pallipes* after 72 hours exposure to MBI-401 SDP

Time	350 mg/L	450 mg/L	550 mg/L	650 mg/L	750 mg/L	Control
24	2.0	2.0	2.0	2.0	2.0	2.0
48	1.7	1.7	2.0	2.0	2.0	2.0
72	1.7	1.7	2.0	2.0	2.0	2.0
% Mortality	15	15	0	0	0	0

Statistical Evaluation

GLM results confirm that the different test concentrations and time did not affect mortality. There was no significant difference in mortality between replicates (Appendix D Table 16). It is thought the mortality incurred in the lower dose temperatures was not a result of Zequanox there were no mortalities in the higher treated chambers. The results from separate two-way ANOVAs indicate that concentration had no effect on temperature, pH, DO and NH_3. Time did affect temperature and NH_3 and did not affect pH, DO and turbidity (Appendix D Table 17).

Validity of the Results

According to OECD 219 guidelines 'Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment', 202 *Daphnia* sp.*,* 'Acute Immobilisation Test' and 203 'Fish, Acute Toxicity Test' this study can be regarded as valid;

— In the control not more than 10% of the crayfish died.

— The dissolved oxygen did drop below 6 mg/L, however, this only occurred on two occasions and therefore in is considered only a minor deviation and did not affect the scientific validity of the test

— The pH was between 6-9 in all test vessels.

— The water temperature did differ by more than ±1.0°C but only by an extra 0.7°C. This is considered only a minor deviation and did not affect the scientific validity of the test.

Conclusion

This study showed that based on nominal concentrations no negative effects to *Austropotatamobius pallipes* occurred at the higher concentrations tested. Based These results coupled with zebra mussel control trials (Meehan et al., 2013 and Meehan et al., 2014b) show that the optimal concentration for controlling zebra mussels (approximately 100 mg a.i./L, with a maximum concentration of 200 mg a.i./L) would have minimal to no impact on *Austropotatamobius pallipes.*

2.5.2.8 *Lymnaea peregra*

The test substance, MBI-401 SDP, was assessed on *Lymnaea peregra* exposed in a 72 hour static renewal toxicity test at concentrations of 500, 600, 700, 800 and 900 mg a.i./L on July 23, 2012. Endpoints are reported as $LC_{50,}$ $LC_{10,}$ and LC_{100} after 72 hours.

Test Results

Mortalities

Using averages, no mortality occurred in the control after 72 hours exposure. No mortality was observed in the 500 mg a.i./L, 600 mg a.i./L and 700 mg a.i./L test concentrations. One mortality was observed in 800 mg a.i./L (6%), and two mortalities were observed in 900 mg a.i./L (14%) (Figure 2.15 and Table 2.14).

The mortalities that occurred in 800 mg a.i/L and 900 mg a.i/L both occurred at the 72 hour count, showing the *Lymnaea peregra* were able to withstand these high concentrations for in excess of 48 hours. During the monitoring period, egg masses were found attached to several of the *L. peregra*. Egg masses were also found attached to the air stones and the glass of test container. Egg masses were even found at 900 mg a.i/L showing that the use of MBI-401SDP did not interfere with the natural life cycle of the *L. peregra.*

Table 2.14 Mean number of live individuals (3 treated replicates, 5 control replicates) and % mortality of L*ymnaea peregra* after 72 hours exposure to MBI-401 SDP.

Time	500 mg/L	600 mg/L	700 mg/L	800 mg/L	900 mg/L
24	5.0	5.0	5.0	5.0	5.0
48	5.0	5.0	5.0	4.7	5.0
72	5.0	5.0	5.0	4.7	4.3
% Mortality	0	0	0	6	14

Statistical Evaluation

GLM results show that test concentrations had no effect on organism survival. This was expected due to the low mortality. Time was confirmed to have no effect on organism survival and there was no significant difference in mortality between replicates (Appendix D Table 18).

The results from separate two-way ANOVAs indicate that time did affect temperature but concentration did not. There was no effect to DO or pH due to time or concentration. Turbidity was affected by time. Ammonia was affected by time and not concentration (Appendix D Table 19).

Validity of the Results

According to OECD 219 guidelines 'Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment', 202 *Daphnia* sp.*,* 'Acute Immobilisation Test' and 203 'Fish, Acute Toxicity Test' this study can be regarded as valid because:

— In the control not more than 10% of the snails died

— The dissolved oxygen did not drop below 6 mg/L

— The pH was between 6 and 9 in all test vessels

— The water temperature did differ by more than ± 1.0°C, but no control organisms were impacted by this difference, so it did not affect the scientific validity of the test

Conclusion

This study indicated that, based on nominal concentrations, the estimated EC_{50} of MB1-401 SDP to *Lymnaea peregra* was 2,082 mg a.i./L, the estimated EC_{10} was 881 mg a.i./L and the estimated EC_{100} was 3,584 mg a.i./L. This study showed that that there were no negative effects to *Lymnaea peregra* at concentrations required to achieve zebra mussel mortality. These results coupled with zebra mussel control trials (Meehan et al., 2013 and Meehan et al., 2014b) show that the optimal concentration for controlling zebra and quagga mussels (approximately 100 mg a.i./L, with a maximum concentration of 200 mg a.i./L) would have minimal to no impact *Lymnaea peregra*.

2.5.2.9 *Salmo trutta* (parr)

The test substance, MBI-401 SDP, was assessed on *Salmo trutta* in a 72 hour semi-static toxicity test at concentrations of 180, 100, 56, 32 and 18 mg a.i./L. Due to the lack of aquaculture facilities for *S. trutta* at the labs at IT Sligo this testing was carried out by Shannon Aquatic Toxicity Laboratory. S. Meehan was onsite for the first treatment where after, water renewal and the water quality recording was carried out by a member of staff. MBI-401 SDP was not renewed during testing and so *Salmo trutta* were only treated once at the beginning of testing. Endpoints are reported as $LC_{50,}$ $LC_{10,}$ and LC_{100} after 72 hours.

Test Results

Mortalities

Using averages, no mortality occurred in the control after 72 hours exposure. All brown trout died in the 180 mg a.i./L, eight mortalities occurred in 100 mg a.i./L test concentration (80%) and seven mortalities were observed in 56 mg a.i./L (70%), one mortality was observed in 32 mg a.i./L (10%) and no mortalities were observed in 18 mg a.i./L(Figure 2.16 and Table 2.15).

The results show that *Salmo trutta* are the most sensitive of all the non target species to treatment with Zequanox. In concentrations 56, 100, and 180 mg a.i./L mortality had occurred by 24 hours. In the wild *Salmo trutta* would not be exposed to these levels of Zequanox, mainly due to the fact that the product would be highly diluted upon discharge back to the source water after treatment of a defined enclosure. As Zequanox has the potential to be used in open water, efforts could be made to remove fish from a treatment area by block area with nets or removing fish from the treatment enclose if an experimental set-up is used similar to that in Tullamore Harbour (Chapter 4).

Table 2.15 Mean number of live individuals (2 treated replicates, 2 control replicates) and % mortality of *Salmo trutta* after 72 hours exposure to MBI-401 SDP.

Time	18 mg/L	32 mg/L	56 mg/L	100 mg/L	180 mg/L
24	5.0	5.0	2.5	3.0	2.0
48	5.0	5.0	2.5	2.0	0.5
72	5.0	4.5	1.5	1.0	0.0
% Mortality	0	10	70	80	100

Statistical Evaluation

A two-way ANOVA was carried out in Minitab instead of a general linear model as individual replicate mortality was not counted by Shannon aquatic toxicity lab therefore a two-way ANOVA conformed that test concentration did have an effect on organism survival. This was expected due to the high mortality observed. Time was also shown to effect organism survival, this was evident as the mortality increased every 24 hours (Appendix D Table 20).

The results from separate two-way ANOVAs indicate that, time had no effect on temperature but concentration did, (the changes in temperature as previously discussed were only minute). There was no effect on DO due to concentration: DO was affected by time; pH was not affected by concentration but was affected by time; turbidity was affected by time NH_4 measurements recorded by Shannon Aquatic Toxicity Lab were only taken for one treatment concentration per day and therefore cannot be included in the ANOVA. (Appendix D Table 21).

Validity of the Results

According to OECD guidelines 203 'Fish, Acute Toxicity Test' this study can be regarded as valid because.

— In the control not more than 10% of the *Salmo trutta* died

— The dissolved oxygen did not drop below 6 mg/L

— The pH was between 6 and 9 in all test vessels

— The water temperature did not differ by more than ± 1.0°C

Conclusion

This study indicated that, based on nominal concentrations, the LC_{50} of MB1-401 SDP to *Salmo trutta* was 85 mg a.i./L, and the LC_{100} was 171 mg a.i./L. As *Salmo trutta* are affected by high and low concentrations of Zequanox further research in the USA is ongoing on trout into the possible reasons why.

2.6 Overall Conclusions

The non target testing demonstrated the progression of the use of MBI-401 FDP to MBI-401 SDP, with results from bridging studies on *Chironomus* and *Ephemerella ignita* indicating that SDP is less harmful. Figure 2.17 displays the comparative effect of MBI-401 FDP and MBI-401 SDP on *Chironomus* and *Ephemerella ignita;* it is clear that for *Chironomus* (all concentrations tested) the effect on mortality from SDP is less than FDP reaching a mortality peak of 28%. For *Ephemerella ignita* between concentrations of 100 mg/L to 400 mg/L the effect from SDP is lower than FDP, at 500 mg/L however a similar high in mortality is reached. These bridging studies eliminated the need for retesting of all organisms as the results show that MBI-401 SDP is less toxic to these freshwater organisms than MBI-401 FDP. Coupled with this, it is important to consider that a real time application requires a treatment of only 150-200 mg/L applied for 12-24 hours (Meehan et al., 2013; Meehan et al., 2014b) The results from these assays show that when using MBI-401 SDP on *Chironomus* and *Ephemerella ignita* at concentrations of 100-200 mg/L mortality was 0% after 24 hours.

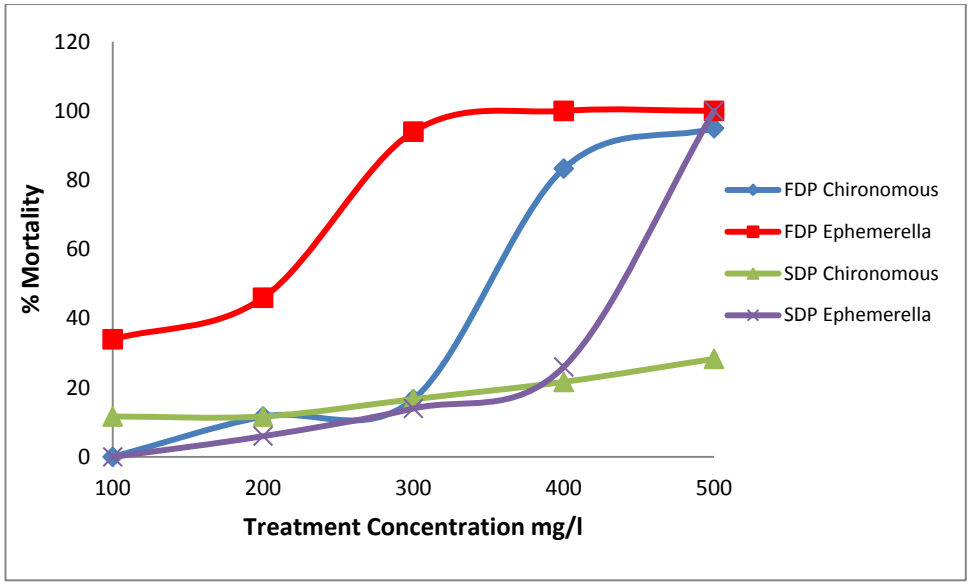

Figure 2.17 Concentration response graph of bridging studies after 72 hours exposure to MBI-401 FDP and SDP

It is important to note that these non target tests were carried out to increase understanding on the potential effect Zequanox may have to organisms it comes in contact with in open waters; the aims were to find the LC_{50} for each individual organism. Ecotoxicology tests are more stringent and test at higher Zequanox concentrations than those used in open water. As different organisms have different levels of sensitivity to Zequanox finding the LC_{50} value allows for direct comparison between species (Hedayati et al., 2010). Since the tests were carried out using 12-24 hour renewals over a 72 hour period, these conditions can be considered extreme and in no way representative of conditions organisms would encounter in open water as in Chapter 4 (Tullamore Harbour trial). Figures 2.7 – 2.16 are representative of final mortality after the 72 hour test period the X axis varies because of the ranging treatment concentration; they were used to determine the LC_{50}'s only. Treatments in the wild would normally occur at 150 mg/L and would never exceed 200 mg/L which is the maximum allowable concentration (Marrone Bio Innovations, 2012b).

Looking at the individual organism mortality tables for mortality after 12-24 hours, levels between 100 and 200 mg/L treatment concentration give a greater indication as to the potential effects of a Zequaonox treatment to non target organisms in open water (Tables 2.5 – 2.14). In all species except *Salmo trutta* mortality was zero to one death between treatment concentrations 100-200 mg/L. Mortality was high for *Salmo trutta*

even at lower treatment concentrations. This means that consideration will have to be given to *Salmo trutta* if Zequanox is to be used where this and salmonid species (namely *S. salar)* are present. This may include the treatment set up; if treating in a man-made enclosure like the one at Tullamore Harbour (Chapter 4) efforts can be made to exclude fish species. In Lough Sheelin, Co. Cavan, Ireland wild stocks of *S. trutta* are present supporting a recreational fishery; here zebra mussels and *Anodonta* are also in abundance (Millane, 2008; Millane et al., 2008). Lough Sheelin is an example of how a potential *Anodonta* restoration treatment as in the Great Lakes Restoration Initiative, would require protection of the native fish species like *S. trutta*. Treatment of the native unionid beds could exclude fish species by using an enclosure (as in Chapter 4) to cover infested beds. The setting up of a treatment, would involve divers which would also inadvertently drive the fish away, due to disturbance in the water. Also there is potential to reduce treatment time even further in *S. trutta* and *S. salar* enriched water. Additional studies would be required to determine by how much treatment times can be reduced without compromising zebra mussel mortality.

Good water quality appears to be the most important factor in keeping organism mortality low, the higher the test concentration the greater the effect to water quality and for some organisms the higher the mortality. Increased Zequanox concentration causes a rise in NH_3 and turbidity, while DO often decreases; it is the change in these parameters that is thought to cause mortality and not the toxicity of Zequanox to these organisms. As a real time application would treat at a max of 200 mg/L for 12-24 it is important to look at the resulting water quality and mortalities. The following organisms were treated at 100-200 mg/L; *A. aquaticus, E. ignita, Chironomus, M. edulis, Anodonta, Daphnia, S. trutta* here after 12-24 hours DO, pH and temperature stayed within limits set out for Salmonid water (Appendix:C, raw data). The NH_3, as previously discussed, was elevated but did stay below 1 mg/L for all species except for one NH_3 reading recorded after 12 hours at a treatment concentration of 200 mg/l for *M. edulis.* As water quality was good, resulting mortality was low, between 0-1 deaths after 12-24 hours (except *S. trutta* which had four and six mortalities). In higher test concentrations it was the smaller organisms treated with MBI-401 SDP e.g. *Daphnia pulex* and *E. ignita* that incurred the most mortality, this is contrary to the larger invertebrate organisms namely *A. pallipes* and *Anodonta,* which can withstand poorer water quality such as high turbidity, low DO and temperature fluctuations. The large

variance in water quality seen is due the large range of treatment concentrations. The water quality measurements at the higher treatment concentrations are in no way representative of a real time treatment.

NPWS requested that this non target testing be carried out prior to any trials/testing to ensure there were no negative impacts to freshwater fauna. They were consulted regarding species chosen; the organisms were chosen from each functional feeding group and were selected based upon the fact that they all play an important role in freshwater food webs. Choosing from all feeding groups was important; if one organism was impacted by Zequanox the repercussions would have a knock on effect to other organisms in the food web, particularly if a keystone species, e.g. *A. pallipes* was involved. Jones et al. (1996) demonstrates the repercussions the removal of organisms can have on the surrounding environment and describes organisms that directly or indirectly control the availability of resources to other species as ecosystem engineers. The taxa fell under the following functional feeding groups; *E. ignita, L. peregra* - benthic grazers, *A. aquaticus*, *Chironomus* – decomposers, *Daphnia*, *Anodonta, M. edulis* - filter feeders, *A. pallipes* - omnivore *S. trutta* – carnivores (Klemetsen et al., 2003; IOWATER, 2005). Some of the organisms tested such as *Ephemerella ignita, Asellus aquatics, Chironomus* and *Daphnia* are preyed upon by fish species like perch (*Perca fluviatilis*), brown trout (*Salmo trutta*), bream (*Abramis brama*) and roach (*Rutilus rutilus*), which may in turn be consumed by birds (Swynnerton and Worthington, 1940; Suter, 1998; Mullarney et al., 1999; Wetzel, 2001). Many of the organisms chosen are benthic and known to co-occur with the zebra mussel (Millane, 2008), meaning that they too would become an inadvertent target of Zequanox. Non target testing was therefore an imperative part of this thesis research, as the introduction of Zequanox to any freshwater ecosystem introduces potential impacts within freshwater food webs therefore different trophic levels were studied for effect caused by Zequanox. The results of this non target testing were given to NPWS as part of an initial agreement with them to insure the safety and protection of native species prior to any open water testing. The results were also presented to the Department of Agriculture in order to gain permission for further testing/trials using Zequanox in Ireland. In addition to making the results available to the NPWS and the Dept. of Agriculture, the results obtained were presented to all other public bodies consulted (Figure 8.1, chapter 8) prior to the Tullamore Harbour and Cairns Hill trials to

demonstrate that the discharge/dispersal of Zequanox would have no negative impacts to the existing fauna. As well as the non target testing playing a key role in securing trial permission in Ireland this testing was also included in an EU dossier for Zequanox approval in Europe providing supplementary testing data and information.

Two trials took place in Ireland (IT Sligo and Cairns Hill/Grand Canal Tullamore Harbour), trial authorisation was granted by the Department of Agriculture as this non target testing demonstrated that there were no negative impacts to Ireland's native freshwater species and ensured that Ireland's most vulnerable species were protected. These results will also allow for further research and development of the product to occur in a European capacity. The results also call for further research into the protection of fish species during Zequanox treatment. This testing will also provide an important point of reference for stakeholders should the use of Zequanox in Ireland ever be licensed. These non target trials have significantly supplemented the EU dossier for European Union submission to gain a license for the use of Zequanox in defined enclosures, therefore eventually allowing Zequanox to be sold and used commercially in Europe.

Chapter 3

Comparing a Microbial Biocide and Chlorine as Zebra Mussel Control Strategies in an Irish Drinking Water Treatment Plant

3.1 Introduction to Paper

A paper outlining the trial was composed and published in 'The Management of Biological Invasions' (volume 4 issue 2) and is incorporated in this chapter with regional context, with the paper in its entirety (as was published) in Appendix E. [http://www.reabic.net/journals/mbi/2013/2/MBI_2013_2_Meehan_etal.pdf]

Cairns Hill drinking water treatment plant is located just outside Sligo town, Ireland. This plant extracts water from Lough Gill for treatment and supplies a broad spectrum of Sligo's water including, high areas of Sligo town, Strandhill, Ballysadare, Collooney, Carraroe, Aghamore, Ballygawley and Ballingtogher. Cairns Hill was refurbished in 2002 and caters for a demand of between 6.000 to 7500 cubic meters per day (Eamon Fox plant manager pers. comm.).

The stages of water treatment at Cairns Hill are as follows:

- Intake screening at lake - with mesh screens, suction pipes and foot valves to stop large material passing through
- High lift pumping from lake to treatment plant house.
- Pre-ozone chamber - fitted with 8 ozone diffusers.
- Microstrainers – two rotating drums covered with micro mesh and backwash pumps which collect grit to discharge back to lake.
- Main ozone chamber – ozone here is produced onsite and has a life span of 10 minutes.
- Forward feed pumps – to lift post ozone water back up to G.A.C (granular activated carbon filtration)
- G.A.C. filters – water passes from the filter inlet channel through the carbon.
- Backwashing G.A.C. filters – air scouring for 3 minutes and water backwashing for 15 minutes, waste water from backwash is then drained out into a holding tank and drained back into the lake.

- Filtered water channel – filtered water is injected with chlorine and fluoride on route to the reservoir.

Figure 1.6 displays the infrastructure of the process listed above. This information was obtained from the water treatment plant on a guided tour. It is the raw water chambers (pre ozone chambers) of the plant that are infested with Zebra mussels. The juveniles are able to pass through the first stage of intake screening and pipe work before encountering the low flow in the chambers where with an abundance of substrate for settlement they grow and repopulate. A biobox trial was carried out onsite at Cairns Hill and IT Sligo to demonstrate the efficacy of Zequanox in controlling zebra mussels at the water treatment plant. The results were then compared to a simultaneous chlorine treatment at the plant.

3.2 Introduction

The zebra mussel *Dreissena polymorpha* (Pallas, 1771), is an invasive, exotic aquatic bivalve, which has greatly affected lakes, canals and other aquatic ecosystems in Ireland (Minchin et al., 2002a; Lucy, 2010; Lucy et al., 2014) since first invading in the early 1990's (Minchin and Moriarty, 1998). The control methods currently used in Ireland, Europe and North America are necessary in industries requiring water abstraction, for example in drinking water treatment plants and land-based fish hatcheries where juvenile zebra mussels settle in water pipe networks and ancillary plants, developing into fully grown zebra mussels (Mackie and Claudi 2010). In such cases, either physical removal and/or chlorine dosed at approximately 2 mg/L is frequently used to control the mussels (Mackie and Claudi, 2010) as is the case in the drinking water treatment plant in Sligo, used in this study. At 2 mg/L chlorine treatments can take up to 21 days to be effective (Mackie and Claudi, 2010). At the Sligo drinking water treatment plant, flow through raw water chambers receiving chlorine treatment are bypassed for the chlorination period and the treated water is released back to the discharged water body. Trihalomethanes can be formed in drinking water as a result of the chlorination of organic matter in the raw water supplies (Coffin et al., 2000) and according to Wright et al (2007) THM formation is enhanced when dead mussels are present. The use of chlorine also presents more risks to the user; oversaturation

of the air can cause the mucous membrane to become irritated and severe coughing can occur (West Virginia Department of Health and Human Resources, 2010). With drinking water plants in particular, high chlorine concentrations in the water may impact the taste and odour (Roche and Benanou, 2007). In the USA, chlorine discharge limits permissible in receiving water should not exceed 19 μg/L more than once every three years on average under the acute toxicity criterion. Under the chronic toxicity criterion, the 4 day average concentration should not exceed 11 μg/L more than once every three years on average (Tikkanen et al, 2001).

Marrone Bio Innovations (MBI), a company specialising in the development and commerce-alisation of natural biocides in Davis, CA, USA, is the commercial license holder of *Pseudomonas fluorescens* strain CL145A; a microbe used to control invasive zebra and quagga (dreissenid) mussels. In 2012, MBI registered and commercialised Zequanox, a spray dried powder comprised of killed *Pseudomonas fluorescens* CL145A cells, in the United States and Canada. *Pseudomonas fluorescens* CL145A cells have been shown to be lethal to dreissenid mussels (Molloy et al., 2013a), but pose minimal to no risk to other aquatic organisms (Molloy et al., 2013c). This bacterial species is present worldwide and commonly found in food. In nature, it is a harmless bacterial species that is known to protect the roots of plants from disease (Marrone Bio Innovations, 2012). It has been shown that killed *Pseudomonas fluorescens* CL145A cells have no negative impacts to aquatic organisms in Irish waters at treatment concentrations required to achieve >80% zebra mussel mortality (Meehan et al., 2014a).

The main objective of this study was to demonstrate the efficacy of MBI 401 FDP (a developmental formulation of Zequanox) at controlling zebra mussels in Ireland. This was done in a biobox trial at a drinking water treatment plant by comparing juvenile settlement pre and post treatment with MBI 401 FDP as well as adult mussel survival after treatment. In addition, these results were compared to juvenile settlement and adult mussel survival after chlorine treatment in the plant's raw water chambers. Water quality, before, during and after treatment with MBI 401 FDP, was also monitored to determine the impact from treatment to source water quality and to the environment.

3.2.1 Sligo Drinking Water Treatment Plant, Ireland

This research study was carried out at a drinking water treatment plant, located on the perimeter of Sligo city in the north-west of Ireland (54°25'07"N, 08°45'22"W). This plant extracts between 6000 to 7500 m^3 of raw water per day for treatment from a nearby lake, Lough Gill (14.3 km^2). The raw water chambers in the plant house are infested with zebra mussels (Figure 3.1). During summer reproduction, the free floating zebra mussel larvae (veligers) are able to pass through the first stage of mesh filtration at the lake abstraction point. The veligers are then pumped 1 km with the influent water, via the intake pipe, and then enter the water chambers in the treatment plant where they settle on the walls and begin to grow. Lough Gill has been infested with zebra mussels since approximately 2004 and high densities were present in the raw water chambers by 2009.

Sligo drinking water treatment plant began using chlorine to treat the zebra mussel infestation in the raw water chambers in 2009 and have been treating once a year, in autumn following the reproductive season. During treatment, the plant is forced to shut down the chambers being treated; this process delays operations for the duration of the treatment (typically seven days) as well as the additional time for the set up and break down of the treatment.

3.3 Materials and Methods

3.3.1 Biobox and Chamber Set Up

Bioboxes are used to monitor mussel settlement in power plants or other similar facilities by mimicking the flow in industrial piping and demonstrating the resulting zebra mussel settlement in piping and water chambers (Mackie and Claudi, 2010). The biobox is connected to the main inflow of raw water to the plant.

Three 200 L bioboxes were placed on a flow through system in the Sligo drinking water treatment plant on the 13th of July 2011 (Figure 3.2). These tanks received water from the water treatment plant's main chambers via gravity flow, with a total flow of 287,000 L over 13 weeks until the 11th of October 2011. Of these three tanks, one was established to serve as the experimental control (tank 1) and the other two (tanks 2 and

3) were to receive MBI 401 FDP treatments. The tanks were covered with heavy plastic with weights on each side to protect from any harsh weather exposure or interference.

Figure 3.1 Zebra mussel infestation in raw water chambers at Sligo drinking water plant (photo by Eamon Fox).

Figure 3.2 Bioboxes outside of Sligo drinking water treatment plant (photo by Sara Meehan).

Three PVC plates (15 cm × 15 cm) were placed in each of the three tanks to allow for natural zebra mussel settlement (Marsden 1992; Lucy 2006). These plates were suspended in the tanks from a metal rod inserted lengthways across the top of the tank. Every week, either the middle or bottom plate was removed (in rotation) and replaced by a new plate so biweekly juvenile settlement rates could be estimated (Marsden, 1992; Lucy, 2006). The top plate was maintained throughout in order to monitor seasonal settlement. Water temperature, dissolved oxygen, and pH were recorded every week in each tank using a handheld Orion 5-star meter.

Three PVC plates (15 cm × 15 cm) were also placed in each of the plant's three raw water chambers on the 13th of July 2011. These plates were suspended lengthways from

the top of each chamber and were held in place by a rope hung from a ladder (Figure 3.3). Of these three chambers, one was established to serve as the experimental control (chamber B) and the other two (chambers A and C) were to receive chlorine treatment. Weekly removal of plates and recording of water quality parameters was the same as for the bioboxes.

Figure 3.3 Bags with adult mussels and PVC juvenile settlement plates attached to the suspension rope, deployed in the drinking water treatment plant chambers (photo by Sara Meehan).

3.3.2 Preparation of Bioboxes and Chambers for MBI 401 FDP and Chlorine Treatment

In addition to measuring and treating juvenile settlement, adult zebra mussels from a wild population in Lough Conn, Co. Mayo were seeded into each of the bioboxes to test whether treatment is effective on all life stages (Mackie and Claudi, 2010). Three mesh bags containing 50 mussels each were suspended in each biobox on the 7th of October

2011; this was three days in advance of treatment to allow the mussels to acclimatise (Figure 3.4). Prior to treatment on the 10th of October 2011, the bioboxes were moved from the water treatment plant to the research facility at IT Sligo (Figure 3.5). The bioboxes were then no longer on a flow-through system. Twenty-four hours prior to MBI 401 FDP treatment, the seeded mussels were checked for mortality and any dead mussels were replaced with healthy, live mussels.

Pre-treatment juvenile settlement on the PVC plates was assessed. The middle and bottom plates in the treated tanks (tanks 2 and 3) were removed prior to treatment due to the low numbers of established mussels. The top plate (which was the plate that accumulated settlement over the duration of the settlement season) was left in the bioboxes for treatment. Treatment was carried out after the Irish seasonal reproductive period (Lucy 2006).

One week after treatment of the bioboxes with MBI 401 FDP, the treatment of the raw water chambers at the drinking water treatment plant took place on the 17th of October 2011. The same methods for assessing adult mortality and juvenile settlement were applied here as with the bioboxes - adult mussels were seeded into the chambers and the top plate was assessed for settlement before repositioning in the chambers.

Figure 3.4 Bags with adult mussels used to assess mortality were suspended in the bioboxes and chambers (photo by Sara Meehan).

Figure 3.5 Bioboxes set up outside of IT Sligo (photo by Sara Meehan).

3.3.3 Application in Bioboxes

MBI 401 FDP (a dry powder) was a 100% active substance (or active ingredient). The powder was mixed on-site with Lough Gill water to create the following stock solution concentration:

$C_1V_1 = C_2V_2$ where

C_1 = target treatment concentration (mg active substance (a.s.)/L)

V_1 = volume of bioboxes (200 L)

C_2 = stock concentration (g a.s./L)

V_2 = volume of stock concentration to be injected (ml).

The target concentration was 200 mg active substance (a.s.)/L. These preliminary tests were carried out with the maximum allowable concent-ration in the U.S. in order to show efficacy and potential impact to water quality.

For each tank treated, 42 g (a.s.) of product was mixed with 0.93 L of water on a stir plate to achieve a stock concentration of 45 g (a.s.)/L. This stock concentration was injected into each tank at a rate of 50 mL/min for 19 minutes to achieve the target concentration of 200 mg a.s./L. The product was fed to the tanks using a peristaltic pump. A mixer was placed in the chambers to keep the product in suspension for the duration of the treatment.

As MBI 401 FDP is comprised of organic material, it is known that turbidity and MBI 401 FDP concentrations are strongly correlated. To confirm that the target concentration of MBI 401 FDP in each treatment tank was reached and maintained, a site specific linear regression was developed to determine the linear relationship between product concentration and turbidity (Figure 3.6). This was done according to MBI standard operating procedure, Turbidity and MOI-401 Active Ingredient Correlation and Application Monitoring (MBI personal communication). Turbidity was monitored throughout the application and post-treatment period with a Hach 2100N turbidimeter.

Once the target concentration was reached, the treated water was held for 8 hours. The application time was based on previous trials carried out by MBI at Davis Dam, Lower Colorado River, and Bullhead City, Arizona, USA. After the 8 hour treatment time, the tanks were rinsed three times and replaced with fresh Lough Gill water that was transported to IT Sligo in 1000 L containers. All MBI 401 FDP treated water was discharged to the sewer.

After all rinses were completed, bioboxes were transported back to the drinking water plant and hooked back up to the flow through system. Adult and juvenile mussels were then checked for mortality, initially daily and eventually once a week until juvenile survival reached zero and adult mussel mortality reached a plateau.

3.3.4 Water Quality in Bioboxes Treated with MBI 401 FDP

Water quality samples were taken before treatment, during treatment at 4 and 8 hours, and for each of the three rinses in treated tank 3 and the control tank. Water quality measurements included: temperature, dissolved oxygen (DO), pH, turbidity, biological oxygen demand (BOD), and total organic carbon (TOC). DO, pH and temperature were measured with an Orion 5 star meter. The analysis of BOD and TOC were subcontracted out to Alcontrol Laboratories. BOD was analysed following MEWAM BOD5 2nd Ed.HMSO 1988/ Method 5210B, AWWA/ APHA, 20th Ed., 1999; SCA Blue Book 130 and TOC was determined using US EPA Method 415.1 & 9060.

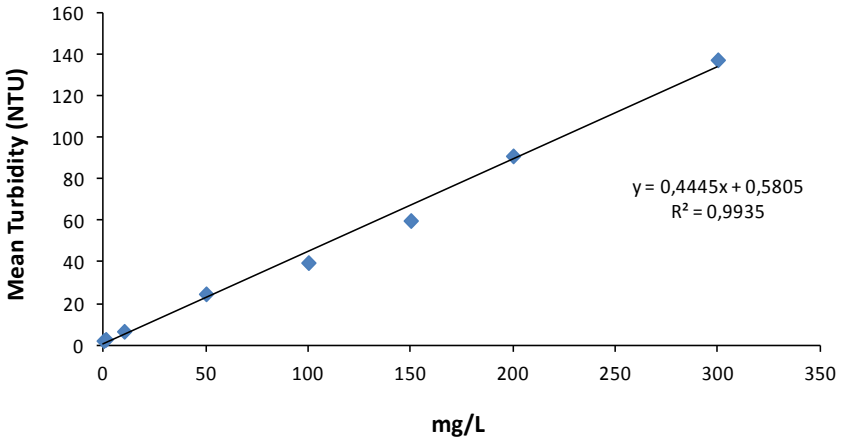

Figure 3.6 Site specific linear regression of MBI 401 FDP concentration and turbidity.

3.3.5 Application in Chambers

On the 17th of October 2011, the raw water chambers were treated with chlorine. The chambers receiving treatment were bypassed meaning that the raw water goes directly to the pre ozone chamber bypassing micro straining. The treatment was carried out by the plant manager where drums of chlorine were slowly poured into the receiving chambers. The chlorine concentration was monitored via a hand held meter to ensure that the concentration of 2 mg/L residual chlorine was maintained in the treated chambers; when the concentration dropped below 2 mg/L more chlorine was added. This treatment was carried out over a total of seven days; adult and juvenile mussels were then checked for mortality, initially daily and eventually once a week until juvenile survival reached zero and adult mussel mortality reached a plateau.

A *t*-test was applied to determine if there was a statistically significant difference between the mortality means of the two treatment methods.

3.4 Results and Discussion

Several long-standing and accepted chemical treatment methods exist for controlling zebra mussels, including chlorination. Chorine however, carries potential impacts for the surrounding environment and potential hazards to the user during its application, all previously stated. A need exists for a control method that has a quick application time and does not pose risks to the receiving water and the user. The results presented below demonstrate the efficacy of MBI 401 FDP in controlling zebra mussels and compares MBI 401 FDP treatment to chlorine treatment.

3.4.1 MBI 401 FDP Treatment - Juvenile Mussels

Juvenile settlement counted biweekly prior to treatment was relatively low reaching a peak of 5,000 juveniles/m^2 in the control biobox on the 10th of August 2011. As the number of settled juveniles is determined by the number of planktonic larva in the water, which in turn is determined by the water temperature (Lucy, 2006; Garton and Haag, 1993), relatively low summer water temperatures in 2011 (reaching < 10°C in August in the bioboxes) may have contributed to low settlement rates. In another Irish study, settlement reached a peak of 170,000 juveniles/m^2 where temperatures where higher and the same methodologies for gathering settlement was used (Lucy et al., 2005). Seasonal plates are also known to underestimate total natural settlement but are considered a good proxy (Lucy et al., 2005).

For the seasonal settlement plates, the control tank had the highest settlement with 4,670 juveniles/m^2, treated tank 2 had 3,670 juveniles/m^2, and treated tank 3 had 2,000 juveniles/m^2 (Figure 3.7). Treated juvenile survival declined rapidly between treatment and day 3; treated tank 2 reached 18% survival by day three and 0% survival seven days after treatment and treated tank 3 reached 16% survival by day three and 0% survival 6 days after treatment. The juvenile survival in the control began to decline between day 3 and 6. It is hypothesised that this decline in the control tank occurred from natural causes, as by day three, juvenile settlement was nearly depleted in treated tanks 2 and 3, whereas in the control tank, juvenile numbers did not begin to decline until after day three. The decline in the control and treated plates after day 3 could be attributed to the regular removal of the plates from the biobox for monitoring settlement and other natural causes. Additionally, according to Nichols (1996), 20% up to 100% natural mortality can occur pre and post settlement. It is hypothesised that the decline in juvenile survival prior to day 3 in treated tanks 2 and 3 was due to MBI 401 FDP treatment.

3.4.2 Chlorine Treatment – Juvenile Mussels

Juvenile settlement measured biweekly in the chambers, prior to treatment, was relatively high in comparison to the biweekly biobox settlement reaching a peak of 14,670 juveniles/m^2 in chamber A on the 4th of August. Although this count is higher than that of the bioboxes it is still relatively low in comparison to the juvenile settlement

measured in the study by Lucy et al. (2005) for the 1st week of August between 2001 to 2003.

Treated chamber C had the highest seasonal settlement with 31,000 juveniles/m^2, treated chamber B had 18,330 juveniles/m^2, and control chamber A had 10,670 juveniles/m^2. Figure 3.8 displays mean juvenile counts in the water chambers before and after treatment with chlorine. Treated juvenile survival declined rapidly between treatment and day 2; treated chamber A reached 12% survival by day two and 0% survival six days after treatment and treated chamber C reached 35% survival by day two and 0% survival 6 days after treatment. The juvenile survival in the control began to decline between treatment and day 2. Although control survival initially declined more rapidly than treated chamber C, overall survival reached 0% more rapidly in the treated chambers, therefore we can attribute this decline in survival to treatment with chlorine, with decline in juvenile survival on the control plate resulting from its removal from the chambers during examination.

3.4.3 MBI 401 FDP Treatment - Adult Mussels

After treatment, adult mussel mortality was monitored every 2–3 days for 16 days and then weekly for four weeks. At the end of the monitoring period on day 48 the control tank had 1.3% mortality, treated tank 2 had 80% mortality, and treated tank 3 had 81% mortality (Figure 3.9). Most of the adult mortality in the bioboxes occurred within the first 16 days after treatment; in treated tank 2 mortality was at 71% and in treated tank 3 mortality was at 76% by day 16. In similar biobox studies conducted in North America and Canada, >90% adult mussel mortality was observed (Figure 3.10). The water temperature during the Irish treatment was 13.8°C and for the post treatment monitoring period the min and max temperature was 13–15°C, in trials conducted in the USA the average water temperature was > 16°C.

3.4.4 Chlorine Treatment - Adult Mussels

Adult zebra mussel mortality after treatment with chlorine was monitored every 2–3 days for ten days and then weekly for five weeks until 80% mortality was reached (the plant's treatment goal). In treated chamber A, by day 16, the adult mortality was at 76.5% reaching 87% by day 49, and in treated chamber C, at day 16, mortality was 79% reaching 83% by day 49 (Figure 3.11).

A *t*-test showed there is no statistically significant difference between the resulting mortalities from treatment with Zequanox and chlorine ($p>0.05$). This confirms that Zequanox is equally effective as chlorine for controlling zebra mussels.

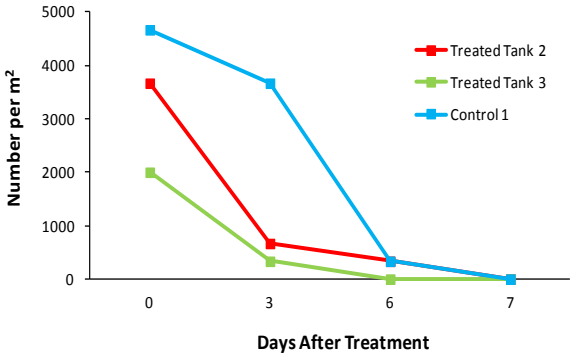

Figure 3.7 Mean number of juvenile mussels in the bioboxes after treatment with MBI 401 FDP.

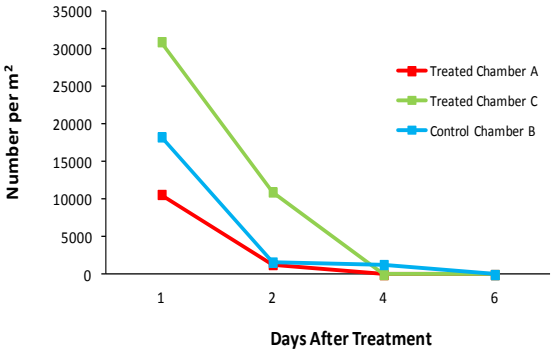

Figure 3.8 Mean number of juveniles in the water chambers after treatment with chlorine.

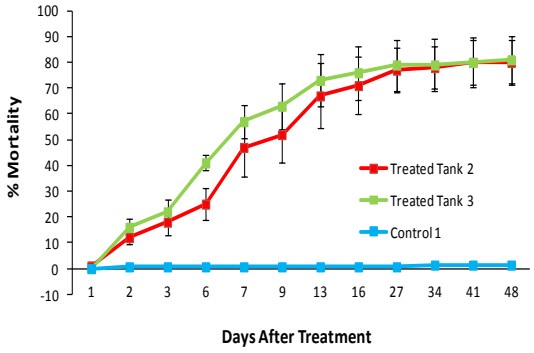

Figure 3.9 Mean mortality (± SD) of adult mussels in bioboxes after treatment with MBI 401 FDP.

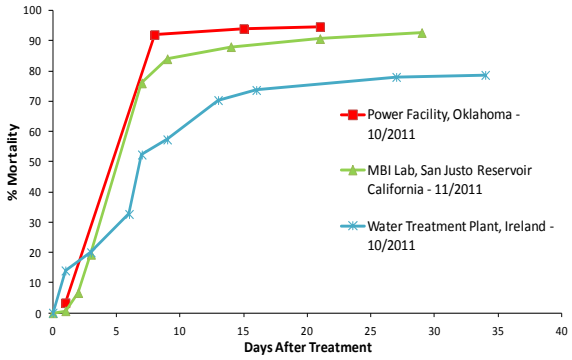

Figure 3.10 2011 biobox trials with MBI 401 FDP in North America and Ireland.

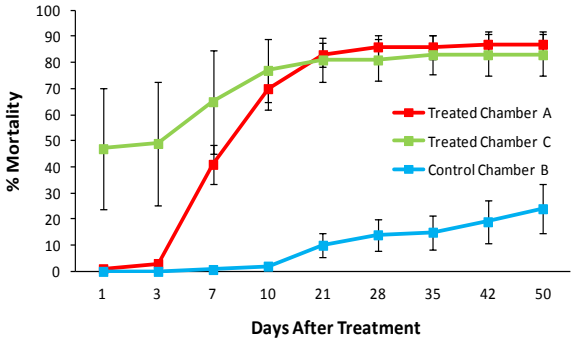

Figure 3.11 Mean mortality (± SD) of adult mussels in chambers after treatment with chlorine.

The low water temperature during chlorination (< 10°C) directly affects the length of time chlorination is required (Rajagopal et al., 2002) and the length of time it takes for mortality to reach > 70%. At the end of the monitoring period mortality in control chamber B was 24%. It is unknown why control mortality reached 24%; nevertheless, the high mortality attained in both the treated chambers indicates the treatment was effective. The rate of adult mussel mortality after chlorine treatment is on par with the mortality after MBI 401 FDP treatment.

Table 3.1 Water quality results before, during (4 and 8 hours) and after (3 rinses) biobox treatment with MBI 401 FDP. R1 = rinse 1, R2 = rinse 2, R3 = rinse 3.

Sample Date	Location	Turbidity (NTU)	Temp (°C)	BOD	TOC	pH	DO
Before Treatment							
	Control Tank 1	3.27	14.6	1.21	15	7.84	9.08
11-Oct	Treated Tank 3	3.18	14.5	2.94	10.6	7.82	9.23
4hr							
	Control Tank 1	2.79	14.5	1.48	15.2	7.76	8.93
11-Oct	Treated Tank 3	80.1	14.5	9	56.2	7.59	9.07
8hr							
	Control Tank 1	2.34	14.7	1.58	10	7.71	8.7
11-Oct	Treated Tank 3	79.3	14.9	8.81	54.6	7.59	8.81
R1							
	Control Tank 1	5.81	15	1.04	9.73	8.04	9.63
11-Oct	Treated Tank 3	7.36	15	2.14	10.3	7.83	9.68
R2							
	Control Tank 1	4.99	16	3.59	9.9	7.86	10.7
12-Oct	Treated Tank 3	4.48	15.9	3.22	9.91	7.89	10.6
R3							
	Control Tank 1	3.99	14.8	2.61	9.59	7.86	9.4
13-Oct	Treated Tank 3	4.35	14.6	1.17	9.41	7.96	9.22

3.4.5 MBI 401 FDP Treatment - Water Quality

Results of water quality parameters recorded in treated tank 3 and control tank 1taken before, during and after MBI 401 FDP treatment are presented in Table 3.1. These results, though gathered from samples in the static bioboxes, give an indication of the effects MBI 401 FDP would have on water quality if used in a similar static treatment in the raw water chambers of the Sligo drinking water treatment plant. However, if used in the plant the treated water would be discharged gradually back to the receiving lake, Lough Gill and would eventually be heavily diluted upon discharge. In treated tank 3, the temperature ranged from 14.5–15.9°C, and pH varied between 7.59 and 7.96. The turbidity ranged between 3.18 and 80.1 NTU. Dissolved oxygen varied between 8.81 and 10.61 mg/L. Biological oxygen demand (BOD) ranged between 1.17 and 9 mg/L and the TOC ranged between 9.42 and 56.2 mg/L.

Measurements of temperature, DO and pH did not differ by more than ± 1 unit before during and after treatment in the bioboxes; therefore, the treatment had little effect on these parameters. Turbidity did increase substantially; however, since turbidity and MBI 401 FDP concentration are strongly correlated, this increase was expected. After the three rinses, turbidity returned to background levels. An increase in turbidity is due to the nature of the product which is primarily composed of particulate organic matter.

A similar trend occurred with the BOD, which also increased to a peak of 9.00 mg/L during treatment at 4 hours and went down to 8.81 mg/L at 8 hours. Over time, it is expected that the BOD measurements would have continued to decrease as the dissolved organic matter degraded (Graham and Gilbert 2012). TOC followed the same pattern as BOD; at 4 hours it increased to 56.2 mg/L and then decreased to 54.6 mg/L at 8 hours. The TOC increased over the 8 hour treatment duration but decreased to background levels after the first rinse. This increase in TOC was expected as the product is primarily particulate organic matter.

3.5 Conclusions

Adult mortality reached 80% after treatment with both chlorine and MBI 401 FDP. The mortality of adults after chlorine treatment reached 80% by day 20. After MBI

401 FDP treatment, mortality was at 76% by day 20 and reached 80% by day 27. Mayer (2011) demonstrated that at lower water temperatures following treatment with *Pseudomonas fluorescens* mortality is slower. This was apparent in this trial when compared to those carried out in the USA (Figure 3.10) mortality at Cairns Hill was slower to occur as the water temperature was lower.

It must be remembered that MBI 401 FDP treatment duration was 8 hours and chlorine treatment duration was 7 days. MBI 401 FDP treatment can begin and end within the working day whereas chlorine treatment is a continuous 24 hours a day treatment, and in this instance, 7 days long. This does not include the set up and breakdown. Chlorine treatments require this longer application time because the zebra mussels recognise chlorine as a harmful substance and shut their valves and cease feeding (Rajagopal et al., 2003). Formulated *Pseudomonas fluorescens* CL145A cells (like those in MBI 401 FDP), however, are not recognised as harmful and the zebra mussels feed readily on them (Marrone Bio Innovations, 2012).

Studies indicate *Pseudomonas fluorescens* CL145A cells specifically target zebra and quagga mussels (Molloy et al., 2013a; Molloy et al., 2013b; Molloy et al., 2013c). In addition to many non-target studies carried out in the USA, (Molloy et al., 2013c) non-target trials carried out at IT Sligo in accordance with OECD and ASTM guidelines on 12 Irish aquatic organisms (some of which were collected from Lough Gill) show that calculated median effective concentration or median lethal concentration values were noted to be in excess of the treatment rates.

Chlorine is a general biocide; with its original purpose being a bleaching agent, chlorine gas was also used as a chemical warfare agent (Winder, 2001). Airborne chlorine gas at a concentration of 3 mg/L causes mild irritation of the mucous membrane (the concentration used in this study fits within this category), above 5 mg/L causes eye irritation, 15–30 mg/L causes a cough, choking and burning, and finally 430 mg/L causes death after just 30 seconds exposure (Winder 2001). *Pseudomonas fluorescens* CL145A cells are designated as "Biosafety Level 1" by the American Type Culture Collection, and are defined as "having no known potential to cause disease in humans or animals" by American Biological Safety Association.

This study shows that MBI 401 FDP was an effective alternative zebra mussel control method and could be used in place of chlorine treatments, or, in conjunction with chlorine treatments in an Integrated Pest Management program (IPM). As an example, for this Sligo water treatment plant, a final chlorine treatment or an MBI 401 FDP treatment at 100–150 mg a.s./L at the end of the season could be performed to control zebra mussels in the system. Followed by a yearly lower treatment concentration to control for any veligers or settled juveniles in the system.

Moving forward, this trial has offered a suitable alternative to chlorine and has shown MBI 401 FDP's effectiveness as a zebra mussel control option.

Chapter 4

Zebra mussel control using Zequanox® in an Irish waterway

4.1 Introduction to Paper

This chapter was published in part in the *'International Conference on Aquatic Invasive Species'* (ICAIS) edition of 'The Management of Biological Invasions' (volume 3, issue 5). It goes through the techniques and results of zebra mussel control using Zequanox in the Grand Canal at Tullamore Harbour. This paper is presented in its entirety in appendix F.
[http://www.reabic.net/journals/mbi/2014/3/MBI_2014_Meehan_etal_correctedproof.pdf].

Following on from the success of the biobox trial at Cairns Hill (Chapter 3) and the successful demonstration of a no negative impact to a number of organisms native to an Irish freshwater ecosystem (Chapter 2), the next step of this research was to look at an *in situ* treatment of invasion. All the research and work carried out thus far has allowed for the progression to an open water treatment, as it was important to first determine the potential effects of Zequanox to organisms present in the Grand Canal.

The Grand Canal at Tullamore Harbour was chosen as a trial site due to its accessibility (boat traffic could be diverted for the two day trial period) and the substantial mussel infestation present along the canal wall. A mesocosm set up was used to contain the treatment to the impacted areas only.

4.2 Introduction

The zebra mussel, *Dreissena polymorpha* (Pallas, 1771), is an invasive, aquatic bivalve mollusc, which has impacted freshwater ecosystems and water abstraction in all invaded countries including Ireland (Minchin et al., 2002b; Lucy, 2010; Lucy et al., 2014). The zebra mussel arrived in Ireland in the early 1990's (Minchin and Moriarty, 1998) in the lower River Shannon on the hulls of boats, most likely attached to used leisure crafts from Britain (Pollux et al., 2003). Inland waterway systems (canals) in Ireland have allowed for movement of the zebra mussel both of its own accord and by accidental movement, largely attributed to boaters and recreational anglers (Minchin et al., 2005). Not only is the zebra mussel causing problems for Ireland's rivers and lakes through their role as ecosystem engineers (Karatayev et al., 2002), but industries are also

suffering from the high costs of controlling these mussels (Aldridge et al., 2004). Currently chlorine is the most commonly used control method (Mackie and Claudi, 2010); however, its use is limited and is only suitable in enclosed systems (intake pipes) as it is a non selective general biocide and is lethal to all living organisms. Presently the only control method for zebra mussels in inland waterways is physical removal, and therefore, there is a need for a more efficient management option.

Marrone Bio Innovations (MBI), a company specialising in the development and commercialisation of natural biocides in Davis, CA, USA, is the commercial license holder for the invasive zebra and quagga mussel (dreissenid) control product Zequanox. The active ingredient in Zequanox is killed *Pseudomonas fluorescens* strain CL145A cells, which is lethal to dreissenid mussels but studies show it has minimal to no impact on other aquatic organisms (Molloy et al., 2013b; Molloy et al., 2013c). *Pseudomonas fluorescens* is present worldwide and commonly found in food. In nature, it is a harmless bacterial species that is known to protect the roots of plants from disease (Marrone Bio Innovations, 2012). Ecotoxicology studies were carried out in IT Sligo and in the USA, where Zequanox was tested on a number of aquatic species. No negative effects were observed at concentrations required to sufficiently control zebra mussels (150 mg active ingredient/L) (Marrone Bio Innovations Ecotoxicology Studies, 2012). Additionally, Molloy et al. (2013c) carried out a number of non target trials using the active ingredient in Zequanox (*Pseudomonas fluorescens* CL145A) and again found no negative impacts to the organisms tested at concentrations required to control zebra mussels.

In March, 2012 the United States Environmental Protection Agency registered Zequanox for use in the USA in enclosed or semi-enclosed systems. In 2011, successful Zequanox trials were conducted within the cooling water system of Davis Dam in Bullhead City, Arizona in the USA, and in 2012 within the cooling water system of DeCew II Generating Station of Ontario Power Generation in St. Catharine's, Ontario, Canada. MBI also conducted a successful open water trial in Deep Quarry in DuPage County, Illinois, USA in 2012; this open water trial was similar to the canal trial described in this report.

Tullamore Harbour is part of the Grand Canal, connecting the east of Ireland to the Shannon River navigation in central Ireland. It was traditionally used for transporting

goods via barge boats, and now is solely used for leisure purposes (Byrne, 2007). The Grand Canal at Tullamore Harbour has a zebra mussel infestation spanning from under the bridge, along the harbour branch of the canal, and into a harbour and dock area (Figure 4.1).

A pilot demonstration trial using Zequanox was conducted under the bridge in the Grand Canal at Tullamore Harbour treating two infested sites either side of the canal wall under the bridge, a control site was chosen further along the docking area (Figure 4.1). The objectives of this trial were to firstly demonstrate an effective method of zebra mussel control in inland waterways and secondly trial a method which could be used for zebra mussel fouled jetties, pontoons and navigational structures

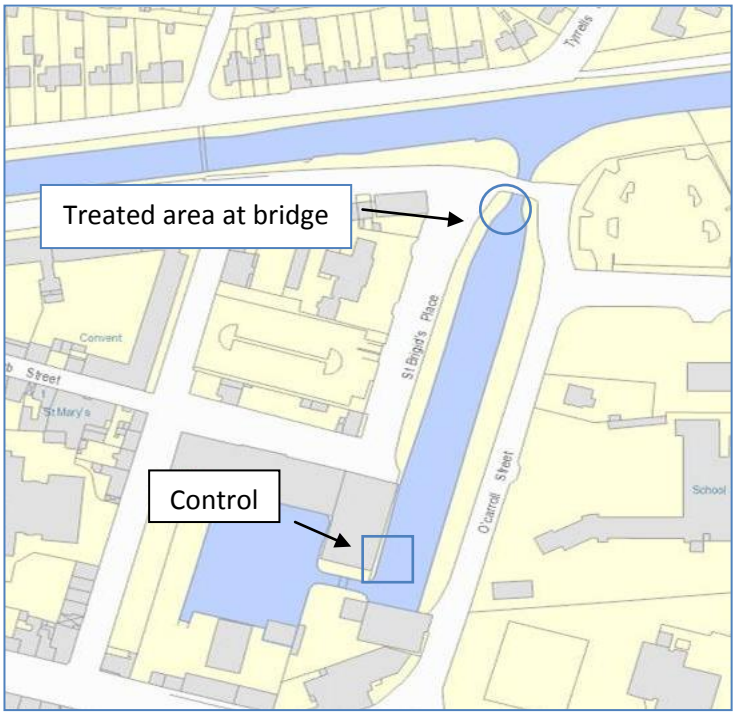

Figure 4.1 Tullamore Harbour, Co. Offaly, Ireland (Ordnance Survey Ireland).

4.3 Materials and Methods

4.3.1 Experimental Set-up

This trial was conducted under the bridge at Tullamore Harbour (53º27’82”N,-7º48’86”W); where dreissenid infested canal walls on both banks were treated with Zequanox to test its effect on settled juveniles, seeded adult mussels and naturally settled adult mussels. The areas of the canal were labeled treatment side 1, treatment

side 2 (treated areas under the bridge) and control. Two impermeable curtains were set up to enclose the treatment area (canal wall). These curtains were comprised of an impermeable material (scaffband), which was weighted down with stainless steel chains at the bottom and attached to aluminum at the sides, with foam used to seal in the containment area (Figure 4.2). The curtains were on average 7.70 m in length, 0.45 m in width and 1.31 m in depth, so that approximately 4.5 m^3 (4500 L) of water was enclosed along each concrete wall. The curtains were set up one day in advance of treatment to allow the mussels to acclimatise and resume normal feeding behavior prior to treatment. Flow in the canal is wind induced and as the weather was calm was not a consideration during this trial (Scobey, 1939).

The infested canal walls under the bridge at Tullamore Harbour were treated with Zequanox at a target concentration of 150 mg active substance (a.s.)/L (active substance is synonymous with active ingredient). The target concentration was maintained for 8 hours. This treatment concentration and duration was based on the results of trials carried out in North America and in Ireland (Meehan et al., 2013).

Figure 4.2 Impermeable curtains used to hold treated water within treatment area along canal walls (photo by Sara Meehan).

4.3.2 Juvenile Mussel Collection

PVC plates were deployed in Lough Key (53°593'04"N, 08°16'46"W) on July 23rd, 2012 to gather juvenile zebra mussel settlement, as this lake is known for high settlement (Lucy, 2005). These plates were removed from Lough Key on September 2nd,

2012 and an initial baseline count was made. These plates were then transported to the Grand Canal at Tullamore Harbour and placed in the two treatment areas and the control area on weighted rope (Figure 4.3). Juvenile plates were counted 24 hours after treatment then daily followed by weekly counts until juvenile settlement reached zero.

Figure 4.3 PVC plates used to monitor juvenile survival (photo by Sara Meehan).

4.3.3 Adult Mussel Collection

Adult zebra mussels were collected from the Grand Canal at Tullamore via a long-handled scraper (Minchin, 2007; Minchin et al., 2002a) and by hand removal from the wall while wading. Healthy mussels were then seeded into three mesh cages (mesh size 3mm), each containing three compartments housing 50 mussels each (Figure 4.4). These mesh cages were attached to bricks via cable ties. Floating rope was then tied to the bricks so the cages could be easily removed from the canal using a boat hook; this method was developed so the cages would not be visible to the public as they were to remain in the canal for an extended period of time. Once the mesh cages were ready, they were left to acclimatise overnight in the canal. One cage was placed in the control area, and one in each treatment area. Mussels were checked for mortality before treatment and any dead ones were removed and replaced with live healthy ones. Mussels were presumed dead if shells were open and did not close after being gently prodded. After treatment seeded adult mussels were counted first daily then weekly for seven weeks.

Figure 4.4 Mesh cages to hold seeded adult mussels (26cm in length) (photos by Bridget Gruber)

4.3.4 Naturally Settled Adult Mussels

The number of naturally settled adult mussels in the two treated areas and the control area was estimated prior to treatment using 25 cm x 25 cm quadrats. Three quadrats per defined area were used to estimate mussel settlement/m^2. Quadrats were placed at random and at different depths by divers. Divers counted the number of live mussels within each quadrat. A record of the exact spot the quadrats were placed was kept by measuring its distance from a pre-determined point along the bank and the depth at which the quadrat was placed. Photographs were also taken so that the same quadrats could be counted again after treatment. Quadrats were re-counted seven weeks after treatment.

4.3.5 Zequanox Application

The curtains were placed in the canal 24 hours prior to treatment to allow the naturally settled mussels to resume normal behavior after the disturbance of the curtain placement. Twenty four hours after the curtains were placed in the canal (before treatment), dissolved oxygen (DO) inside the curtained areas had significantly reduced and was approximately 3 mg/L lower than the DO outside of the curtains. This was likely due to the natural diurnal cycle and flow restriction. Therefore, treatment side 1 was aerated with bubblers until the curtains were removed to ensure DO stayed at background levels, whilst on treatment side 2, DO was not controlled and no aeration occurred. This experimental design allowed us to quantitatively determine if observed mortality could be attributed to Zequanox, or whether the observed mortality could be

attributed to low DO levels. It also allowed us to infer if water quality conditions impacted zebra mussel ingestion of Zequanox.

Zequanox, a dry powder formulation (as registered in the US), was used to treat the canal walls. The powder was mixed on-site with canal water to create the following stock solution concentration:

$C_1V_1 = C_2V_2$ where

C_1 = target treatment concentration (mg a.s./L)

V_1 = volume of treatment area (4500 L)

C_2 = stock concentration (100 g a.s./L)

V_2 = volume of stock concentration to be applied (L)

For each curtained off area a total of 675 g a.s. of Zequanox was mixed with 6.75 L of canal water using a small hand blender to achieve a concentrated product solution of 100 g a.s./L. This solution was slowly poured into the curtained off area so as to evenly distribute the product. Once all the product was in the water, a wooden paddle was used to gently mix the treated water to achieve an even distribution of product within the treated area. As turbidity and treatment concentration have a linear relationship (Meehan et al., 2013), turbidity inside the curtains was monitored throughout the application process using a Hach 2100Q portable turbidimeter to ensure the target concentration was reached and maintained.

As flow in the canal increased, nominal leakage of product from within the curtain occurred and concentrations within the treatment area decreased. This leakage likely occurred due to an increase in wind speed or the passing of a barge along the canal. In order to maintain a target concentration of 150 mg a.s./L, additional product was mixed in two stages and added.

After the 8 hour treatment period in which Zequanox concentrations were maintained at 150 mg a.s./L, the curtains were then held in place for a further 16 hours (but no additional product was added) making the hold time 24 hours in total. This additional hold time allowed for natural degradation of the product. Studies indicate that, once Zequanox is wetted, it biodegrades rapidly and the efficacy significantly decreases after

8 hours in water and after 24 hours in water, it is no longer efficacious. After the 24 hour hold time, the curtains were removed and, based on water quality measurements, the product dispersed to non-detectable levels within the canal system.

4.3.6 Water Quality Measurements

Turbidity inside the treatment area was monitored throughout the application and post-treatment period with a Hach 2100Q portable turbidimeter; as turbidity and concentration are correlated this ensures that the target concentration was reached and maintained throughout the application period, and that Zequanox had dispersed to non-detectable levels after the curtains were removed.

Additional water quality measurements were taken before treatment, during treatment (at 4 and 8 hours), 24 hours after treatment before the curtain was removed, and 24 hours after the curtain was removed. These water quality measurements included: temperature, dissolved oxygen (DO), pH, turbidity, biological oxygen demand (BOD), and total organic carbon (TOC).

Dissolved oxygen, pH and temperature were measured with an Orion 5 star meter. The analysis of BOD and TOC was subcontracted out to Alcontrol Laboratories. Method 5210B, AWWA/APHA, 20th Ed., 1999; SCA Blue Book 130 was used to determine BOD. US EPA Method 415.1 & 9060 was used to determine TOC.

4.4 Results

4.4.1 Juvenile Mussels

Figure 4.5 and Table 4.1 show the mean juvenile counts for the treatment and the control areas. Juvenile numbers were high (over 8,000/m^2) 48 hours in advance of the trial. Between 48 hours and the first count carried out after treatment, survival dropped considerably for both the treated juveniles and the control juveniles. After this initial drop, juvenile survival in the treated areas continued to decrease, while juvenile survival in the control area stayed approximately the same between 05/09/12 and 07/09/12.

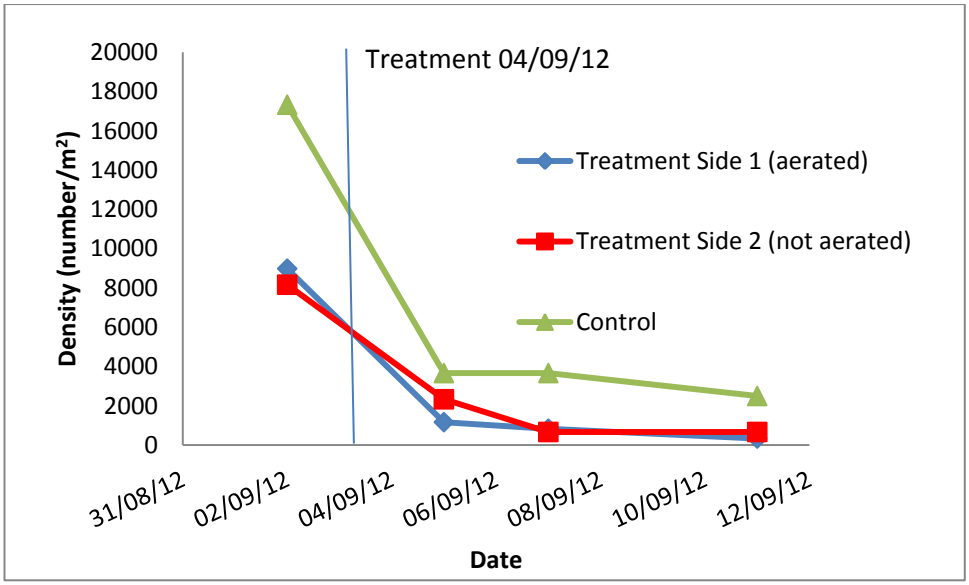

Figure 4.5 Mean density of juveniles before and after Zequanox treatment

Table 4.1 Mean density of juveniles before and after Zequanox treatment with standard deviation (juveniles/m^2)

Date	Treatment Side 1 (aerated)	SD	Treatment Side 2 (not aerated)	SD	Control	SD
02/09/2012	8983	4820	8167	2593	17333	3300
05/09/2012	1167	236	2333	943	3666	2828
07/09/2012	833	236	667	0	3667	0
11/09/2012	333	471	667	471	2500	1179
% Survival	4		8		14	

4.4.2 Adult Mussels

Seeded Adult Mussels

After 55 days, treatment side 1 had 75% seeded adult mussel mortality and treatment side 2 had 56% mortality. The mortality in the control was 9% (Figure 4.6).

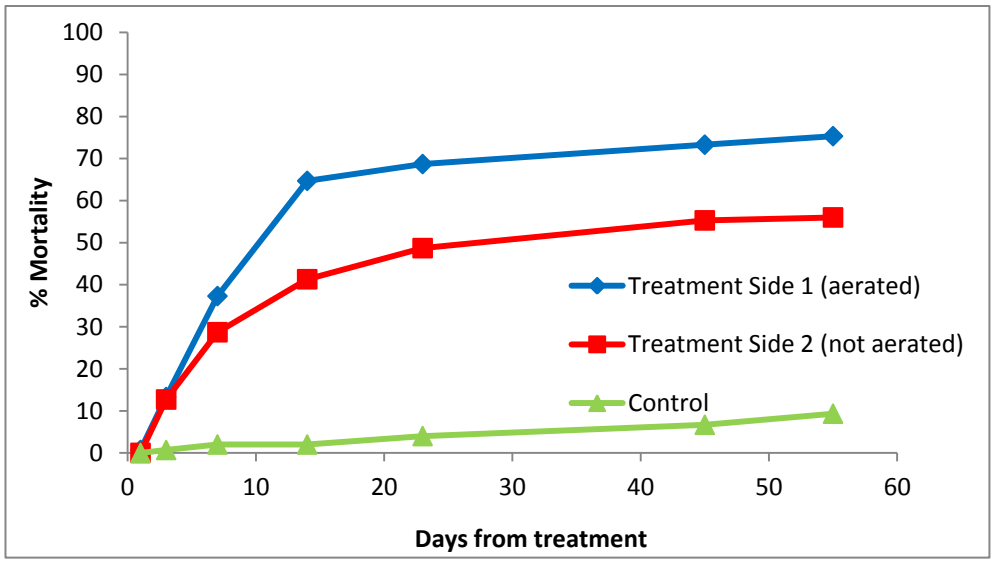

Figure 4.6 Seeded adult mussel mortality after treatment with Zequanox

Naturally Settled Adult Mussels

Table 4.2 shows the mean number of naturally settled mussels before and after treatment within the treatment areas and the control. The mean numbers of live adult mussels decreased by approximately 46% in treatment side 1, and by approximately 65% in treatment side 2. The mean number of live mussels decreased by 15% in the control area (this amounts to one less mussel observed in the control area after treatment).

Table 4.2 Mean density of naturally settled adult mussels (live adult mussels/m^2) before and after Zequanox treatment with standard deviation (SD)

Date	**Treatment Side 1 (aerated)**	SD	**Treatment Side 2 (not aerated)**	SD	**Control**	SD
03/09/2012	1000	662	272	136	69	37
22/10/2012	539	272	96	34	59	24
% Mortality	46		65		15	

4.4.3 Water Quality

In treatment areas 1 and 2 the temperature ranged from 17.8 to 18.6°C; in the control area the temperature ranged from 17.1 to 19.6°C (Table 4.3). In treated areas, pH varied between 7.58 and 8.03, similar to the range seen in the control area (7.76 -7.88). Dissolved oxygen in treatment side 1 (aerated side) ranged from 5.6 to 7.68 mg/L. Dissolved oxygen levels in treatment side 2 (not aerated) ranged from 2.38 to 7.58 mg/L. In treatment side 2, 24 hours after treatment, DO dropped to 2.38 mg/L. Once the curtain was removed DO levels increased to 7.58 mg/L (background levels). Biological oxygen demand in the treated areas ranged between < 2 and 103 mg/L. Total organic carbon ranged from 20.7 to 49.5 mg/L. The turbidity in the treated areas before treatment was < 3 NTUs. During treatment, the turbidity in the treated areas increased and ranged between 59.9 and 127 NTUs. Approximately 24 hours after treatment, prior to curtain removal, turbidity decreased to 26.5 and 32.3 in the treated areas. Once the curtains were removed, within 24 hours, turbidity decreased to 9.31 and 8.19 NTUs. The turbidity of the control throughout the 48 hour monitoring period ranged from 3.74 to 8.78 NTUs.

Table 4.3 Water quality measurements before, during (4 and 8 hours) and after treatment (before and after curtain removal)

Sample Date & Time	Location	Turbidity (NTU)	Temp (°C)	BOD (mg/l)	TOC (mg/l)	pH	DO (mg/l)
Before treatment							
4-Sep, 09:30	Control	4.97	17.1	<2	22.8	7.76	7.22
04-Sep, 09:30	Treated 1	2.72	17.8	<2	21.7	8.03	5.6
04-Sep, 09:30	Treated 2	2.97	17.9	<2	20.7	7.82	3.84
4 hrs into treatment							
04-Sep, 14:00	Control	4.26	19.3	<2	21.6	7.85	8.2
04-Sep, 15:00	Treated 1	109	18.4	91.5	49.5	7.59	7.15
04-Sep, 14:10	Treated 2	125	18.2	71	43.3	7.68	4.29
8 hrs into treatment							
04-Sep, 18:09	Control	3.74	19.6	<3	21.1	7.83	8.44
04-Sep, 18:56	Treated 1	127	18.5	103	31.9	7.93	7.63
04-Sep, 18:20	Treated 2	59.9	18.6	28.6	23.1	7.85	5.08
24 hrs after treatment; before curtain removal							
05-Sep, 07:45	Control	8.78	17.9	<2	20.8	7.87	6.85
05-Sep, 08:00	Treated 1	26.5	18.3	13.1	22.3	7.84	7.68
05-Sep, 07:55	Treated 2	32.3	18.5	17.1	25.3	7.58	2.38
24 hrs after curtain removal							
06-Sep, 12:00	Control	5.12	17.9	3.2	21.6	7.88	7.18
06-Sep, 12:00	Treated 1	9.31	18	3.65	22.7	7.63	7.42
06-Sep, 12:00	Treated 2	8.19	18.1	<2	22.3	7.85	7.58

4.5 Discussion

4.5.1 Juvenile Mussel Survival

Juvenile survival on the treated plates and the control plates initially declined after treatment. After this decline, control survival leveled out and survival on the treated plates continued to drop. There is no way to determine if any of the mortality during the initial decline in survival is due to Zequanox treatment therefore it must be assumed that

it is due to outside influences namely the transportation of the plates to the treatment site. However the continued decline of settlement on the treated plates was due to Zequanox as the control survival was maintained. These results parallel studies conducted by MBI at Davis Dam (Arizona, US) where a decline in juvenile survival on settlement plates treated with Zequanox was observed, and a study carried out in Sligo, Ireland (a demonstration trial for a water treatment plant) where juvenile survival after treatment with Zequanox decreased (Meehan et al., 2013). It is also important to note that seasonal plates are known to underestimate total natural settlement but are considered a good proxy (Lucy et al., 2005). The initial high mortality in both the treated and control plates is not representative of what would happen in a real time application as there would be no movement of settlement plates from one site to the other. Therefore further research is necessary to examine the effects of Zequanox on settled juveniles *in situ*.

4.5.2 Adult Mussel Mortality

Seeded mussel mortality was observed in treatment side 1 (aerated) and 2 (not aerated); however, mortality was greater on treatment side 1 (75%) than side 2 (56%). Several factors may have contributed to this difference. The lower DO levels on treatment side 2 may have disturbed the mussel's feeding, by causing them to shut their valves as a response to unfavorable conditions as is the case with intermittent chlorination (Rajagopal et al., 2003). Zequanox must be ingested by the mussels to have an effect. Mixing and aeration may also have contributed to the difference in mortality, making Zequanox more bioavailable throughout the treatment area. On treatment side 2 only hand mixing aided in the distribution of the product whereas aeration on treatment side 1 may have helped to more evenly distribute Zequanox.

A decrease in naturally settled mussels after treatment with Zequanox was observed; however, in contrast to the seeded mussel mortality, more mortality occurred in treatment side 2 (65%) than in treatment side 1 (46%). This may have been due to the aeration bubblers and air tubing on treatment side 1 being located close to the wall thus disturbing the mussel's feeding and causing them to shut their valves and cease feeding. The seeded adult mussels on the aerated side 1 were located at the bottom of the canal away from the direct interference from the aeration system this would account for the difference in mortality between the seeded and naturally settled mussels.

4.5.3 Water Quality

No negative impact from Zequanox treatment to temperature or pH was observed. The temperature range seen in the treated and control areas was consistent with the natural diurnal and seasonal cycles in Ireland. The slightly higher temperatures in the control area was likely due to that area being in direct sunlight while the treated areas were under the bridge and therefore had less sun exposure. The difference in sunlight had no apparent impact on pH levels. The zebra mussels in this study (seeded and naturally settled) at all sites were present at depths of between 1.0 -1.5m and due to low water transparency were at naturally low light levels. In fact the divers required torch light to take samples on both sampling dates. Therefore sunlight is not considered a varying environmental factor in this study.

During treatment, the turbidity in the treated areas increased (since Zequanox is made up of organic material, turbidity was expected to increase significantly) and ranged between 59.9 and 127 NTUs. After treatment was terminated, but prior to curtain removal, turbidity, as expected, began to decrease due to natural degradation of the product. Once the curtains were removed, within 24 hours, turbidity dropped to control levels.

Aeration sufficiently controlled DO levels in treatment side 1. In treatment side 2, 24 hours after treatment, DO dropped to 2.38 mg/L. This was expected as Zequanox is comprised of dead bacterial cells that degrade in the natural environment causing a decrease in DO, particularly in low flow environments. However, once the curtain was removed and flow was restored, DO increased to background levels.

TOC increased in treated areas four hours into the treatment; however, by eight hours TOC levels were decreasing to background levels. This increase again was expected because Zequanox is primarily made up of particulate organic matter, TOC levels decreased as degradation of the product took place. Since Zequanox is organic in nature, biological oxygen demand also followed a similar pattern, increasing at 4 hours into treatment and then decreasing as time passed and Zequanox was degrading.

Environmental monitoring before, during, and after treatment indicated there was minimal impact to water quality in the canal. Though TOC, BOD, and turbidity temporarily increased during treatment in the enclosed treatment areas, by 8 hours,

measurements were decreasing and returned to background levels 24 hours after treatment once Zequanox had naturally biodegraded.

4.6 Conclusions

Presently the only zebra mussel control option for canals in Ireland is mechanical removal. This study shows that Zequanox effectively controlled up to 75% of zebra mussels in an Irish canal. Though Zequanox is not yet registered in the EU, it has potential as an alternative control option for Irish waterways; the results of the study show that when Zequanox is applied under the correct conditions (sufficient DO levels and minimal disturbance to the mussels) it can be an effective zebra mussel control method for inland waterways and structures.

Future recommendations for a similar trial would include aeration in all enclosures ensuring that the aeration occurs a sufficient distance from settled mussels so as to cause minimal disturbance to the mussels. Also, settlement plates should be removed less frequently and allowed more time to acclimatise after plate transportation so as to avoid high levels of control mortality. This trial was the first canal treatment with Zequanox and the methods used here support further development of similar application techniques for static, contained, and open water treatments.

Chapter 5

Comparison of European and North American Zebra Mussel Populations Response to Zequanox®

5.1 Introduction

Many similarities and dissimilarities exist between North American and European zebra mussel populations in terms of temperature ranges in the waters they inhabit and their consequent filtration rates and reproductive cycles (Marsden, 1992; Mackie and Schloesser, 1996; Lucy and Sullivan 2001; Garton et al., 2013). It is considered that for implementing successful control of zebra mussels with Zequanox, the treatment of early stages as well as adults is a key approach for long-term effectiveness. Therefore the reproductive pattern within the specific waters must be understood. Reproduction occurs annually in both North American and European mussel groups, following spawning in late spring/early summer mainly when the temperature reaches >12°C (Borcherding, 1991; Garton and Haag, 1993; Nichols, 1996, Ram et al., 1996; Karatayev et al., 1998). The timing of spawning and temperatures at which it occurs are highly variable between different mussel populations (Borcherding, 1991; Garton and Haag, 1993) and can occur at temperatures up to 22°C (Borcherding, 1991; Sprung,1989 cited by Nichols, 1996, Ram et al., 1996, Lucy, 2005). In parts of North America as the waters are warmer this initial spawning threshold of 12ºC is reached earlier (Borcherding, 1991) than in Ireland where water temperatures can still be <12ºC in May (Lough Key) (Lucy, 2005). In Ireland the typical reproductive period lasts from June to September (Lucy, 2006). It is noted that where water temperatures are warmer and persist for longer, spawning can occur earlier than colder waters and last past the summer months (Garton and Haag, 1993; Nichols, 1996) making it not as seasonal in nature as in Europe and more northerly North American populations e.g. in the Great Lakes where the climate is either continental or temperate (Nichols, 1996; Ram et al., 1996). In Ireland, Zequanox treatments for this research were timed after the zebra mussel reproductive season to target early life stage juvenile settlement as well as adult mussels.

Filtration rates like reproduction capabilities are correlated with water temperature and increase as water temperatures rise (Borcherding, 1991; Holland, 1993; Neuman et al.,

1993; Nichols, 1996; Fanslow et al., 1995; Costa et al., 2008). Rajagopal et al. (2002) found that between autumn and spring during low water temperatures where zebra mussels were not in the reproductive phase, they were more tolerant of biocides due to less exposure to toxins because of their lower metabolic and filtration rates. At a temperature of 15◦C Kraak et al. (1994) found that filtration rates per mussel were on average 100 ml/h. Fanslow et al. (1995) found that at 13◦C filtration rates were 46 ml/h and overall the highest filtration rates were found between 10◦C and 20◦C. This demonstrates that the treatment timing for zebra mussels is imperative in order to maximise mortality and that this timing is linked to water temperatures both in terms of filtration rates and reproductive cycles. Therefore in warmer parts of North America (Colorado) Zequanox can be applied across all seasons as the mussels are continually spawning and are metabolically active. However at the end of the zebra mussel reproductive period in Ireland (September) (Lucy, 2006) the timing of treatment to target juveniles and adults has a narrow window as water temperatures are decreasing rapidly (18◦C in September to 4◦C in December) meaning that maximum mortalities may not be achieved because the mussels are not as metabolically active and consequently do not ingest as much Zequanox.

Trials to test the effectiveness of Zequanox in combating *in situ* zebra mussel invasions have previously been carried out in North America and Ireland. To briefly review this research, the first in Ireland was a biobox trial at a drinking water treatment plant in County Sligo (Meehan et al., 2013) to mimic the effect a Zequanox treatment would have inside infested raw water chambers. This treatment resulted in 80% adult mortality. The second in Ireland was an open water trial at the Grand Canal, Tullamore Harbour, where treatment of the infested walls of the canal took place, resulting in average; 55% mortality of settled adults and 66% mortality of seeded adults (Meehan et al., 2014b). Similar biobox trials were carried out in North America at Lake Mead Fish Hatchery in Henderson, Nevada, USA where 81.9% mortality was achieved, and an *in situ* trial similar to the Irish one was carried out at Deep Quarry Lake, Illinois, USA and achieved 97.1% mortality (Marrone Bio Innovations, unpublished). The difference in the final mortality is apparent, with North American trials resulting in higher levels of mortality. Water temperatures during Zequanox application and the post monitoring period were noted as a possible reason. Zebra mussels are more metabolically active in warmer weather and therefore will consume more Zequanox. During the biobox trial at

the drinking water treatment plant Sligo, water temperature ranged from 13.8°C – 15◦C. Water temperatures ranged from 17.1◦C - 19.6◦C during the Tullamore Harbour trial. For the biobox trial at Lake Mead Fish Hatchery in North America water temperature was on average 24.8◦C and for the open water trial in Deep Quarry Lake, Illinois water temperatures ranged from 27.2◦C - 29.2◦C. A clear pattern is evident; increasing water temperature is positively correlated with increasing mortality due to Zequanox. It was necessary to test this hypothesis in order to ensure the variance in mortality was not due to a difference in biology between the two populations. The variance in natural water chemistry between Ireland and North America was also accounted for to determine whether it contributed to mortality.

The main objectives of this trial were to determine the Zequanox response relationship of zebra mussels collected from Ireland (Lough Conn, Co. Mayo) with those collected from North America (San Justo Reservoir, San Benito County, California), treated under identical conditions and to calculate and compare clearance rates for both populations. This will determine if the difference in treatment response is due to the biology of the mussels or due to the treatment conditions (temperature). If the lower water temperature in European tests is deemed the cause of the difference in mortalities, recommendations can be made to optimize treatments in Europe.

5.2 Materials and Methods

This research was carried out in two labs, the North American mussel assay was conducted at Marrone Bio Innovations, Davis, CA, USA as a collaboration between the author and research associates employed by MBI. The European mussel assay was carried out at the Centre for Environmental Research Innovation and Sustainability (CERIS) in the Institute of Technology Sligo.

5.2.1 Zequanox Application

Zequanox (Marrone Bio Innovations, Davis CA.), a dry powder formulation (as registered in the US), was used to treat the zebra mussels. The powder was mixed on-site with US EPA (United States Environmental Protection Agency) standard dilution water (United States Environmental Protection Agency, 2002) to create the following stock solution concentration:

$C_1V_1 = C_2V_2$ where

C_1 = target treatment concentration milligrams active ingredient per liter (mg a.i./L)

V_1 = volume of treatment area (0.250 Litres [L])

C_2 = stock concentration grams active ingredient per liter (g a.i./L)

V_2 = volume of stock concentration to be applied (L)

1.1025 grams (g) was mixed with 110.25 millilitres (ml) of water and the appropriate amount was dispensed using a pipette into each treatment chamber to achieve the desired treatment concentration; a separate batch of product was made up for the clearance study.

5.2.2 Jar Assays

The bioassays to determine the response relationship of European and North American zebra mussels was performed according to *MBI-RD-0002-SOP Dreissena Jar Bioassay Standard Operating procedure* (MBI personal communication). North American zebra mussels were collected from suspended PVC plates in San Justo Reservoir, from a depth of 3 - 4.5 metres (m) on August 23, 2013 and given three weeks to acclimatise in the lab. European zebra mussels were collected from Lough Conn using a long handled scraper (Minchin et al., 2002a) on August 25, 2013 and left to acclimatise for eight days. The European mussels (N = 60) were on average 16.8 mm in length and 0.84 g in weight. The North American mussels (N = 60) were on average 21.23 mm in length and 1.64 g in weight.

Test chambers were 500 ml jars containing 250 ml of water, with 20 mussels per chamber. Mussels were picked at random for their test chambers therefore representing population differences. There was a minor difference in test chamber set up, i.e. test chambers at MBI Davis were housed in a closed treatment cabinet (Figure 5.1), and at IT Sligo in a water bath, covered with plastic sheeting keeping mussels in the dark (to encourage feeding) (Horgan and Mills, 1997) (Figure 5.2). Zebra mussels were treated in triplicate at concentrations; 50 mg a.i. /L, 100 mg a.i. /L, 150 mg a.i. /L, and 200 mg a.i./L. The assay also included three untreated controls. For both assays the test water was made in accordance with U.S. Environmental Protection Agency standard dilution water (US EPA, 2002) and conducted at a temperature of 20°C±1 °C (ambient lab

temperature at MBI Davis). A water bath and fans were used to maintain this temperature during treatment at IT Sligo, where after mussels were placed in a 20°C±1 °C incubator. To insure the correct temperature was maintained during treatment at MBI, the temperature of three random jars was recorded every hour for the first eight hours and then again at 24 hours, by turning off the lights to minimally disrupt mussel feeding. At IT Sligo a temperature probe was continually kept in one of the test chambers and temperature recorded every hour. A *t*-test was applied to determine if there was a statistically significant difference between the mortality means of the two populations.

Figure 5.1 Treatment cabinet with water bath

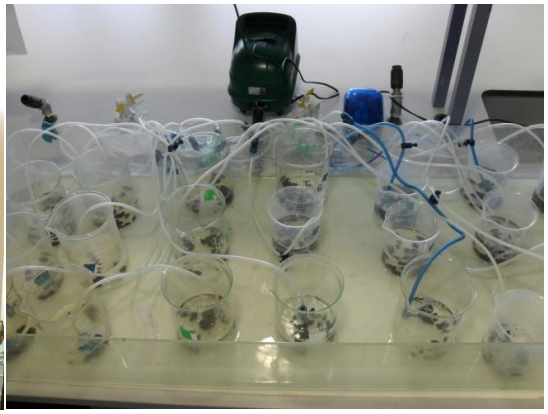

Figure 5.2 Test chambers in water bath (photos by Sara Meehan).

5.2.3 Clearance Rates

To compare how much product was cleared by the two mussel populations, three beakers were set up at each test location at 18 °C ± 1 °C containing 20 mussels each and treated with 200 mg a.i/L. Average length and weight was recorded for each population. As Zequanox is comprised of organic material, it is known that turbidity and Zequanox concentrations are strongly correlated, therefore turbidity readings were taken every hour for eight hours and a final reading at 24 hours. For both populations all the lights in the lab were switched off to minimally disrupt mussel feeding, the test chambers were gently swirled and 15 mL of treated water removed using a pipette. The sample was placed into a clean cuvette and turbidity measured using a Hach Turbidimeter. The sample was then poured back into the corresponding jar to retain the treatment volume.

A site specific linear regression was developed to determine the linear relationship between product concentration and turbidity (Figure 5.3) (This was done according to *MBI-RD-4008-SOP Turbidity and MOI-401 Active Ingredient Correlation and Application Monitoring Procedure* [MBI personal communication]). Using the linear regression equation (Figure 5.3), concentration of product left in each jar every hour for the first eight hours and then again at 24 hours was calculated. The average clearance rate of the mussels in L/hour was then determined from the reduction of Zequanox concentration as a function of time using the formula;

FR = [V/n*t]*[(ln Conc0 – ln Conct) – (ln Conc0’ – ln Conct’)] (Coughlan, 1969) where;

FR = Filtration Rate (L/mussel/hr)

V = Suspension Volume (L)

n = Number of Mussels (mussels)

t = Length of Time between Measurements (hr)

Conc0 = Initial Concentration (mg a.i/L)

Conct = Final Concentration (mg a.i/L)

Conc0’ = Initial Control Concentration (mg a.i/L)

Conct’ = Final Control Concentration (mg a.i/L)

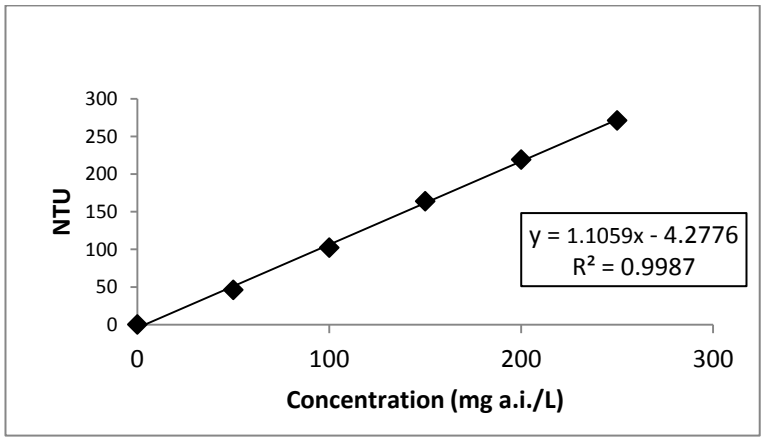

Figure 5.3 Turbidity (NTU) Linear Regression showing validity of the relationship between Zequanox and turbidity.

A *t*-test was applied to determine if there was a difference in clearance rates between the European and North American mussel populations.

5.3 Results

5.3.1 Jar Assays

An increase in mortality was correlated with an increase in treatment concentration for both European and North American assays (Figure 5.4). Zebra mussel mortality increased from 23% at 50 mg a.i/L to 88% at 200 mg a.i/L for the North American assay. The European zebra mussel mortality increased from 33% at 50 mg a.i/L to 77% at 200 mg a.i/L, however in this instance the mortality in jars treated at 150 mg a.i/L was higher at 85%. As can be seen in Figure 5.4 there is no distinctive mortality pattern between the two mussel populations. For the European mussels, mortality was higher than the North American mussel assay at treatments of 50 mg a.i/L and 150 mg a.i/L, whereas in the in the latter the mortality was higher in treatments of 100 mg a.i/L and 200 mg a.i/L. A *t*-test showed there is no statistically significant difference between the means of the two mussel populations, $p>0.05$.

The standard deviation was high in some instances, 29% for North American mussels treated at 150 mg a.i/L and 28% for the European mussels treated at 100 mg a.i/L. A standard deviation of ≤ 15% is usually achieved: in 2013 MBI analysed the variability of past in-house assays carried out in 2011 and 2012 and found that 75 % of summer variability values and 71% of winter variability values fell between ±0 and ±15% mortality. The control mortality values for this study stayed below 10 % (≤3%) which is required for most assays to be valid according to numerous OECD test guidelines (OECD; 1992, 2004a, 2004b, 2011).

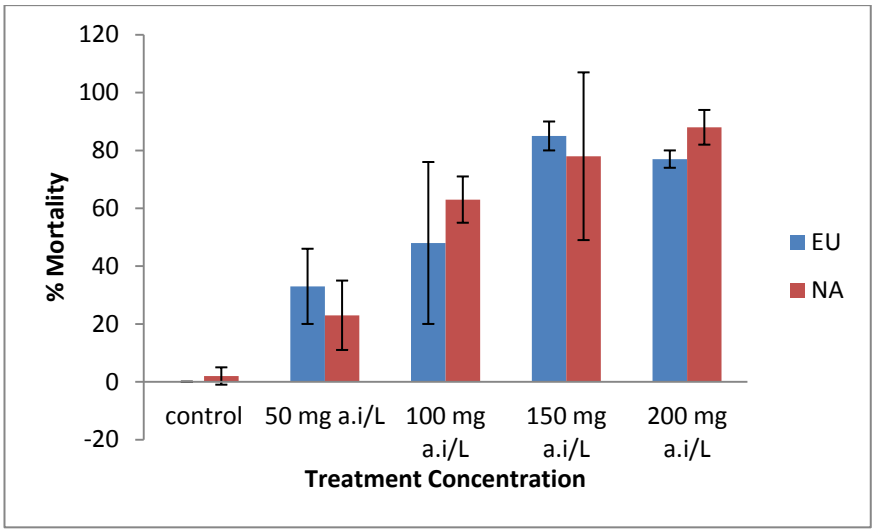

Figure 5.4 Percentage mortalities of North American (NA) and European (EU) Zebra mussels treated with MBI-401

5.3.2 Clearance Study

One hour after treatment a large difference in turbidity and product concentration was noted between the European and North American test beakers (Figure 5.5 Table 5.1). The higher turbidity in the European test chambers indicated a lower filtration rate here compared to the North American mussels. This pattern continued until the turbidity reading at 24 hours where the turbidity readings were similarly low for both the European and North American mussels. Natural degradation of Zequanox was evident from the control test chamber, where turbidity was reduced from 202.2 to 127.7 NTU. Zequanox is composed primarily of particulate organic matter therefore natural degradation over time occurs resulting in loss of product and therefore a reduction in turbidity readings. The calculated clearance rates as per Coughlan (1969) shows that on average the North American mussels were clearing slightly more product than the European mussels. The average clearance rate per European mussel was 2.3 ml of water per hour and for the North American mussels was 3.8 ml of water per hour. The *t*-test applied showed no statistically significant difference ($p>0.05$) difference between clearance rates from the two populations; although the North American population filtered more Zequanox in the first hour, the filtration rates gradually lowered over time.

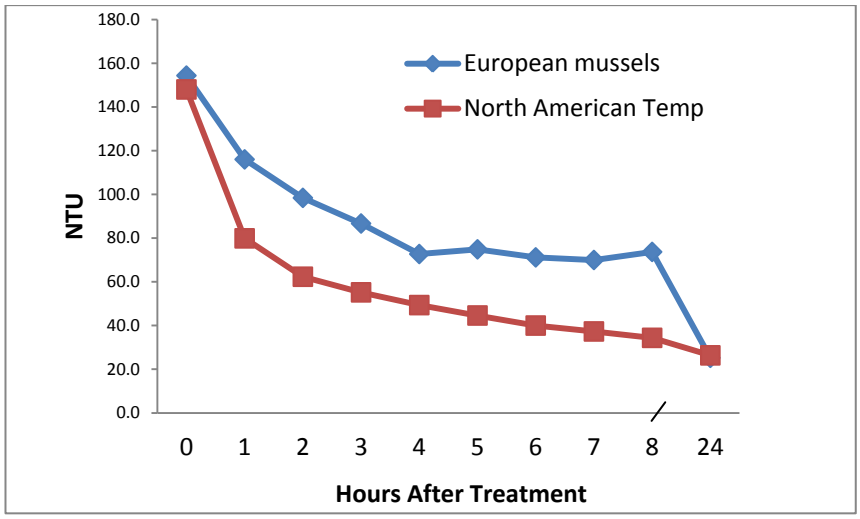

Figure 5.5 Turbidity (NTU) of treated jars after Zequanox application.

Table 5.1 Average Zequanox concentration in jars with European (EU*) and North American (NA*) mussels and without (Zero) mussels treated with 200 mg a.i/L Zequanox

Average Concentration (mg a.i/L)			
	EU* Average	**NA* Average**	**Zero Mussels**
Hour 0	202.2	202.2	202.2
Hour 1	108.8	76.1	171.2
Hour 2	92.8	60.2	157.6
Hour 3	82.2	53.8	151.3
Hour 4	69.6	48.5	146.7
Hour 5	71.5	44.2	138.6
Hour 6	68.2	40.0	137.7
Hour 7	67.1	37.6	136.8
Hour 8	70.5	34.9	131.4
Hour 24	26.7	27.7	127.7

*European/North American

5.4 Discussion

The maximum allowable concentration of Zequanox as designated by the US EPA is 200 mg/L (Marrone Bio Innovations, 2012b). This concentration has proven to control the zebra mussels with minimal to no impacts on non target organisms (Meehan et al, 2013; Meehan et al 2014a; Meehan et al 2014b). Treatments are now currently carried out at 150 mg/L as this lower concentration still effectively controls the zebra mussel (Meehan et al., 2014b). The concentrations tested here were used to help understand the response of the two mussel populations to Zequanox at both high and low treatments.

At the lowest concentration tested (50 mg a.i/L), the European zebra mussels appeared to have a higher sensitivity to Zequanox than the North American mussels, however, at the highest treatment concentration (200 mg a.i/L) the North American zebra mussels appeared to be more sensitive to Zequanox. Therefore it is determined that any noted differences are due to natural variability and Zequanox affects both mussel populations equally when temperature is controlled. This may account for the higher levels of mortality observed in trials carried out in North America when compared to those in Ireland. Water temperatures were higher in North American test sites meaning the mussels are more metabolically active and ingest more product (Fanslow et al., 1995; Costa et al., 2008; Meehan et al., 2013, Meehan et al., 2014b).

In order to successfully transition the use of Zequanox from North America to Europe the treatment timing of both populations should be considered separately. In Europe the water temperature should be incorporated into the treatment plans of any further *in situ* trials in order to maximize the outcome. In Lough Key, Ireland, Lucy et al. (2005) noted that the rate of adult spawning peaks from the last week in June to the last week in August; with the highest amount of settlement occurring during the entire month of August (in 1998-2003). A two-treatment regime is recommended in order to target adult mussels (post spawning) and newly settled veligers. This treatment, where possible, should coincide with the warmest water temperature. A treatment of 150 mg a.i/L could be applied to target adults when the water is at its warmest, and a second treatment at the lower concentration of 10-40 mg a.i/L to target veligers at the end of the reproductive period. If two treatments are impractical, a single treatment at the end of August start of September, to coincide with the conclusion of the reproductive cycle

while the water temperature is still high would suffice (Meehan et al., 2013, Meehan et al., 2014b, Lucy, 2006).

As there was no significant difference between mortality rates from the two populations, it could be expected that the clearance rates would be the same. However the North American mussel population cleared Zequanox more rapidly than the European population. Here the difference in wet weight between the two mussel groups must be taken into consideration. The North American assay had a greater wet weight per mussel. The mean blotted wet weight of the North American mussels was 1.64 g and for the European mussels was 0.84 g. The North American mussels were longer in length thus accounting for the larger wet weight. This difference in biomass could account for the lower turbidity readings (higher clearance rate) from the North American mussels, as the population with the largest biomass filtered more product; Costa et al. (2008) notes that mussel size directly effects filtration rate. Fanslow et al. (1995) noted similar effects as this study where in Lake Huron, North America; the lower the population biomass the lower the filtration impact.

5.5 Conclusions

It is recommended to repeat this study to further confirm that there is no difference in mortality between North American mussels and European mussel of the same wet weight, treated under the same conditions (temperature and water quality) with Zequanox. In order to further improve the validity of the experiment, the study could also be undertaken in Europe with North American mussels and in North America with European mussels. This study provided preliminary evidence that mortality is comparable when populations are treated at the same temperature using the same water (Figure 5.4). The results indicate that for Zequanox treatment in a colder climate such as Ireland it is necessary to consider the water temperature and therefore the time of year treatment occurs in order to achieve maximum mortality. The results of this test can be used as a reference point for any further treatments in order to maximise the outcome.

Chapter 6

Zequanox® as a Control Agent for Juvenile Zebra Mussels

6.1 Introduction

The biobox study at Cairns Hill drinking water treatment plant (Chapter 3) and the open water treatment at Tullamore Harbour (Chapter 4) gave inconclusive results regarding juvenile survival following Zequanox treatment. A successful juvenile treatment was assumed from the low survival on the treated plates and the high adult mortalities. However as a large number of the control juveniles did not survive it could not be proven. An effective control method will successfully control its target at all life stages. Successful juvenile treatments have been previously assumed from past trials at a Sligo drinking water treatment plant (DWTP) and Tullamore Harbour (Meehan et al., 2013; Meehan et al., 2014b). This success was derived from the low juvenile survival on the treated plates and the high adult mortalities. However with these treatments a large number of the control juveniles did not survive and therefore it could only be assumed.

For both the Cairns Hill and Tullamore Harbour trials, juvenile survival on both the treated and control plates was greatly reduced immediately after treatment (Chapters 3 and 4). After this initial reduction, survival on the treated plates continued to decline, whereas survival on the control plates plateaued (Figure 3.7 and 4.5). However, approximately seven days after treatment, survival on both the control and treated plates was reduced to <15%. As this decline included the control plates, the initial decline was likely due to the movement of the plates from the settlement site to the treatment site, recorded as the immediate count after treatment took place. It is also known that from 20% up to 100% natural mortality can occur pre and post settlement (Sprung, 1993; Claudi and Mackie, 1994; Borcherding 1995; Nichols, 1996; Karatayev et al., 1998; Kobak, 2013) making further research on the post treatment survival of juveniles necessary.

In industrial settings, for instance power plants or drinking water treatment facilities, early detection of zebra mussels is key to mussel reduction. Monitoring for veligers or settled juveniles is the best process for deciding on the timing of treatments. Control of these life stages is easier as lower doses of Zequanox will kill these early life stages and unlike adult treatments, physical removal is not necessary following juvenile treatment

due to the small shell size. Therefore further research on the post treatment survival of juveniles was necessary due to the importance of treating early life stages coupled with the lack of knowledge on definitive juvenile mortality.

The objectives of this study were to;

- Gather sufficient juvenile settlement and treat the settlement plates with Zequanox to determine the exact Zequanox effect on juveniles
- Determine if mortality on the control plates is reduced when juveniles on treated plates are allowed sufficient time after treatment for die off to occur
- Provide further data to assess the results of juvenile mortality from the Tullamore and Cairns Hill treatments, that could not be proved due to the high levels of control mortality
- Give recommendations for the use of settlement plates in future trials

6.2 Materials and Methods

6.2.1 Settlement Plates

Settlement plates were used to gather natural mussel settlement in Lough Arrow and Lough Key. The methodology used by Meehan et al. (2013) (adapted from Mardsen, 1992; Lucy and Sullivan, 2001; Lucy et al., 2005) was followed for plate set up and deployment. Plates were deployed on July 23, 2013 during the peak reproductive period for Irish zebra mussels (Lucy et al., 2005) to optimise potential settlement (Mardsen, 1992). Two lakes were chosen to deploy plates: Lough Key (53°98'59" N 08°23'19" W) and Lough Arrow (54°04'22" N 08°20'04" W). Three 15cm^2 plates were attached to 3 m long weighted rope as the maximum density of veligers occurs at 3-7 m (Mackie et al., 1989; Marsden, 1992; Lucy, 2005). Two sets of rope were tied to a buoy at each location in adequate water depth (>4m) (Figure 6.1 and Figure 6.2). Water temperature (°C) was recorded when the plates were both deployed and collected.

The plates were collected on September 11 and 12, 2013, seven weeks after their deployment, allowing sufficient time to gather settlement. The plates were collected and returned to the lab at IT Sligo in plastic boxes therefore causing minimal disturbance. One side of the plates was monitored throughout this trial. The plates from Lough

Arrow had low relative settlement so were discarded, the plates from Lough Key had sufficient levels to proceed with treatment.

On arrival at the lab the plates were placed in lightly aerated, eight litre aquaria, where again the unused side faced down keeping the side to be monitored free from damage. One plate was placed in each tank with three litres of water. Test water was made according to US EPA (2002) standard dilution water and was approximately 16-20ºC throughout testing. In total three aquaria served as the treated chambers and two served as controls. The plates were then left to acclimatise overnight before the baseline count. A *t*-test was carried out on the slope coefficient in excel to determine if there was a statistically significant difference between mortality on the control plates and treated plates.

Figure 6.1 Buoy in Lough Key, rope was tied to eyes on either side (photo by Sara Meehan).

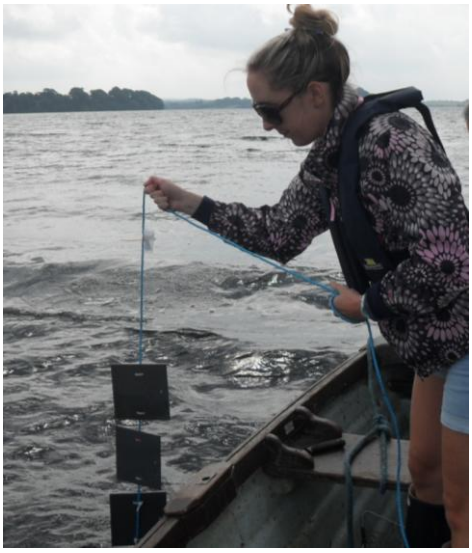

Figure 6.2 Settlement plates on weighted rope being lowered into the water after they were tied onto buoy (photo by Frances Lucy).

6.2.2 Application of Zequanox

The settlement plates were treated with Zequanox on September 13, 2013. The powder was mixed in the lab with US EPA standard dilution water to create the following stock solution concentration:

$C_1V_1 = C_2V_2$ where

C_1 = target treatment concentration (mg a.i./L)

V_1 = volume of treatment area (3 L)

C_2 = stock concentration (g a.i./L)

V_2 = volume of stock concentration to be applied (L)

A total of 3.78 g a.i. of Zequanox was mixed with 378 ml of dilution water using a stir bar to achieve a concentrated product solution of 200 g a.i./L.

120ml of the Zequanox solution was applied to the aquaria to achieve a treatment of 200 g a.i./L, the product remained in the test chambers for 24 hours, the controls were not treated. Following treatment, the plates were moved to new aquaria with fresh water to minimise disturbance. The plates were then left for six days before their first survival count to allow for settlement die off and were recounted twice after that on September 24 and 26; until survival on most the treated plates was at zero and counting ceased. In theory this process should allow for any juveniles affected by Zequanox treatment sufficient time to die and fall off the plates so they are not counted as live ones, giving a false positive for mortality.

For the most part dead juveniles were easily distinguishable by the absence of live tissue. On a few occasions where it was hard to tell whether or not tissue was present, neutral red dye was used. This dye is absorbed by live tissue, therefore in the absence of tissue inside the shell the dye would not be absorbed. As juveniles fall off settlement plates quickly after death (Meehan et al., 2013) it was assumed that any tissue that absorbed the dye was alive. Neutral red dye is normally used in water as opposed to directly on the tissue, determining dead or alive by the uptake of the dye through filtering (Nagabhushanam, 1956; Horvath and Lamberti, 1999). However as this was an

ongoing count a drop of dye was placed on the juveniles suspected to be dead and care was taken not to contaminate others.

6.3 Results

Figure 6.3 displays the decline in juvenile survival after treatment. Tanks 1 and 2 reached zero survival less than eleven days after treatment, Tank 3 did not reach zero survival, however by day 13 only two live mussels remained on the plate and counting ceased. Table 6.1 shows the final percentage survival and the estimated number of mussels on the plates per m^2 (as per Marsden, 1992). Survival on the control plates dropped initially after treatment from 9,000 and 10,000 juveniles/m^2 to 7,666 and 5,333 juveniles /m^2 on the first count at day 6. Survival on the control plates continued to decrease throughout the monitoring period, this decline however was minimal and survival did not reach zero as in the Cairns Hill and Tullamore trial (Chapters 3 and 4). Water temperature was 22.5 °C in Lough Key (July) when plates were deployed and 17.9°C (September) when plates were collected. A *t*-test showed that there is a statistically significant difference in mortality between the control and treated plates.

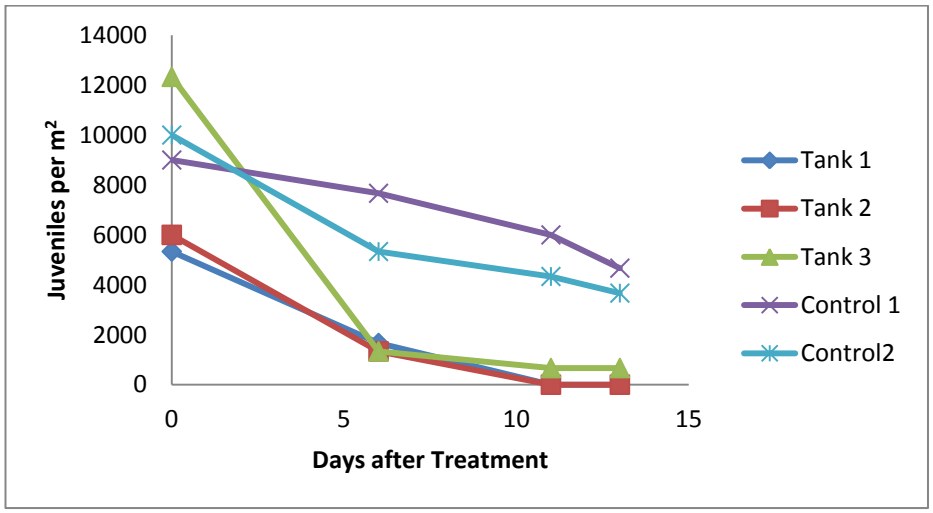

Figure 6.3. Juvenile mortality per m^2 after Zequanox treatment

Table 6.1. Juvenile survival before and after treatment with Zequanox

Date	Tank 1 (juveniles/m^2)	Tank 2 (juveniles/m^2)	Tank 3 (juveniles/m^2)	Control 1 (juveniles/m^2)	Control 2 (juveniles/m^2)
13/09/2013	5330	6000	12330	9000	10000
19/09/2013	1670	1330	1330	7670	5330
24/09/2013	0	0	670	6000	4330
26/09/2013	0	0	670	4670	3670
% Survival	0	0	5.40	51.90	36.70

6.4 Discussion

Settlement on the plates taken from Lough Key corresponds with previous settlement data from Lough Key. Lucy (2006) found that cumulative juvenile settlement gathered the 2nd week in September 1998-2003 was <20,000 juveniles/m^2, close to the settlement gathered in Lough Key for this study (Table 6.1).

It is evident that the results obtained in this trial are much more conclusive then those from Chapter 3 and 4 mainly as the control survival was higher. A decline in control survival did however occur in this study but it was less significant and slower than the decline on the control plates from Cairns Hill and Tullamore Harbour. For example on the control plates at Tullamore Harbour juvenile numbers declined from 17,330 juvenile/m^2 (48 hours before treatment) to 3,670 juvenile/m^2 (24 hours after treatment). The removal of the plates from their original location and overnight transport to Tullamore, followed by their immediate count gave a higher baseline number than could have been achieved if the plates had acclimatised in their new environment prior to the baseline count. After this, survival on the Tullamore control plates continued to decline and eventually diminished to 14% survival. When this is compared to the survival seen from this in-house assay (Table 6.1.), it is evident that limiting the time for plate removal when counting reduces mortality probably caused by air expose and disturbance.

The younger the life stage of the zebra mussel the more sensitive they are to outside disturbances meaning that a certain amount of mortality naturally occurs with juvenile zebra mussels (Nichols, 1996; Sprung, 1993). Some causes are: predation by fish, filtering by copepods or by adult *Dreissen*a, bacterial infection, and shortage of food

(MacIsaac et al., 1991; Sprung, 1993; Molloy et al., 1997). Relating this to a lab environment this predisposition of sensitivity coupled with water turbulence from refreshing and measuring water quality would cause additional mortality (Rehmann et al., 2003). Horvath and Crane (2010) also note from laboratory trials with another sensitive life stage of the zebra mussel (larvae) that the longer they remained in the lab the higher their control mortality.

Some mortality still occurred on the control plates from this in house assay (on average 44%), however it is evidently less compared to Tullamore (86%) and Cairns Hill (100%). Figure 6.4 clearly shows that the control decline from this in house assay was gradual compared to the initial sharp decline in survival from Cairns Hill and Tullamore. The control mortality at Cairns Hill and Tullamore likely occurred due to the removal of the plates for counting but this study shows that a reduction in mortality is directly linked to a reduction in the amount of times the plates are removed.

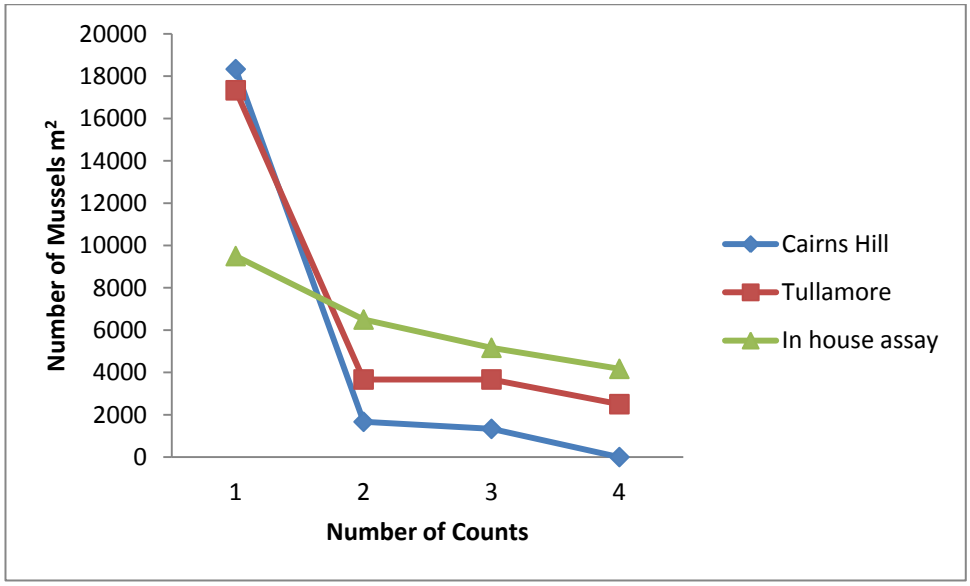

Figure 6.4 Control survival

The decline in juvenile survival after Zequanox treatment was rapid. For example tank 3 dropped from 12,333 to 1,333 juvenile/m^2 six days after treatment; a decrease of 89%, compared to a decrease of 15% in control tank 1. The huge difference in mortality further demonstrates that treatment with Zequanox was the reason for this drop in survival.

This assay was carried out to provide support for Chapters 3 and 4, to demonstrate that if the settlement plates in those studies had been allowed to aclimatise in Tullamore Harbour and in the bioboxes at IT Sligo after they were transported, and had their removal after treatment been minimal, survival on the control plates would not have declined to < 15% along with the treated plates. Therefore it can be hypothesized that the majority of the mortality on the treated plates at Cairns Hill and Tullamore was the result of Zequanox treatment, and the early mortality on the control plates was due to air exposure and other disturbance.

6.5 Conclusions and Recommendations

The results of this trial mean that further consideration needs to be given to measuring juvenile survival after treatment. Ideally juvenile settlement would be gathered from the location where the treatment occurs therefore eliminating further mortality from the transportation of plates. If settlement must be gathered away from a test site, a five day acclimation period when plates are re-homed to the wild and a 1-2 day acclimation period when plates are moved to a lab is advisable. Also counting of survival on plates must be kept to a minimum to ensure the mortality counted is from Zequanox treatment and not the removal of the plates.

This trial supports the conclusions drawn from Chapters 3 and 4 that the early mortality of the juveniles on the treated plates (at Cairns Hill and Tullamore) was mainly due to Zequanox treatment and that the mortality of juveniles on the control plates was due to removal of the plates for counting and/or natural die off.

Chapter 7

Trouble Shooting: Zequanox® Research

7.1 Introduction

The aim of this trouble shooting chapter is to demonstrate how errors in testing allowed for knowledge expansion and the progression and development of methods and related laboratory skills. In many instances, as in most research projects, errors that occurred allowed for the improvement of methodologies. As ASTM and OECD ecotoxicology guidelines were not strictly followed, trial and error occurred in the initial stages of method development. Progressing Zequanox use from small scale lab experiments to large scale open water treatments also incurred some unexpected results which required further exploration. It must be remembered that all initial experiments and experience with Zequanox during this research was in a small scale laboratory setting. A certain amount of trial and error was expected when transitioning to larger open water treatments. Trouble shooting has broadened my scientific knowledge and armed me with a practical approach to problem solving; both of which are important to a research career. Moreover, the scientific information provided will increase the capacity to further develop research both on Zequanox and for zebra mussel life-stage studies.

7.2 Non Target Testing

Troubleshooting for the non target testing involved both the taxa test organisms and Zequanox. As the Zequanox formulation was under development, there were still some unknowns regarding its effect on NH_3. As always when working with live organisms, the outcome can never be guaranteed and a certain degree of trial and error was expected prior to the non target testing.

7.2.1 Zequanox Build Up and Residue in Test Chambers

During ecotoxicology studies, Zequanox build up occurred in the test chambers due to the particulate nature of the product (50% inerts). Zequanox build up was also linked to water changing and product renewal (some tests only allowed removal of 90% of water due to the small size of the organisms). A residual build up of Zequanox may have caused a decrease in DO and an increase in NH_3 over time as the organic matter in Zequanox degraded.

7.2.2 *Ephemerella ignita*

During the non target testing, *Ephemerella* were initially clinging to either air stones or to one another, therefore the lack of substrate for attachment may have caused mortality in the initial trial optimisation. To mimic the natural system as much as possible, stones were placed in the test chambers during testing, to provide the mayfly with an appropriate substrate (Olsen, 2001; Lyman, 1956).

Due to the fact that testing was carried out in springtime and that they were at different developmental stages, some of these mayflies went through their final moult and emerged as adults during method optimisation. Netting was then placed over the test chambers to catch the adult winged mayfly, and they were treated as alive for the purpose of statistical analysis. So although this meant there was some loss in test organisms, this was in fact a useful scientific outcome demonstrating that Zequanox did not interfere with the *Ephemerella* life cycle.

Temperature fluctuated during method optimisation testing. Therefore different methods to control temperature were tried and tested such as dry ice, ice packs, fans and leaving windows open in the laboratory. It was found that fans on a timer system adequately controlled the room temperature. Here the timer was set so that the fan turned on at specific intervals, depending on what temperature was required for testing. Method optimisation was carried out to ensure that using fans kept water temperature in the test chambers within $\pm 1^{o}$C of the optimum temperature.

7.2.3 *Chironomus plumosus*

Chironomus were difficult to see in the highly concentrated jars due to their small size and light colour, and during initial method optimisation some *Chironomus* were accidentally poured down the sink. It was decided to use only five organisms to make counting easier. In some cases where specimens were missing *Chironomus* had become intertwined with Zequanox accumulations. Here any missing *Chironomus* in the Zequanox build up was sorted under the microscope with forceps and the chironomids were retrieved. Stone substrates were placed in later tests for *Chironomus* for attachment and shelter as in the mayfly tests. Here Zequanox would often build up on the stones trapping the *Chironomus* making them difficult to locate, the stones were also placed under the microscope to locate any missing *Chironomus.*

There was a small amount of mortality in the control test chambers for *C. plumosus.* A possible reason for this could have been disturbance from the daily water change. Here water was poured slowly out of the test chambers and slowly back in so as to minimise disturbance. However, it is possible that simply removing the *C. plumosus* and placing them in new test chambers may reduce interruption and in turn reduce control mortality.

7.2.4 *Anodonta*

Anodonta had to be retained in the laboratory prior to testing, for over two weeks, due to a delay in Zequanox product arrival. Here the water was changed every other day, with lake water, to ensure the mussels were supplied with some food and kept in good water quality. *Anodonta* were also kept in 7 L laboratory aquariums so they had more space than their test chambers. This conditioning actually ensured that only healthy *Anodonta* were selected for testing and in fact no mortalities were observed for *Anodonta.*

7.2.5 Negative Correlation - *Asellus aquaticus* and *Austropotamobius pallipes*

There was negative correlation between mortality and concentration for *Asellus aquaticus and Austropotamobius pallipes*. For *A. aquaticus* mortality occurred at both 100 mg/l and at 400 mg/l where three and one deaths occurred respectively. As all of the mortality occurred in one test chamber at 100 mg/l it is suspected that perhaps a pathogen spread amongst the organisms, as water quality parameters, particularly DO, for this test chamber were within acceptable limits. A repeat test is recommended to further confirm this fact.

For *A. pallipes* mortality occurred at the two lowest treated concentrations, 350 mg/l and 450 mg/l, with one mortality in each. It was suspected that mortality occurred at these low concentrations due to the small size of the two organisms that died. For this study, as *A. pallipes* are a protected species in Ireland due to their low numbers, the amount of crayfish that could be collected was limited. If this experiment was to be carried out again in a situation where crayfish availability was not limited it is recommended to select organisms of the same size thus increasing robustness of the test.

7.2.6 Mortality - *Salmo trutta*

High levels of mortality were recorded for *S. trutta*, 100% mortality was achieved at 180 mg/l. It is well known that fish are sensitive to elevated ammonia levels, and was likely the contributing factor for this high mortality. It is recommended that a repeat lab study is carried out, whereupon if similar mortality levels are achieved, a mesocosm study would be the next stage to examine Zequanox effect. In a mesocosm study ammonia levels would most likely be reduced because of the larger test environment.

7.2.7 Future Expansion

It is also an important and useful aspect of this chapter to explore future expansion of these studies in order to progress and deepen the knowledge and understanding for use of Zequanox. Having more controls in future studies could further substantiate the results of non target testing and increase confidence. Controls for turbidity and organic matter would allow the researcher to explore organic load as a possible cause of mortality in the higher test concentrations. This would in turn confirm that at low concentrations Zequanox is not toxic to non target organisms and mortality in higher concentrations is from organic load and not due to the toxicity of Zequanox.

7.3 Tullamore Harbour, Trial Improvements

As this study was the first of its kind in Ireland, it will serve as a good baseline for those wishing to repeat or expand on this research. Therefore it is imperative to record lessons learnt in order to aid knowledge expansion. To begin it is necessary to repeat this study using the same set up in a different location. Will a treatment of this nature be successful in another water body? With that, as discussed in section 7.6 dissolved oxygen needs to be considered and the method which it is delivered conducted in such a way so as to cause minimal disturbance to the naturally settled wall mussels. The results from Tullamore Harbour show that where dissolved oxygen was administered the wall mussel mortality decreased but the seeded mussel mortality increased. Therefore it is necessary to keep the tubing for the DO away from the wall so as not to disturb the settled mussels. Alternative methods to deliver the DO can be explored such as using an aerator that sits on the bottom delivering oxygen upwards. If these aerators were placed away from the wall and a short distance from seeded mussels DO would increase and the mussels would not be impacted from its administration.

For the control it is recommended to use the same curtain structure as used to enclose the treated areas. Due to limitations in this study that was not possible. This would determine if the use of the curtain causes any additional mortality in the treated areas. It also allows for a more thorough examination of water quality between treated areas and controls. It would be useful to examine water quality outside enclosed areas to determine if using a curtain and therefore blocking the flow of water has any negative impact to the water quality apart from dissolved oxygen levels.

7.4 Settlement Studies

Settlement plates during testing were moved from the lake (Lough Key) where settlement was gathered to a separate test location; this was the case in both the Tullamore Harbour trial and Cairns Hill biobox study (Chapters 3, 4). In each of these studies both control plate mortalities as well as treated plate mortalities had low survival. Although, it could be safely assumed that the control mortalities were caused by the removal and transportation of the test plates further scientific confirmation was still required.

Laboratory trials were carried out to determine if whether allowing the plates time to acclimatise in their new environment before treatment could reduce control mortality (Chapter 6). Further studies on controls under different environmental conditions would provide more information on survival of juvenile zebra mussels independent of Zequanox treatment.

The results of this research further confirmed that the mortality achieved in the Cairns Hill and Tullamore Harbour trials could be attributed to Zequanox. Also this study allowed for recommendations for any future studies of this kind and will provide a reference point for other researchers when carrying out juvenile settlement studies.

7.5 European and North American Mussel Comparisons

Comparisons between similar biobox and open water treatments carried out in North America and Ireland gave different rates of mortality. Mortality was higher in North America than in Ireland: it was assumed that this was due to test water being warmer in North America, meaning the mussels are more metabolically active and therefore ingest more Zequanox. However, this theory required further examination.

Jar assays were carried out at both MBI and IT Sligo under the same conditions, proving that there is no difference in mortality between the two mussel populations when treated under identical conditions. The results of this test showed only a 10% difference in mortality when treated under identical conditions. This test provided information regarding treatment timing in order to optimise mortality in EU treatments, making the methods standardised where possible.

This trial however, required further trouble shooting due to a fault in the air condition system at MBI which increased water temperatures causing high control mortality. A retest had to be carried out followed by further retesting at MBI and IT Sligo following the loss of activity of the product.

It is recommended to carry out repeats of this trial in order to increase confidence in results. In addition, testing North American mussels in Ireland and Irish mussels in America will further confirm and increase confidence in the results. If similar results are found in these repeated lab studies, to further advance this trial, a temperature controlled biobox study could be carried out in America and Ireland. From this it would be possible to make further recommendations regarding the timing of treatment in industry.

It is also important to consider weight difference between the two populations. Zebra mussels with the same wet weight should be selected for the North American and European studies. This would also allow comparisons to be made using traditional zebra mussel filtration studies (Karatayev et al., 1997).

7.6 Use of Zequanox - Lab to Ecosystem Approach

It must be remembered that Zequanox use in the lab at IT Sligo mainly consisted of non target testing where Zequanox was applied to small amounts of water at low to high concentrations. In the lab either an electronic stir plate or a test tube stirrer was easily used to evenly dilute the product. Adjustments to this method of mixing needed to be made in order to apply large quantities in the field. For the Cairns Hill trial, as Zequanox was applied to 200 L bioboxes, it was still possible to mix using a stir plate with 1 L beakers. Here Zequanox was fed from inside the lab to the bioboxes located outside, through the laboratory window using a peristaltic pump. For Tullamore Harbour, Zequanox treatment was on a much larger scale (4,500 L) and methods had to be reviewed. It was first decided to use an over-head mixer to mix the product. However

due to the larger volumes of water required (6.75 L), this process did not mix the Zequanox fast enough. It was then decided to use a hand blender, this allowed for the quicker removal of all clumps of Zequanox due to the operator being able to target specific masses within the mix.

In the Tullamore Harbour Trial, 24 hours after the curtains were placed, a large drop in DO was recorded inside the curtains. It was suspected that this low DO would result in lower mortality levels, however this could not be proven. As a precaution, it was decided to aerate one treatment side only in order to compare the effect of low DO versus normal DO levels on mussel mortality. It was found that for the seeded adult mussels, mortality was approximately 20% greater with aeration, however for the naturally settled mussels mortality was approximately 20% greater in the non aerated side. From this troubleshooting episode, it is apparent that for any future open water trials aeration will have to be further considered and used to optimise mortality.

7.7 Conclusions

The addition of a trouble shooting chapter for this research was developed to demonstrate method development during this research PhD, particularly for non target testing. Troubleshooting allows the researcher to critically assess and improve all aspects of the scientific process from experimental design to execution, whilst continually improving test methods and their own personal skill set.

Chapter 8

Conclusions

Before a new product can be introduced into an existing ecosystem, one core research question must be asked: what are the potential impacts to the environment? This PhD research has followed a systematic step by step approach in answering this question whilst also testing the efficacy of the product. This methodical stepwise approach was as follows; (1) testing the effect of Zequanox on native species; (2) a trial showing its potential effect to industry; (3) trialling Zequanox in a natural ecosystem and assessing its impact on receiving water quality. This conclusion takes into account the role and input of regulatory stakeholders in the development of this research. In addition to the science presented in this thesis, the regulatory process was an integral part of the research process; therefore this conclusion aims to integrate this process with the scientific process and outputs.

Invasive species not only affect the ecology of the environment they invade but they also directly affect ecosystem services; which are critical to the functioning of the earth (Costanza et al., 1997). As it is often difficult to quantify ecosystem services in terms of capital, their value to mankind is often underestimated. Consequently the general public are often unaware of the direct impact of invasive species not only to the environment but also to the economy. The involvement of various stakeholder groups during this research increased general awareness of this key area of ecosystem services. It was imperative to the success of this research to consult with and inform all regulatory stakeholders, whether or not permission was required from them to carry out a trial. At the time of this research MBI were seeking licensing approval for the commercial use of Zequanox in Europe and this research made up part of the application for EU approval. As well as providing scientific data, this research spread awareness about Zequanox through the Cairns Hill and Tullamore Harbour trials due to relevant stakeholder interactions and by presenting the results at various conferences. Figure 8.1 gives an outline of the steps that were followed and the public bodies consulted prior to this research. The Irish National Parks and Wildlife Service (NPWS) were first consulted regarding the non target organisms that were to be tested; concerns were raised for two particular organisms, namely *A. pallipes* and *Anodonta*, due both to their conservation status and their vulnerability to zebra mussel invasion. The species that were tested in

this research were selected following direct communication with the NPWS. Prior to any open water trials using Zequanox in Ireland, a trial authorisation had to be granted by the Department of Agriculture Food and the Marine. For this, meetings ensued where the Dept. of Agriculture Food and the Marine requested the results of the non target trials to first ensure there were no negative impacts to native fauna. Ahead of any decisions regarding Europe's first Zequanox open water trial at Tullamore Harbour, Inland Fisheries Ireland (IFI), Waterways Ireland (WI) and the Environmental Protection Agency (EPA) were consulted to make them aware of the plans. Data was presented to display the early results of the non target testing and to explain the potential effects to the aquatic environment of the proposed trials. Work was presented from previous studies carried out in the USA and every effort was made to fully explain the implications of Zequanox treatment.

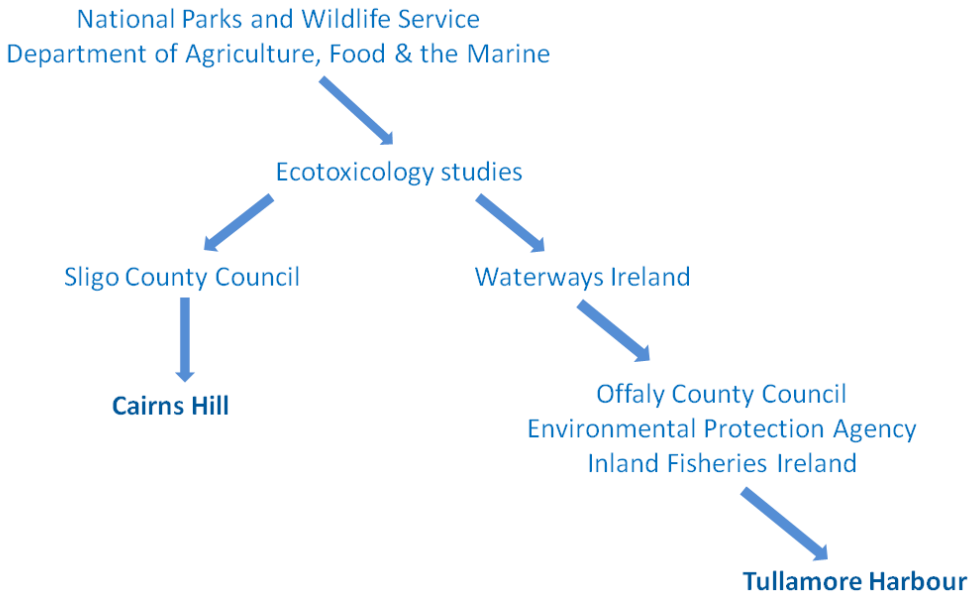

Figure 8.1 Flow chart outlining the sequence in which agencies were consulted and contacted prior to and during this research

Chlorine is the most widely accepted and traditionally used product for zebra mussel control. It is well documented that chlorine affects human health and aquatic ecosystems as it is generally toxic (in fact harmful to every aquatic organism it comes in contact with) (Winder, 2001). Thus posing the obvious question, why is it still being used? Unfortunately chlorine continues to be considered acceptable due to its historic long-term use in the treatment of water for potable use.

Through meetings with stakeholders and public bodies it was clear that there is an aspiration to find a less toxic alternative control method, than chlorine. Therefore in order to transition to the use of a biopesticide like Zequanox it is imperative to first present evidence to the stakeholders that Zequanox does not interfere with the normal functioning of the ecosystem. Non target trials are the first step of this process; testing from each functional feeding group demonstrates the effect to the food chain. Although Zequanox was confirmed to have no negative impacts on native and endangered species from Ireland's waters at concentrations required to achieve >80% zebra mussel mortality, negative effects of chlorine to freshwater biota are well documented (Brungs, 1973). This evidence provides a strong reason to replace chlorine in the treatment of zebra mussels and was well received by various stakeholders.

Conservation stakeholders (in particular NPWS and EPA) were particularly interested in the no negative impacts to two of Irelands threatened species, *Anodonta* and *Austropotamobius pallipes* and thus testing the effects to these species was vital.

The research presented in this thesis provides basic independent evidence for non toxic effect of Zequanox to native organisms. This is fundamentally important in progressing this new substance into practical application as a control product. It is important to carefully consider infestation on a case by case basis, to design a treatment to suit individual situations. Due to the fact that use and knowledge of this product is novel to Europe all treatments had to be contained as per agreement with National Parks and Wildlife Services in accordance with trial permits issued. In order to use Zequanox in industry it is necessary to determine potential impacts to water quality and safe treatment concentrations.

The biobox trial was set up to mimic the parallel effects of chlorine and Zequanox treatment in industry. A drinking water treatment plant (Cairns Hill) with a long-term severe zebra mussel infestation in the raw water chambers was chosen. The biobox treatment was carried out in parallel with a chlorine treatment of the raw water chambers allowing for the comparison of treatment time, mortality rates and the direct comparison of potential effects and benefits. Again stakeholder involvement and agreement was key. Numerous meetings were carried out with the plant manager and Sligo County Council seeking permission to run this trial. It was agreed that the bioboxes could be hooked up to the main inflow of the plant and they could be

monitored and maintained in the weeks running up to and after Zequanox treatment. Permission was not however granted to carry out Zequanox treatment of the bioboxes on site at the plant as concerns were expressed by Sligo County Council over the discharge of Zequanox to the source water (Lough Gill) after treatment. Therefore treatment was carried out on IT Sligo grounds and Zequanox discharged to the sewer. This trial successfully demonstrated that Zequanox has the potential to replace chlorine in industry. This means there is potential to reduce the man power and hours previously required for zebra mussel removal in industry, thus reducing costs and water supply issues. Furthermore the use of Zequanox would stop the high volume of chlorine discharge back to the source water thus reducing any potential impacts to the freshwater ecosystem such as the formation of trihalomethanes (Coffin et al., 2000; Meehan et al., 2013).

It is well known and has been discussed in detail in this thesis that zebra mussels not only cause problems in industry but also in open water. Currently in open water physical removal remains the only non toxic control method to the environment. As Zequanox proved non toxic to all native organisms tested (except perhaps *S. trutta)* at concentrations required to gain >80% zebra mussel mortality, the next logical step is to explore the control of zebra mussels in open water using Zequanox. The first open water trial for Zequanox in European waters was at Tullamore Harbour. Prior to this trial permission was first sought from WI as any experimental trials in the Grand Canal falls under their remit. The results of the non target testing were presented along with those of the Cairns Hill trial and additional trial results and non target testing from the USA. The possession of an abundance of data on Zequanox trials and non target testing helped to secure trial permission from WI. From the meetings with WI it was agreed that a small ecological survey of the Grand Canal at Tullamore Harbour would be carried out and that it was extremely important to consider and consult/inform all the relevant stakeholders. From here meetings ensued with the Inland Waterways Association of Ireland, the local ranger from NPWS, a senior ecologist from the EPA and the Offaly county council heritage officer. A press briefing was drafted for Waterways Ireland using non scientific language and another press release was drafted for IT Sligo. A notice was also submitted for inclusion in the Waterways Ireland newsletter stating when the Tullamore Harbour dock would be closed due to trial

commencement. This trial proved successful and information about Zequanox use reached a wide audience.

These three aspects of this thesis provide strong evidence that Zequanox can be used safely and effectively in industry. The Tullamore Harbour trial demonstrated that there is potential to use Zequanox in open water, but further research is necessary first. Through this research and the publication of peer-reviewed papers, both stakeholder awareness and openness for exploring other non traditional control methods has increased.

As this research progressed other questions and aims arose from results of the Cairns Hill and Tullamore Harbour trials. A knowledge gap existed on whether there was a difference in mortality rates between North American and European mussels treated with Zequanox and a more definitive result was required to show the effect of Zequanox on settled juveniles. Coupled with this, Zequanox originates in the USA where all test methods were developed, this research tested the hypothesis on whether the product is more effective in North American waters. This PhD redeveloped and researched the original test methods in order to apply them in a European context although it is recognised that more research is required.

This PhD research presents the results from innovative trials new to Europe. Some of the testing was based on tests/trials previously carried out in the USA and adapted to an Irish setting; other testing like the Tullamore trial was the first of its kind. The trouble shooting chapter demonstrates the evolution of the non target testing and the development of methods in order to bring Zequanox use from the lab to ecosystem successfully. All the results obtained were presented independently of previous trials carried out in the USA by Marrone Bio Innovations as they were undertaken by independent research. The main outcomes of this research were as follows; knowledge sharing of applicable techniques for Zequanox use in Europe; identification of the effects to the environment and non target organisms; awareness of stakeholders through trials of the potential use of Zequanox to remove the zebra mussel. Finally this research has demonstrated the potential to use Zequanox to replace other non selective zebra mussel control methods such as chlorine, therefore reducing the impact of zebra mussel control to the environment. The conclusions of this research are as follows;

1) Zequanox is not harmful to most native Irish organisms in the lab at concentrations required to control zebra mussels.
2) Zequanox can potentially be used safely in industrial settings.
3) Potential exists for Zequanox use in open water. However further exploration is necessary.
4) It is likely that when temperature is increased so is mussel mortality from Zequanox treatment.
5) Juvenile zebra mussel mortality can be reduced in experiments by using best practice in handling methods.

It is again important to note that this research was the first of its kind in Europe and serves as a strong baseline study for the assessment and utilisation of Zequanox for zebra mussel control. With further research it is possible that Zequanox will become a routine control method for zebra mussels both in industry and open waters.

References

Ackerman, J. D., Sim, B., Nichols, S. J. and Claudi, R., 1994. A review of the early life history of zebra mussels (*Dreissena polymorpha*): Comparisons with marine bivalves. Can. *J. Zool*. **72**:1169- 117.

Aigbavbiere, E., 2010. How does predation from fish influence the benthic invertebrates' species composition in the *Phragmites australis* and *Chara* vegetation of Lake Takern ? Master Thesis Department of Physics, Chemistry and Biology Linköpings university SE-581 83 Linköping, Sweden.

Aldridge, D.C., Elliott, P. and Moggridge, D.G., 2004. The recent and rapid spread of the zebra mussel (*Dreissena polymorpha*). Biol. Conserv. **119**, 253–261.

Aldridge, D.C., Elliott, P. and Moggridge, G.D., 2006. Microencapsulated biobullets for the control of biofouling zebra mussels. *Environ. Sci Technol*. **40**, 975-979.

Apperson, C., Waldvogel, M. and Bambara, S., 2006. Extension Entomology http://www.ces.ncsu.edu/depts/ent/notes/Urban/midges.htm [Last accessed 29th October 2013].

Araujo, R., Valladolid, M. and Gómez, I., 2010. Life cycle and density of a newcomer population of zebra mussels in the Ebro River, Spain. *In:* Van Der Velde, G., Rajagopal, S. and Bij de Vaate, A. (eds.) *The Zebra Mussel in Europe*. Margraf publishers, pp. 183-189.

Arim, M., Abades, S.R., Neill, P.E., Lima, M. and Marquet, P.A., 2006. Spread dynamics of invasive species. *Proceedings of the National Academy of Sciences of the United States of America* **103(2)**, 374-378.

ASTM E1192 – 97, 2008. Standard Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates and Amphibians.

ASTM E729 – 96, 2007. Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates and Amphibians.

ASTM E2455 – 06, 2013. Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels.

ASTM E724 – 98, 2004. Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs.

ASTM E1711 – 95, 2008. Standard Guide for Measurement of Behaviour During Fish Toxicity Tests.

Baglinière, J.L. and Maisse, G., 1991. *Biology and Ecology of the Brown and Sea Trout.* Praxis Publishing, Chichester, UK, pp. 286.

Başçınar1, N.S, Düzgüneş, E., Mısır1, S., Polat, H. and Zengin, B., 2009. Growth and Flesh Yield of the Swan/duck Mussel [*Anodonta cygnea* (Linnaeus, 1758)] (Bivalvia: Unionidae) in Lake Çıldır (Kars, Turkey). *Turkish Journal of Fisheries and Aquatic Sciences* **9,** 127-132.

Bash, J., Berman, C. and Bolton, S., 2001. Effects of Turbidity and Suspended Solids on Salmonids. Washington State Transportation Commission and U.S. Department of Transportation.

VanSlooten, K. B. and Tarradellas, J., 1994. Accumulation, depuration and growth effects of tributyltin in the freshwater bivalve *Dreissena polymorpha* under field conditions. *Environmental toxicology and chemistry*, **13(5)**, 755-762.

Benson, A.J, 2013, Chronological history of zebra and Quaga mussels (*Dreissenidae*) in North America , 1988-2010. *In:* Schloesser, D.W. and Nalepa, T.F. (eds.) Q*uagga and zebra mussels: biology, impacts and control, second edition.* CRC Press, Taylor & Francis Group, Boca Raton, pp. 9-31.

Bij de Vaate, A., Jazdzewski, K., Ketelaars, H.A.M., Gollasch, S. and Van der Velde, G., 2002. Geographical patterns in range extension of Ponto-Caspian macroinvertebrate species in Europe. Canadian Journal of Fisheries and Aquatic Sciences **59(7)**, 1159-1174.

Boeckman, C.J. and Bidwell, J.R., 2013. Density, growth and reproduction of zebra mussels (*Dreissena polymorpha*) in two Oklahoma reservoirs. *In:* Schloesser, D.W. and Nalepa, T.F. (eds.) Q*uagga and zebra mussels: biology, impacts and control, second edition.* CRC Press, Taylor & Francis Group, Boca Raton, pp. 369-381.

Bootsma, H.A and Liao, Q., 2013. Nutrient cycling by Dreissenid mussels controlling factors and ecosystem response. *In:* Schloesser, D.W. and Nalepa, T.F. (eds.) Q*uagga and zebra mussels: biology, impacts and control, second edition.* CRC Press, Taylor & Francis Group, Boca Raton, pp. 555-574.

Borcherding, J., 1991. The annual reproductive cycle of the freshwater mussel *Dreissena polymorpha* Pallas in lakes. *Oecologia* **87(2)**, 208-218.

Broennimann, O., Treier, U. A., Müller-Schärer, H., Thuiller, W., Peterson, A. T. and Guisan, A., 2007. Evidence of climatic niche shift during biological invasion. *Ecology Letters*, **10(8),** 701–709.

Bundschuh, M., Appeltauer, A., Dabrunz, A. and Schulz, R., 2012. Combined effect of invertebrate predation and sublethal pesticide exposure on the behavior and survival of *Asellus aquaticus* (crustacea; isopoda)**.** *Archives of Environmental Contamination and Toxicology* **63(1),** 77-85.

Burrows, R. E., 1964. Effects of accumulated excretory products on hatchery-reared salmonids. Bureau of Sport, Fisheries and Wildlife Service. Research Report 66, pp. 12.

Byrne, M., 2007. The Grand Canal and Tullamore. Offaly Historical and Archaeological society.http://www.offalyhistory.com/articles/78/1/The-Grand-Canal-amp-Tullamore/Page1.html [last accessed 27th August 2013].

Byrne, A., Moorkens, E.A. anderson, R., Killeen, I.J. and Regan, E.C., 2009. Ireland Red List No. 2 – Non- Marine Molluscs. National Parks and Wildlife Service, Department of the Environment, Heritage and Local Government, Dublin, Ireland, pp. 49.

CAISIE project, 2008. Research and Control Programme for *Lagarosiphon major* in Lough Corrib 2008. http://caisie.ie/wp-content/uploads/2010/01/Lagarosiphon-report-20083.pdf [last accessed 3rd December 2013].

Chase, M.E. and Bailey, R.C., 1999. The ecology of the zebra mussel (*Dreissena polymorpha*) in the lowere Great Lakes of North America: I. Population dynamics and growth. *Great Lakes Research* **25(1),** 107-121.

Clare, J. 2002. "Daphnia: An aquarist's guide". http://animaldiversity.ummz.umich.edu/accounts/Daphnia_magna/#E3A3960A-3F05-11E2-A8AD-002500F14F28. [last accessed 5th October 2014].

Claudi, R., Mackie, G.L., 1994. Practical *manual for zebra mussel monitoring and control*. Published by CRC Press, Boca Raton, Florida, pp. 227.

Coffin, J.C, Ge, R., Yang, S., Kramer, P.M, Tao, L. and Pereira, M.A., 2000. Effect of trihalomethanes on cell proliferation and DNA methylation in female B6C3F1 mouse liver. *Toxicologicol Sciences* **58(2),** 243–52.

Conn D.B., Simpson, S.E., Minchin, D. and Lucy F.E., 2007. Occurrence of Conchophthirus acuminatus (Protista: Ciliophora) in *Dreissena polymorpha* (Mollusca: Bivalvia) along the River Shannon, Ireland. *Biological Invasions* **10(2)**, 149-156.

Cope, W. G., Bartsch, M. R. and Marking, L. L., 1997. Efficacy of candidate chemicals for preventing attachment of zebra mussels (*Dreissena polymorpha*). *Environmental Toxicology and Chemistry* **16(9)**, 1930-1934.

Costa, R., Aldridge, D.C. and Moggridge, G.D., 2008. Seasonal variation of zebra mussel susceptibility to molluscicidal agents. *Journal of Applied Ecology*, **45,** 1712-1721.

Costa, R., Moggridge, G.D. and Aldridge, D.C., 2012. Improved Mussel Control Through Microencapsulated BioBullets. *In* Rajagopal, S., Jenner, H.A., Venugopalan, P. (eds.) *Operational and Environmental Consequences of Large Industrial Cooling Water System*s. pp. 273-286.

Costanza, R., d'Arge, R., deGroot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P. and van den Belt, M., 1997. The value of the world's ecosystem services and natural capital. *Nature* **387**, 253–260.

Coughlan, J., 1969. The estimation of filtering rate from the clearance of suspensions. *Marine Biology* **2(4)**, 356-358.

Eddy, F. B., 2005. Ammonia in estuaries and effects on fish. *Journal of Fish Biology* **67(6)**, 1495–1513.

Edelist, D., Golani, D., Rilov, G. and Spanier, E., 2012. The invasive venomous striped eel catfish *Plotosus lineatus* in the Levant: possible mechanisms facilitating its rapid invasional success. *Marine Biology* **159,** 283–290.

Effler, S.W., S.R. Boone, L., Walrath and S.L. Ashby., 1997. Mobilization of Ammonia and Phosphorus by Zebra Mussels (*Dreissena polymorpha*) in the Seneca River, New York. *In:* D'Itri, F. (ed.) *Zebra Mussels and Aquatic Nuisance Specie* Ann Arbor Press, Chelsea, Michigan, pp. 187-207.

Elliott, J.M., 1975. The Growth Rate of Brown Trout (Salmo trutta L.) Fed on Maximum Rations. *Journal of Animal Ecology* **44(3),** 805-821.

Environmental Protection Agency, 2001. Parameters of water quality Interpretation and standard. Published by the Environmental Protection Agency, PO Box 3000, Johnstown Castle, Co. Wexford, Ireland, pp. 143.

The Ephemeroptera Recording Scheme, http://www.ephemeroptera.pwp.blueyonder.co.uk/7465/ [last accessed 14th March 2013].

European Commission, 2013. Proposal for a regulation of the European parliament and of the council on the prevention and management of the introduction and spread of invasive alien species. Brussels, 9.9.2013COM 620 final.

The European Communities (Birds and Natural Habitats) Regulations 2011. S.I. No. 477 of 2011. http://www.npws.ie/media/npwsie/content/files/Birds%20and%20Habitats%20Regulations%20SI%20477%20of%202011.pdf [last accessed 11th November 2013].

European Communities (Quality of Salmonid Waters) Regulations, 1988. *Irish Statute Book.* S.I. No. 293/1988 http://www.irishstatutebook.ie/1988/en/si/0293.html [last accessed 4th March, 2013].

Fanslow, D.L., Nalepa, T.F. and Lang, G.A., 1995. Filtration rates of zebra mussels (*Dreissena polymorpha*) on natural seston from Saginaw Bay, Lake Huron. *Journal of Great Lakes Research* **21(4)**, 489-500.

Fisher, S.W., Gossiaux, D.C., Bruner, K.A., Landrum, P.F., 1993. Investigations of the toxicokinetics of hydrophobic contaminants in the zebra mussel (*Dreissena polymorpha*). *In:* Nalepa, T.F. and Schloesser, D.W. (eds.) *Zebra mussels: biology, impacts and controls*. Ann Arbor, MI: Lewis Publ. Boca Raton., pp. 465–90.

Fishlock, P., 2011. http://www.fishingnet.com/brown_trout.htm [last accessed 26th February 2011].

Forbes, V. E. and Forbes, T. L., 1994. *Ecotoxicology in theory and practice*, volume 2. Chapman and Hall, London, pp. 247.

Fowler, J. Cohen, L. and Jarvis, P., 1998. Practical statistics for field biology. Second edition John Wiley, New York, pp. 259.

Füreder, L., Gherardi, F., Holdich, D., Reynolds, J., Sibley, P. and Souty-Grosset, C., 2010. *Austropotamobius pallipes*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.2. <www.iucnredlist.org>. [Last accessed 19th March 2014].

Gargan, G. and O'Grady, M. F., 1992. Feeding relationships of trout *Salma trutta* L., Perch *Perca fluviatilis* L. and Roach *Rutilus rutilus* (L.) in Lough Sheelin, Ireland. *Irish fisheries Investigations series A* (Freshwater) No. 35.

Garton, D. and Haag., 1993. Seasonal reproduction cycles and settlement patterns of *Dreissena polymorpha* in western Lake Erie.). *In:* Nalepa, T.F. and Schloesser, D.W. (eds.) *Zebra mussels: biology, impacts and controls*. Ann Arbor, MI: Lewis Publ. Boca Raton., pp. 111-128.

Garton, D.W., McMahon R. and Stoeckmann, A.M. 2013. Limiting environmental factors and competitive interactions between zebra and quagga mussels in North America. *In:* Schloesser, D.W. and Nalepa, T.F. (eds.) Q*uagga and zebra mussels: biology, impacts and control, second edition.* CRC Press, Taylor & Francis Group, Boca Raton, pp. 383-402.

GeoChemBio Daphnia spp. http://www.geochembio.com/biology/organisms/daphnia/ [Last accessed 29th May 2013].

Graczyk, T.K., Conn, D.B., Lucy, F., Tamang, L., Moura, L.N. and Da-Silva, A.J., 2004. Human waterborne parasites in zebra mussels (Dreissena polymorpha) from the Shannon River drainage area, Ireland. *Parasitology Research* **93(5)**, 385–391.

Graham, R. and Gilbert, J., 2012. MBI-401 SDP: Assessment of Ready Biodegradable by Measurement of CO2 Evolution. Unpublished Report.

Great Lakes Restoration Initiative (GLRS) Evaluating the Safety and Efficacy of *Pseudomonas fluorescens* Strain CL145A to Control Dreissenid Mussels. http://cida.usgs.gov/glri/projects/invasive_species/zm_control.html [last accessed 7th March 2013].

Griffiths, R.W., 1993. Effects of zebra mussels (*Dreissena polymorpha*) on the benthic fauna of Lake St. Clair.). *In:* Nalepa, T.F. and Schloesser, D.W. (eds.) *Zebra mussels: biology, impacts and controls*. Ann Arbor, MI: Lewis Publ. Boca Raton, pp. 415– 437.

Gross, C., 1997. Long term experience with non fouling coatings and other means to control macrofouling. *In:* D'Itri, F. (ed.) *Zebra Mussels and Aquatic Nuisance Specie* Ann Arbor Press, Chelsea, Michigan, pp. 329-342.

Gu, J.D., Maki, J.S. and R. Mitchell., 1997. Microbial biofilms and their role in the induction and inhibition of invertebrate settlement. *In:* D'Itri, F. (ed.) *Zebra Mussels and Aquatic Nuisance Specie* Ann Arbor Press, Chelsea, Michigan, pp. 343-357.

Harrington, D.K., Van Benschoten, J.E., Jensen, J.N., Lewis, D.P., Neuhauser, E.F., 1997. Combined use of heat and oxidants for controlling adult zebra mussels. *Wat Res* **31**:2783–2791.

Holdich, D.M., Peay, S., Foster, J., Hiley, P.D. and Brickland, J.H., 2006. Studies on the white clawed crayfish (*Austropotamobius pallipes)* associated with muddy habitats. *Bull. Fr. Pêche Piscic.* 380–381, 1055–1078.

Holland, R.E., 1993. Changes in Planktonic Diatoms and Water Transparency in Hatchery Bay, Bass Island Area, Western Lake Erie Since the Establishment of the Zebra Mussel. *Journal of Great Lakes Research* **19(3)**, 617-624.

Hebert, P.D.N., Muncaster, B.W., Mackie, G.L., 1989. Ecological and genetic studies on *Dreissena polymorpha* (Pallas): a new mollusc in the Great Lakes. *Canadian Journal of Fisheries and Aquatic Sciences* **46(9),** 1587–1591.

Hedayati, A., Safahieh, A., Savari, A. and Ghofleh Marammazi, J., 2010. Detection of mercury chloride acute toxicity in Yellowfin sea bream (Acanthopagrus latus). *World J Fish Mar Sci*. ***2*(1)**, 86-92.

Hendry, K. and Cragg-Hine, D., 2003. Ecology of the Atlantic Salmon. Conserving Natura 2000 Rivers Ecology Series No. 7. English Nature, Peterborough.

Hering, D., Buffagni, A., Moog, O., Sandin, L., Sommerhäuser, M., Stubauer, I., Field, C., Johnson, R., Pinto, R., Skoulikidis, N., Verdonschot, P. and Zahrádková, S., 2003. The development of a system to assess the ecological quality of streams based on macroinvertebrates–design of the sampling programme within the AQEM project. *International Review of Hydrobiology* **88(3-4),** 345-361.

Higgins, T.M., Grennan J.M. and Kieran McCarthy, T., 1998. Effects of recent zebra mussel invasion on water chemistry and phytoplankton production in a small Irish lake. *Aquatic Invasions* **3(1),** 14-20.

Horgan M.J. and Mills E.L., 1997. Clearance rates and filtering activity of zebra mussel (*Dreissena polymorpha*): implications for freshwater lakes. *Canadian Journal of Fisheries and Aquatic Sciences* **54(2)**, 249-255.

Horvath, T.G. and Crane, L., 2010. Hydrodynamic forces affect larval zebra mussel (*Dreissena polymorpha*) mortality in a laboratory setting. *Aquatic Invasions* **5(4)**, 379-385.

Horvath, T.G. and Lamberti, G.A., 1999. Mortality of zebra mussel, *Dreissena polymorpha*, veligers during downstream transport. *Freshwater Biology* **42(1)**, 69-76.

Horvath, T. G., Lamberti, G. A., Lodge, D. M. and Perry, W. L., 1996. Zebra mussel dispersal in lake-stream systems: source-sink dynamics. *Journal of the North American Benthological Society* **15**, 564–575.

Hulme P.E., Bacher S., Kenis M., Klotz S., Kühn I., Minchin D., Nentwig W., Olenin S., Panov V., Pergl J., Pyšek P., Roques A., Sol D., Solarz W. And Vilà M., 2008.

Grasping at the routes of biological invasions: a framework for integrating pathways into policy. *J. Appl. Ecol*. **45(2),** 403-414.

Inland Fisheries Ireland, 2013. Socio-Economic Study of Recreational Angling in Ireland. http://www.fisheriesireland.ie/media/tdistudyonrecreationalangling.pdf [last accessed 19th March 2014].

Inland Fisheries Ireland. Fishing in Ireland. http://www.fishinginireland.info/trout/ [last accessed 16th February 2014].

IOWATER, 2005. Benthic Macroinvertebrate Indexing. *IOWATER volunteer water monitoring program.* http://www.iowater.net/Publications/BenthicManual.pdf. [last accessed 23rd March 2014].

Islam, M.N., Port G.R. and McLachlan, A.J., 2001. The biology of *Lymnaea peregra* (Muller) (Gastropoda: Pulmonata: Basommatophora) with special reference to the effects of herbicides on its reproduction. *J. Biological Sci*. **1**, 532-540.

Jones, C. G., Lawton, J. H. and Shachak, M., 1996. Organisms as ecosystem engineers. In *Ecosystem Management*. Springer New York, pp. 130-147.

Karatayev, A.Y., Burlakova, L.B. and Padilla, D.K., 1997. The Effects of *Dreissena polymorpha* Invasion on Aquatic Communities in Eastern Europe. *Journal of Shellfish Research* **16(1)**, 187-203.

Karatayev A.Y., Burlakova, L. and Padilla, D.K., 1998. Physical factors that limit the distribution and abundance of Dreissena polymorpha (Pallas). *Journal of Shellfish Research* **17(4)**, 1219–1235.

Karatayev, A.Y., Burlakova L.B. and Padilla D.K., 2002. The impact of zebra mussels on aquatic communities and their role as ecosystem engineers. *In:* Leppäkoski, E., Gollasch, S. and Olenin, S. (eds.) *Invasive aquatic species of Europe.* Kluwer Scientific Publishers, pp. 433-446.

Karatayev, A.Y., Burlakova L.E. and Padilla D.K., 2006. Growth rate and longevity of *Dreissena polymorpha* (Pallas): a review and recommendations for future study. *Journal of Shellfish Research* **25(1),** 23-32.

Karatayev, A. Y., Padilla, D. K., Minchin, D., Boltovskoy, D. and Burlakova, L. E., 2007. Changes in global economies and trade: the potential spread of exotic freshwater bivalves. *Biological Invasions*, *9*(2), 161-180.

Keller, R.P**.**, Geist, J., Jeschke, J.M. and Kühn, I., 2011. Invasive species in Europe: ecology, status and policy. *Environmental Sciences Europe* **23(1),** 1-17**.**

Killeen, I., and Aldridge, D. 2011. *Anodonta cygnea*. The IUCN Red List of Threatened Species. Version 2014.2. www.iucnredlist.org. [Last accessed 5th October 2014].

Klemetsen, A., Amundsen, P.A., Dempson, J.B., Jonsson, B., Jonsson, N. O'Connell, M.F. and Mortensen, E., 2003. Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpines* (L.): a review of aspects of their life histories. *Ecology of Freshwater Fish* **12(1),** 1–59.

Kobak, J., 2013. Behaviour of juvenile and adult zebra mussels (*Dreissena polymorpha*). *In:* Schloesser, D.W. and Nalepa, T.F. (eds.) Q*uagga and zebra mussels: biology, impacts and control, second edition.* CRC Press, Taylor & Francis Group, Boca Raton, pp. 331-344.

Kraak, M.H.S., Toussaint, M., Lavy, D. and Davids, C., 1994. Short-term effects of metals on the filtration rate of the zebra mussel *Dreissena polymorpha*. *Environmental Pollution* **84(2)**, 139-143.

Kryger, J. and, Ulrick Riisgard, H., 1988. Filtration rate capacities in 6 species of European freshwater bivalves. *Oecologia*. **77(1)**, 34-38.

Kumar A., Correll R., Grocke S. and Bajet C., 2010. Toxicity of selected pesticides to freshwater shrimp, Paratya australiensis (Decapoda: Atyidae): Use of time series acute toxicity data to predict chronic lethality: *Ecology and Environmental Safety* **73(3)**, 360-396.

Lake Champlain Zebra Mussel Monitoring Programme http://www.vtwaterquality.org/lakes/htm/lp_lczebramon.htm. [last accessed 29th October 2013]

Lance, E., Brient, L., Bormans, M. and Gérard, C., 2006. Interactions between cyanobacteria and Gastropods I. Ingestion of toxic *Planktothrix agardhii* by *Lymnaea*

stagnalis and the kinetics of microcystin bioaccumulation and detoxification. *Aquatic Toxicology* **79(2)**, 140–148.

Laruelle, F., Molloy, D.P. and Roitman, V.A., 2002. Histological analysis of trematodes in Dreissena polymorpha: their location, pathogenicity and distinguishing morphological characteristics. *Journal of Parasitology* **88(5),** 856-863.

Leach, J. H., 1993. Impacts of the zebra mussel *(Dreissena polymorpha)* on water quality and fish spawning reefs in western Lake Erie. *In:* Nalepa, T.F. and Schloesser, D.W. (eds.) *Zebra mussels: biology, impacts and controls*. Ann Arbor, MI: Lewis Publ. Boca Raton., pp. 381-397.

Le Page, W.L. 1993. The impact of *Dreissena polymorpha* on waterworks operations at Monroe, Michigan: a case history. *In:* Nalepa, T.F. and Schloesser, D.W. (eds.) *Zebra mussels: biology, impacts and controls*. Ann Arbor, MI: Lewis Publ. Boca Raton., pp. 333– 348.

Lopes-Lima, M. 2014. *Anodonta anatina*. The IUCN Red List of Threatened Species. Version 2014.2. www.iucnredlist.org. [Last accessed 5th October 2014].

Lucy, F. and Sullivan, M., 2001. The Investigation of an Invasive Species, the Zebra Mussel Dreissena polymorpha in Lough Key, Co. Roscommon, 1999. *Desktop Study No. 13.* Irish EPA, Johnstown Castle Estate, Wexford, Ireland, pp. 38.

Lucy, F. (2005) *The dynamics of Zebra Mussel (Dreissena polymorpha) populations in Lough Key, Co. Roscommon, 1998-2003*. Ph.D thesis, IT Sligo, pp. 304.

Lucy, F., Sullivan M. and Minchin D., 2005. Nutrient Levels and the Zebra Mussel Population in Lough Key. Prepared for the Environmental Protection Agency December 2005, pp. 25.

Lucy F., 2006. Early life stages of Dreissena polymorpha (zebra mussel): the importance of Long-term datasets in invasion ecology. *Aquatic Invasions* **1(3)**, 171-182.

Lucy F.E., Graczyk T.K., Tamang L., Miraflor A., Minchin D., 2008. Biomonitoring of surface and coastal water for Cryptosporidium, Giardia and human-virulent microsporidia using molluscan shellfish. Parasitol Research **103**, 1369–1375.

Lucy, F.E., 2010. Zebra Mussels: Review of ecology and impacts since invasion in Ireland. *In:* Mackie G, Claudi R (eds.) Monitoring and control of macrofouling molluscs in freshwater systems. 2nd Edition, CRC Press, Boca Raton, FL., pp. 386–389.

Lucy, F.E., Burlakova, L.E., Karatayev, A.Y., Mastitsky, S.E. and Zanatta, D., 2014. Zebra mussel impacts on Unionids: A synthesis of trends in North America and Europe. *In:* Schloesser, D.W. and Nalepa, T.F. (eds.) Q*uagga and zebra mussels: biology, impacts and control, second edition.* CRC Press, Taylor & Francis Group, Boca Raton, pp 623-646.

Ludyanskiy, M.L., McDonald, D. and MacNeill, D., 1993. Impact of the zebra mussel, a bivalve invader. *Bioscience* **43**, 533–54.

Lyman, F. E., 1956. Environmental factors affecting distribution of mayfly nymphs in Douglas Lake, Michigan. *Ecology* **37(3),** 568-576.

Lyons, L.A., Petrille, J.C., Donner, S.P., Fobes, R.L., Lehmann, F., Althouse P.W., Wall L.T., Post R.M. and Buerger W.F., 1990. New treatment employing a molluscicide for macrofouling control of zebra mussels in cooling systems. *Proceedings American Power Conference,* 52, 1012-1021.

MacIssac, H., Sprules, W.G. and Leach, J.H., 1991. Ingestion of small-bodied zooplankton by zebra mussels (*Dreissena polymorpha*): can cannibalism on larvae influence population dynamics? *Canadian Journal of Fisheries and Aquatic Science* **48(11)**, 2051-2060.

MacIsaac, H.J., Lonnee, C.J. and Leach, J.H., 1995. Suppression of microzooplankton by zebra mussels: Importance of mussel size. *Freshwater Biology* **34(2)**, 379-387.

Mackie, G.L., Gibbons, W.N., Muncaster, B.W. and Gray. LM. 1989. The zebra mussel, *Dreissena polymorpha*: A synthesis of European experiences and a preview for North America. Ontario Ministry of the Environment, Water Resources Branch, Queen's Printer for Ontario, Toronto, Ontario, pp.76.

Mackie GL, Schloesser DW. 1996. Comparative biology of zebra mussels in Europe and North America: An overview. *American Zoologist* **36(3),** 244–258.

Mackie, G.L. and Claudi, R. 2010. Monitoring and control of macrofouling mollusks in freshwater systems. CRC Press, Boca Raton, FL., pp. 508.

Maguire, C., Rosell, R., Roberts D., 2006. Management of the Impacts of Zebra Mussels in Northern Ireland and Determination of Effects on Fish Populations in Lough Erne through Alterations of the Food Web. *Environment and Heritage Service Research and Development Series* **06**, pp. 25.

Marrone Bio Innovations Ecotoxicology Studies, 2012. http://www.marronebioinnovations.com/lightray/site/wp-content/uploads/2012/09/ZQ_EcotoxStudies_0413_web.pdf. [Last accessed 16th September 2013].

Marrone Bio Innovations, 2012a http://www.marronebioinnovations.com/products/brand/zequanox/. [Last accessed 20th March 2014].

Marrone Bio Innovations, 2012b. Product Label: MBI-401 SDP. EPA Reg. No. 84059-15. http://www.epa.gov/pesticides/chem_search/ppls/084059-00015-20120309.pdf. [Last accessed 20th March 2014].

Marsden, J.E., 1992. Standard Protocol for Monitoring and Sampling Zebra Mussels. Illinois Natural History Survey Biological Notes 138, pp. 40.

Matthews, M. A., McMahon, R. F., 1999. Effects of temperature and temperature acclimation on survival of zebra mussels *(Dreissena polymorpha)* and Asian clams *(Corbicula fluminea)* under extreme hypoxia. *Journal of Molluscan Studies* **65(3)**, 317-325.

May, G. E., Gelembiuk G. W., Panov V. E., Orlova M. I., & Lee C. E., 2006. Molecular ecology of zebra mussel invasions. *Molecular Ecology* **15(4)**, 1021-1031.

Mayer, D.A., 2011. Evaluation of a biopesticide against invasive species for native species restoration. A dissertation submitted to the University at Albany, State University of New York. http://udini.proquest.com/view/evaluation-of-a-biopesti cide-pqid:2557999051/. [Last accessed 21st May 2013].

Mayer, C.M., Burlakova, L.E., Eklöv, P., Fitzgerald, D., Karatayev, A., Ludsin, S.A., Millard, S., Mills, E.L., Ostapenya, A.P., Rudstam, L.G., Zhu, B. and Zhukova, T.V., 2014. Benthification of freshwater lakes exotic mussels turning ecosystems upside down. *In:* Schloesser, D.W. and Nalepa, T.F. (eds.) Q*uagga and zebra mussels: biology, impacts and control, second edition.* CRC Press, Taylor & Francis Group, Boca Raton, pp. 575-585.

McCarthy, T.K., Fitzgerald, J. and O'Connor, W., 1997. The occurrence of the zebra mussel *Dreissena polymorpha* (Pallas, 1771), an introduced biofouling freshwater bivalve in Ireland. *Ir. Nat. Jr.* **25**(**11/12),** 413-416.

McMahon, R., Ussery, T. A. and Clarke, M., 1994. Review of Zebra Mussel Control Methods. Technical note ZMR -2-14 Prepared and published by the Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, 3909 Halls Ferry Road, Vicksburg, MS 39180-6199, pp. 12.

McMahon, R. F., Matthews, M. A., Ussery, T. A., Chase, R. and Clarke, M., 1995. Further studies of heat tolerance of zebra mussels: Effects of temperature acclimation and chronic exposure to lethal temperatures. Technical Report EL-95-9, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS, pp. 25.

Mead, D.F., Adams D.J., 1997. An Effective Zebra Mussel Control Program. *In:* D'Itri, F. (ed.) *Zebra Mussels and Aquatic Nuisance Specie* Ann Arbor Press, Chelsea, Michigan, pp. 275-282.

Meehan, S., Lucy, F.E., Gruber, B., Rackl, S., 2013. Comparing Zequanox® and chlorine as zebra mussel control strategies in an Irish drinking water treatment plant. *Management of Biological Invasions* **4(2),** 113-122.

Meehan, S., Shannon, Gruber, B. & Rackl S.A., & Lucy, F.E. 2014a. Zequanox® its Effectiveness in Non Target Ecotoxicology Studies. *Ecotoxicology and Environmental Safety* **107**, 148-153.

Meehan, S., Gruber, B., & Lucy, F.E. 2014b, Zebra mussel control using Zequanox® in an Irish waterway. *Management of Biological Invasions* **5(3),** 279-286**.**

Millane, M., 2008. Impact of the zebra mussel *Dreissena polymorpha* (Pallas 1771) on the ecological integrity of Lough Sheelin, Ireland. Ph.D Thesis, University College Dublin, pp. 263.

Millane, M., Quinn, M.K. and Champ, T., 2008. Impact of the zebra mussel invasion on the ecological integrity of Lough Sheelin, Ireland: distribution, population characteristics and water quality changes in the lake. *Aquatic Invasions* **3(3),** 271-281.

Minchin, D. and Moriarty, C., 1998. Zebra mussels in Ireland. *Fisheries Leaflet* 177, Marine Institute, Dublin, pp. 11.

Minchin, D. and Gollasch, S., 2002. Vectors-How exotics get around. *In:* Leppakowski, E., Gollasch, S. and Olenin, S. (eds.) *Invasive aquatic species of Europe. Distribution, impacts and management*. Kluwer Academic Publishers, Dordrecht, pp. 183-192.

Minchin D., Lucy F. and Sullivan M., 2002a. Monitoring of zebra mussels in the Shannon-Boyle navigation, other navigable regions and principal Irish lakes. *Marine Environment and Health Series* **5**, pp. 17.

Minchin, D., Lucy, F. and Sullivan, M., 2002b. Zebra Mussels: Impacts and spread. *In:* Leppakowski, E., Gollasch, S. and Olenin, S. (eds.) *Invasive aquatic species of Europe. Distribution, impacts and management*. Kluwer Academic Publishers, Dordrecht, pp. 135-146.

Minchin, D., Maguire, C. and Rosell, R., 2003. The zebra mussel (*Dreissena polymorpha Pallas*) invades Ireland: Human mediated vectors and the potential for rapid intranational dispersal. *Biology and Environment: Proceedings of the Royal Irish Academy* 103B: 23-30.

Minchin, D., Lucy, F., Sullivan, M., 2005. Ireland a New Frontier for the Zebra Mussel *Dreissena Polymorpha* (pallas.) *Oceanological and Hydrobiological Studies* **34(1)**, 19-30.

Minchin, D., 2007. Rapid coastal survey for targeted alien species associated with floating pontoons in Ireland. Aquatic Invasions **2(1),** 63-70.

Minchin, D. and Boelens, R., 2010. *Hemimysis anomala* is established in the Shannon River Basin District in Ireland. *Aquatic Invasions* **5(1),** 71-78.

Molloy, D. P., Karatayev, A. Y., Burlakova, L. E., Kurandina, D. P. and Laruelle, F., 1997. Natural Enemies of Zebra Mussels: Predators, Parasites and Ecological Competitors. *Reviews in Fisheries Science* **5**, 27-97.

Molloy D.P., Mayer D.A., Gaylo M.J., Morse J.T., Presti K.T., Sawyko P.M., Karatayev A.Y., Burlakova L.E., Laruelle F., Nishikawa K.C. and Griffin B.H., 2013a. A biopesticide for the control of zebra and quagga mussels (Bivalvia: Dreissenidae) *Journal of Invertebrate Pathology* **113(1),** 104–114.

Molloy D.P., Mayer D.A., Giamberini L. and Gaylo M.J., 2013b. Mode of action of *Pseudomonas fluorescens* strain CL145A, a lethal control agent of dreissenid mussels (Bivalvia: Dreissenidae) *Journal of Invertebrate Pathology* **113(1),** 115-21.

Molloy D.P., Mayer D.A., Gaylo M.J., Burlakova1 L.E., Karatayev A.Y., Presti1 K.T., Sawyko P.M., Morse J.T. and Paul E.A. 2013c. Non-target trials with Pseudomonas fluorescens strain CL145A, a lethal control agent of dreissenid mussels (Bivalvia: Dreissenidae). *Management of Biological Invasions* **4(1),** 71–79.

Mooney, H.A., Cleland E.E., 2001. The evolutionary impact of invasive species. National Academy of Sciences colloquium, "The Future of Evolution". **98**, 5446–5451.

Morton, B., 1982. Some aspects of the population structure and sexual strategy of Corbicula cf. fluminalis (Bivalvia: Corbiculacea) from the Pearl River, People's Republic of China. *Journal of Molluscan Studies*, **48(1),** 1-23.

Moss, B., Stephen, D., Balayla, D. M.,et al. 2004. Continental-scale patterns of nutrient and fish effects on shallow lakes: synthesis of a pan-European mesocosm experiment. *Freshwater Biology* **49**, 1633–1649.

Mubiana, V. K., Vercauteren, K., & Blust, R., 2006. The influence of body size, condition index and tidal exposure on the variability in metal bioaccumulation in *Mytilus edulis*. *Environmental Pollution* **144,** 272-279.

Mullarney, K., Svensson, L., Zetterstrom, D. and Grant, P.J., 1999. Collins Bird Guide. London, United Kingdom: Collins Publishing, pp. 416.

Nagabhushanam, .R, 1956. The rate of water filtration in the marine wood-boring mollusc, *Martesia striata* linn. *Proceedings of the Indian Academy of Sciences - Section B* **43 (4),** 223-227.

Neumann, D., Borcherding, J. and Jantz, B., 1993. Growth and seasonal reproduction of Dreissena polymorpha in the Rhine River and adjacent waters. *In:* Nalepa, T.F. and Schloesser, D.W. (eds.) *Zebra mussels: biology, impacts and controls*. Ann Arbor, MI: Lewis Publ. Boca Raton., pp. 95-109.

The National Biodiversity Data Centre. http://invasives.biodiversityireland.ie/zebra-mussel-continues-spread/. [last accessed 20th November 2013].

Nichols, S.J., 1996. Variations in the Reproductive Cycle of Dreissena Polymorpha in Europe, Russia and North America. *American Zoologist* **36(3),** 311-325.

OECD, 1992. Test guideline 203 Fish, Acute Toxicity Test. Published by: OECD Publishing.

OECD, 2004a. Test guideline 219 Sediment-Water Chironomid Toxicity Test Using Spiked Water. Published by: OECD Publishing.

OECD, 2004b. Test guideline 202 *Daphnia sp.,* Acute Immobilisation Test. Published by: OECD Publishing.

OECD, 2011. Test Guideline 235 *Chironomus* sp., Acute Immobilisation Test. Published by: OECD Publishing.

Ogbonna, F. J. and Chinomso, A. A., 2010. Determination of the concentration of ammonia that could have lethal effect on fish pond. *Journal of Engineering & Applied Sciences* ***5*(2).**

Olsen, L., Sunesen, J. and Pedersen, B., 2001. *Small freshwater creatures*. Oxford University Press, Oxford, pp. 229.

Padilla, D. K. and Williams, S. L., 2004. Beyond ballast water: aquarium and ornamental trades as sources of invasive species in aquatic ecosystems. *Frontiers in Ecology and the Environment* **2(3),** 131-138.

Payne, B.S. and Lowther, D., 1992. Technical note ZMR -1-02. Larval monitoring in a chlorine treatment program to prevent zebra mussel settlement in hydropower facilities. Prepared and published by the Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, 3909 Halls Ferry Road, Vicksburg, MS 39180-6199, pp. 2.

Peix, A., Ramı´rez-Bahena M-H., Vela´zquez E., 2009. Historical evolution and current status of the taxonomy of genus Pseudomonas Infection. *Genetics and Evolution* **9(6)**, 1132–1147.

Pollux, B., Minchin, C., Van Der Velde, G., Van Alen, T., Van Der Staay, S.Y.M. and Hackstein, J., 2003. Zebra mussels (*Dreissena polymorpha*) in Ireland, AFLP-fingerprinting and boat traffic both indicate an origin from Britain. *Freshwater biology* **48(6),** 1127-1139.

Porter, K. G., Orcutt, Jr, J. D. and Gerritsen, J., 1983. Functional Response and Fitness in a Generalist Filter Feeder, Daphnia Magna (Cladocera:Crustacea). *Ecology* **64(4),** 735-742.

Rajagopal, S., Van der Velde G. and Jenner, HA., 1997. Response of zebra mussel, Dreissena polymorpha to elevated temperatures in The Netherlands. *In:* D'Itri, F. (ed.) *Zebra Mussels and Aquatic Nuisance Specie* Ann Arbor Press, Chelsea, Michigan, pp. 257-273.

Rajagopal S, Van Der Velde G and De Vaate AB (2000) Reproductive Biology of the Asiatic Clams Corbicula fluminalis and Corbicula fluminea in the River Rhine. *Archiv Fur Hydrobiologie* **149,** 403-420

Rajagopal, S., Van der Velde, G. and Jenner, H.A., 2002. Effects of low-level chlorination on zebra mussel, *Dreissena polymorpha*. *Water Research* **36,** 3029–3034.

Rajagopal, S., Van der Velde, G., Van der Gaag, M. and Jenner, H.A., 2003. How effective is intermittent chlorination to control adult mussel fouling in cooling water systems? *Water Research* **37,** 329–338.

Ram, J. L., Fong, P. P. and Garton, D. W., 1996. Physiological aspects of zebra mussel reproduction: Maturation, spawning and fertilization. *American Zoologist* **36(3)**, 326-338.

Randall, D.J. and Tsui, T.K.N., 2002. Ammonia toxicity in fish. *Marine Pollution Bulletin* **45**, 17–23.

Reeders, H. H., Bij de Vaate, A. and. Noordhuis, R., 1993. Potential of the zebra mussel (Dreissena polymorpha) for water quality management. *In:* Schloesser, D.W. and Nalepa, T.F. (eds.) Q*uagga and zebra mussels: biology, impacts and control, second edition.* CRC Press, Taylor & Francis Group, Boca Raton, pp. 439-451.

Rehman, C.R., Stoeckel, J.A. and Schneider, D.W., 2003. Effect of turbulence on the mortality of zebra mussel veligers. *Canadian Journal of Zoology* **81(6)**, 1063-1069.

Rejmánek, M. and Richardson D. M., 1996. What attributes make some plant species more invasive? *Ecology* **77(6),** 1655–61.

Reynolds, J. D., 1998. Conservation Management of the White-Clawed Crayfish *Austropotamobius pallipe. Irish Wildlife Manuals* No.1.

Reynolds, J.D. and Donohoe, R., 2001. Crayfish predation experiments on the introduced zebra mussel *Dreissena polymorpha*, in Ireland and their potential for biocontrol. *Bull. Fr. Pêche Piscic* **361**, 669-681.

Reynolds, J.D., O'Connor, W., O'Keeffe, C., Lynn, D., 2010. A technical manual for monitoring white-clawed crayfish Austropotamobius pallipes in Irish lakes. Irish Wildlife Manuals, No 45. National Parks and Wildlife Service, Department of the Environment, Heritage and Local Government, Dublin, pp. 23.

Ricciardi, A., Whoriskey, F.G. and Rasmussen, J.B., 1995a. Predicting the intensity and impact of *Dreissena* infestation on native unionids bivalves from *Dreissena* field density. *Canadian Journal of Fisheries and Aquatic Science* **52(12)**, 1449-1461.

Ricciardi, A., Snyder, F.L., Kelch, D.O. and Reiswig, H.M., 1995b. Lethal and sublethal effects of sponge overgrowth on introduced dreissenid mussels in the Great Lakes-St. Lawrence River system. *Canadian Journal of Fisheries and Aquatic Sciences* **52**, 2695-2703.

Ristola, T., 2000. Assessment of sediment toxicity using the midge *Chironomus riparius* (Diptera: Chironomidae). University of Joensuu, PhD dissertations in biology no. 5. ISSN 1457-2486, pp. 101.

Roche, P. and Benanou, D., 2007. Impact of chlorination on the formation of odour compounds and their precursors in the treatment of drinking water. TECHNEAU. Available at http://www.techneau.org/fileadmin/files/Publications/Publications/Deliverables/D5.3.8.pdf [Last accessed 8th January 2013].

Rosell, R.S., Maguire, C.M. and Mc Carthy, T.K., 1999. First settlement of zebra mussels *Dreissena polymorpha* in the Erne system, Co. Fermanagh. *Biology and Environment*: *Proceedings of the Royal Irish Academy* **98B(3),** 191–3.

Ryther, J.H., 1954. Inhibitory Effects of Phytoplankton upon the Feeding of Daphnia Magna with Reference to Growth, Reproduction and Survival. *Ecology* **35(4)**, 522-533.

Salles, A., 2000. Ephemeroptera Mayflies. http://www.ucmp.berkeley.edu/arthropoda/uniramia/ephemeroptera.html [last accessed 13th March 2013].

Scobey, F. C., 1939. Flow of water in irrigation and similar canals (No. 652). US Dept. of Agriculture, Washington, D.C.

Schloesser, D., Nelepa, T. and Mackie, G.L., 1996. Zebra mussel infestation of unionids bivalves (Unionidae) in North America. *American Zoologist* **36(3)**, 300-310.

Sousa1, R., Antunes, C. and Guilhermino1, L., 2008. Ecology of the invasive Asian clam *Corbicula fluminea* (Müller, 1774) in aquatic ecosystems: an overview *Ann. Limnol. - Int. J. Lim.* **44(2),** 85-94.

Sprung, M. 1989. Field and laboratory observations of Dreissena polymorpha: Abundance, growth, mortality and food demands. *Arch. Hydrobiol*. **115(4),** 537-561.

Sprung, M. 1993. The other life: an account of present knowledge of the larval phase of *Dreissena polymorpha. In:* Nalepa, T.F. and Schloesser, D.W. (eds.) *Zebra mussels: biology, impacts and controls*. Ann Arbor, MI: Lewis Publ. Boca Raton., pp. 39-53.

Staley, M., 2007. Maryland Fish Facts. Department of natural resources. http://www.dnr.state.md.us/fisheries/fishfacts/browntrout.asp. [Last accessed 5th March 2012].

Stańczykowska, A. and Planter M. 1985. Factors affecting nutrient budget in lakes of the R. Jorka watershed (Masurian Lakeland, Poland). X. Role of the mussel *Dreissena polymorpha* (Pall.) in N and P cycles in a lake ecosystem. Ekologia Polska **33(2),** 345-356.

Stokes, K., O'Neill, K. and McDonald, R., 2004. *Invasive species in Ireland.* Report to Environment & Heritage Service and National Parks & Wildlife Service by Quercus, Queens University Belfast, Belfast, Environment & Heritage Service, Belfast and National Parks & Wildlife Service, Dublin, pp. 151.

Suter, W., 1998. The Effect of Predation by Wintering Cormorants Phalacrocorax carbo on Grayling Thymallus thymallus and Trout (Salmonidae) Populations: Two Case Studies from Swiss Rivers. *The Journal of Applied Ecology* **32(1)**, 29-46.

Sweka, J. A. and Hartman, K. J., 2001. Influence of turbidity on brook trout reactive distance and foraging success. *Transactions of the American Fisheries Society* **130(1)**, 138-146.

Swynnerton, G. H. and Worthington, E. B., 1940. Note on the Food of Fish in Haweswater (Westmorland). *Journal of Animal Ecology*. **9(2),** 183-187.

Taucher, J., Schulz, K.G., Dittmar, T., Sommer, U., Oschlies, A. and Riebesell, U., 2012. Enhanced carbon overconsumption in response to increasing temperatures during a mesocosm experiment. *Biogeosciences* **9(9)**, 3531–3545.

Taylor, G. T. and Zheng, D., 1997. Preliminary report on use of marine natural products as repellents for zebra mussels. *In:* D'Itri, F. (ed.) *Zebra Mussels and Aquatic Nuisance Specie* Ann Arbor Press, Chelsea, Michigan, pp. 523-531.

Ten Winkel, E. H. and C. Davids. 1982. Food selection by *Dreissena polymorpha* Pallas (Mollusca: Bivalvia). *Freshwater Biology* **12,** 553-558.

Tikkanen, M.W., Schroeter, J.H., Leong, L.Y.C. and Ganesh, R., 2001. Guidance Manual for the Disposal of Chlorinated Water. Awwa Research Foundation and

American Water Works Association. http://www.vita-d-chlor.com/specs/ AWWARFDechlorGuides.htm. [Last accessed 8thJanuary 2012].

United States Environmental Protection Agency. 1999. Wastewater technology fact sheet: Chlorine disinfection. U. S. Environmental Protection Agency, Washington, DC. EPA/832-F99-062, pp. 7.

United States Environmental Protection Agency, 2001. Final Report: Interlaboratory Variability Study of EPA Short term Chronic and Acute Whole Effluent Toxicity Test Methods. Office of Water, Washington, USA. EPA 821-B-01-004, pp. 153.

United States Environmental Protection Agency, 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms 5th Edition. U.S. Environmental Protection Agency, Office of Water (4303T), 1200 Pennsylvania Avenue, NW, Washington, DC 20460, pp. 266.

United States Environmental Protection Agency, 2013. Aquatic life ambient water quality criteria for ammonia-freshwater. Office of Science and Technology, Office of Water, U.S. Environmental Protection Agency.

University of Minnesota. Department of Entomology http://www.entomology.umn.edu [last accessed 25th February, 2011].

Vailati, G., Bacchetta, R. and Mantecca, P., 2001. Sexual maturity in young Dreissena polymorpha from Lake Como (N. Italy). *Invertebrate reproduction & development*, **40(2-3),** 181-192.

Vanderploeg, H.A., Liebig, J.R., Carmichael, W.W., Agy, M.A., Johengen, T.H., Fahnenstiel, G.L. and Nalepa,T.F., 2001. Zebra mussel (*Dreissena polymorpha*) selective filtration promoted toxic *Microcystis* blooms in Saginaw Bay (Lake Huron) and Lake Erie. *Can. J. Fish Aquat. Sci.* **58**, 1208–1221.

Vanderploeg, H.A., Wilson, A.E., Johengen, T.H., Bressie, J.D., Sarnelle, O., Liebig, J.R., Robinson, S.D. and Horst, G.P., 2013. Role of selective grazing by *Dreissenid* mussels in promoting toxic *Microcystis* blooms and other changes in phytoplankton composition in the Great Lakes. *In:* Nalepa, T.F. and Schloesser, D.W. (eds.) *Zebra mussels: biology, impacts and controls*. Ann Arbor, MI: Lewis Publ. Boca Raton., Chapter 32.

Van Waarde, A., 1983. Aerobic and anaerobic ammonia production by fish. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* **74(4)**, 675-684.

Vinogradov, G.A., Smirnova, N.F., Sokolov, V.A. and Bruznitsky, A.A., 1993. Influence of chemical composition of the water on the mollusk *Dreissena polymorpha*). *In:* Nalepa, T.F. and Schloesser, D.W. (eds.) *Zebra mussels: biology, impacts and controls*. Ann Arbor, MI: Lewis Publ. Boca Raton., pp. 283-293.

Waller D.L., Rach J.J., Cope W.G. and Marking L.L., 1993. Toxicity of Candidate Molluscicides to Zebra Mussels *(Dreissena polymorpha)* and Selected Non target Organisms. *Journal of Great Lakes Restoration* **19(4)**, 695-702.

Waller, D.L. and Fisher S.W., 1996. Prevention of zebra mussel infestation and dispersal during aquaculture operations. *Progressive Fish Culturist* **58(2)**, 77-84.

Water Framework Directive, 2005. Alien Species Risk Assessment Methodology, guidance for practioners on the methodology to be applied in Ireland's river basin districts. Paper by the Working Group on Characterisation and Risk Assessment Surface water guidance document. http://www.wfdireland.ie/Documents/Characterisation%20Report/Background%20Information/Review%20of%20Env%20Impacts/Surface%20Water%20Risk%20Ass/Alien_Species_Practitioners_Guidance.pdf. [last accessed 14th November 2013].

Watts, M.C. and Bigg, G.R., 2001. Modelling and the monitoring of mesocosm experiments: two case studies. *Life Sciences Journal of Plankton Research* **23(10)**, 1081-1093.

Weltje, L. and Bruns, E., 2009. *The chironomid full life-cycle test* .Validation report. First draft. Available online, http://www.oecd.org/chemicalsafety/testing/44082677.pdf. [last accessed 14th March 2014].

West Virginia Department of Health and Human Resources, 2010. Class 1 Water Operator Course Manual. Available at: http://www.wvdhhr.org/oehs/eed/swap/training&certification/drinking_water/documents/ClassIManual.pdf. [Last accessed December 17 2012].

Wetzel, R.G., 2001. Benthic animals and fish communities. *In:* Wetzel, R.G. (ed.) *Limnology lake and river ecosystems, third Edition*. *Academic Press, New York, N.Y.*, pp. 665-730.

Wilson, A.E., 2005. Effects of zebra mussels on phytoplankton and ciliates: a field mesocosm experiment. *Life Sciences Journal of Plankton Research* **25(8)**, 905-915.

Winder, C., 2001. The Toxicology of Chlorine. *Environmental Research* **85(2),** 105–114.

Wright, D.A., Magee, J.A., Setzler-Hamilton, E.M., Chalker-Scott, L. and Morgan, G.L., 1997. Use of high energy monochromatic UV light to kill dreissenid larvae. *In:* D'Itri, F. (ed.) *Zebra Mussels and Aquatic Nuisance Specie* Ann Arbor Press, Chelsea, Michigan, pp. 467-475.

Wright, P. A., Iwama, G. K. and Wood, C. M., 1993. Ammonia and urea excretion in Lahontan cutthroat trout (Oncorhynchus clarki henshawi) adapted to the highly alkaline Pyramid Lake (pH 9.4). *Journal of Experimental Biology* **175(1)**, 153-172.

Yu, N. and Culver, D.A., 1999. Estimating the effective clearance rate and refiltration by zebra mussels, Dreissena polymorpha, in a stratified reservoir. *Freshwater Biology* **41(3),** 481–492.

Zhu, B., Fitzgerald, D.G., Mayer, C.M., Rudstam, L.G. and Mills, E.L., 2006. Alteration of Ecosystem Function by Zebra Mussels in Oneida Lake: Impacts on Submerged Macrophytes. *Ecosystems* **9(6)**, 1017–1028.

Appendix A: 'Ecotoxicological Impact of Zequanox®, a Novel Biocide, on Selected Non-Target Irish Aquatic Species'. This paper is presented as it appears in the journal of 'Ecotoxicology and Environmental Safety', 2014, Volume 4, Issue 2, Pages 113-122.

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Ecotoxicological impact of Zequanox®, a novel biocide, on selected non-target Irish aquatic species

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ABSTRACT

Effective, species-specific zebra mussel control is needed urgently for Ireland's freshwater bodies, which became infested with non-native zebra mussels in the 1990s. Zequanox®, a newly commercialized product for zebra and quagga mussel control, is composed of dead *Pseudomonas fluorescens* CL 145A cells. This paper describes ecotoxicology tests on three representative native Irish freshwater species: *Anodonta* (duck mussel), *Chironomus plumosus* (non-biting midge), and *Austropotamobius pallipes* (white-clawed crayfish). The species were exposed to Zequanox in a 72-h static renewal toxicity test at concentrations of 100–750 mg active ingredient per liter (mg a.i./L). Water quality parameters were measured every 12–24 h before and after water and product renewal. After 72 h, endpoints were reported as LC_{10}, LC_{50}, and LC_{100}. The LC_{50} values derived were (1) *Anodonta*: $\geq$500 mg a.i./L (*2*) *C. plumosus*: 1075 mg a.i./L, and (3) *A. pallipes:* $\geq$750 mg a.i./L. These results demonstrate that Zequanox does not negatively affect these organisms at the concentration required for $>$80percent zebra mussel mortality (150 mg a.i/L) and the maximum allowable treatment concentration in the United Sates (200 mg a.i./L). They also show the overall species-specificity of Zequanox, and support its use in commercial facilities and open waters.

1. Introduction

The zebra mussel *Dreissena polymorpha* (Pallas, 1771), is an invasive, exotic aquatic bivalve that has had a major impact on the ecosystems of many Irish lakes, rivers, and canals since first invading in the early 1990s (Minchin et al., 2002; Lucy et al., 2005; Mackie and Claudi, 2010; Lucy et al., 2014). Internationally, chlorine is the most commonly used zebra mussel control product for treating water pipes and chambers in commercial facilities (Minchin and Moriarty, 1998; Mackie and Claudi, 2010; Meehan et al., 2013). No treatment methods currently exist for zebra mussel control in open water; therefore, these invaders continue to spread, causing a decline in Ireland's native freshwater unionid mussels, *Anodonta* (Lucy et al., 2005).

Pseudomonas fluorescens CL145A is a soil bacterium that has been shown to cause mortality in zebra and quagga mussels (Meehan et al., 2013; Molloy et al., 2013a). The commercial product discussed here is Zequanox® (manufactured by Marrone Bio Innovations, Inc.), where the active ingredient is dead *P. fluorescens* CL145A cells. Zequanox is primarily made up of particulate organic matter, and can therefore create a turbid environment. Zequanox has proven to be lethal to zebra and quagga mussels (Molloy et al., 2013b), which readily ingest Zequanox via filter feeding because they do not recognize it as a potentially harmful substance. Zequanox kills the mussels by destroying their digestive systems (Molloy et al., 2013b).

A Zequanox treatment concentration of 150 mg active ingredient per liter (mg a.i./L) for 8 h successfully achieved $>$75percent zebra mussel mortality in a trial carried out at Tullamore Harbour under optimal conditions (high DO) (Meehan et al., 2014). Greater than 80percent zebra mussel mortality has also been achieved in a trial carried out at a water treatment plant in Sligo (Ireland) at a treatment concentration of 200 mg a.i/L for 8 h (Meehan et al., 2013). According to the United States Environmental Protection Agency (USEPA) registration, the maximum allowable treatment concentration is 200 mg a.i./L for a total of 12 h for the spray dried Zequanox formulation (Marrone Bio Innovations, 2012a; Marrone Bio Innovations, 2012b).

Trials were performed at the New York State Museum to observe the effect of *P. fluorescens* on a small number of invertebrates, fish, and freshwater mussels. These trials found that the active ingredient was not harmful to the subject species (Molloy et al., 2013b).

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It is imperative, however, to test the commercial product Zequanox for its effect on other non-target organisms, especially those found in zebra mussel-infested ecosystems where Zequanox may be applied either in open water or within industrial systems and equipment (where abstracted water would be treated with Zequanox within the facility, then discharged back to source watercourses). Before Zequanox can be used in Europe, its target specificity to zebra and quagga mussels must be tested and confirmed using species native to European ecosystems. The objectives of these non-target ecotoxicology studies were to further evaluate the target specificity of Zequanox with respect to species protected in European aquatic ecosystems and to determine the impact that the resulting water quality may have on the organisms tested. These objectives were achieved by carrying out 72-h acute toxicity tests to determine the LC_{50} of the tested species (concentration at which 50percent mortality of the test species is observed) (Stephen, 1997).

The three taxa selected for this paper fit within different functional feeding groups: freshwater unionid mussels, *Anodonta* (filter feeder); non-biting midge larva, *Chironomus plumosus* (decomposer); and the white-clawed crayfish *Austropotamobius pallipes* (omnivore).

Anodonta (Bivalvia: Unionidae) like the zebra mussel, are primary consumers in the food chain (Başçınarl, 2009). In Ireland there two possible species of *Andonta* (*A. anatina* and *A. cygnea*) present, however no molecular investigations have been undertaken to distinguish between the two (Lucy et al., 2014) and therefore are referred to as *Anodonta* throughout. In Ireland, *Anodonta* are widespread in freshwater systems, but have been largely extirpated in waters where zebra mussels have invaded, attaching to *Anodonta* shells (Fig. 1) and competing with them for food (Minchin and Moriarty, 1998; Lucy et al., 2014). *Anodonta* was chosen because of its significant decrease in numbers since the introduction of the zebra mussel (Lucy et al., 2005). In addition, there is potential to use Zequanox for restoring native freshwater unionid populations; this application is under investigation in the Great Lakes, United States of America (USA) (Great Lakes Restoration Initiative (GLRS)). Methods under development incorporate treatment and restoration structures in native beds. Studies also include assessment of the acute toxicity of Zequanox on freshwater fish and unionids native to the Great Lakes.

C. plumosus (the non-biting midge) is native to Ireland and is a commonly occurring genus of the diverse family Chironomidae, which has 364 known species in Ireland (Heneghan and Murray, 1987). *Chironomus* was chosen as a genus from one of the insect families most commonly used in ecotoxicology tests because their endocrine systems are well documented and they are widely distributed throughout Europe. *Chironomus* can live in most climates and is tolerant of a wide range of water qualities (Armitage et al., 1995; Apperson et al., 2006).

Finally, *A. pallipes* was chosen because it is a protected species in Ireland under Annex IV and Annex II(b) of the European Union (EU) Habitats Directive and International Union for Conservation of Nature and Natural Resources (IUCN) Red List. *A. pallipes* can be found in lakes, streams, rivers or canals, and often co-occurs in lakes with zebra mussels. *A. pallipes* require good water quality and can be sensitive to turbid environments (Reynolds, 1998), further making them an important test species due to the turbid nature of Zequanox.

2. Materials and methods

2.1. Test design (Anodonta)

Anodonta were collected from Trinity Lake, County (Co.) Cavan via snorkeling. The mussels were acclimatized in the lab for 13 days prior to treatment. The selected organisms were all healthy, that is, they all either had their shells closed or were filtering when placed in the aerated 1000 ml test chambers (glass beakers) (Fig. 2). The sizes ranged from 51 to 162 mm (mm). The *Anodonta* were kept in Lake Trinity water that was collected fresh and changed every day for the duration of the test. Water quality parameters (pH, dissolved oxygen [DO], ammonia [NH_3], temperature in degrees C [°C]) and turbidity in NTU), of the collected water was measured daily prior to its use using an Orion 5 star plus meter and a 2100 N Hach Turbidity meter. The water for the test chambers was changed every 12 h and Zequanox was renewed.

The Zequanox formulation used in the *Anodonta* testing was an earlier developmental formulation than that used for the *C. plumosus* and *A. pallipes* testing; due to the fact that the product was still in the research and development stage. The primary difference being that the newer formulation contains 50percent active ingredient, while the earlier development formulation used in the *Anodonta* testing was 100percent active ingredient. Both formulations are dried powders. Testing was conducted and reported based on active ingredient to correct for the difference in these two formulations.

Test concentrations were based on previous tests on unionids carried out by The New York State Museum (Molloy et al., 2013b), in which five species of unionid mussels (*Pyganodon grandis*, *Pyganodon cataracta*, *Lasmigona compressa*, *Strophitus undulatus*, *Elliptio complanata*, and *Lampsilis radiata*) were exposed to live cells of *P. fluorescens* CL145A at concentrations of 100 and 200 mg a.i./L. Concentrations of 100, 200, 300, 400, and 500 mg a.i./L of Zequanox and controls were tested to determine the LC_{50}. Each concentration tested contained two organisms, and was replicated three times; also included were five control test chambers with three organisms each.

Observations of mortality were made at 12, 24, 36, 48, 60, and 72 h. Mortality was determined by gently prodding any open shells during water changing. If the shells remained open they were presumed dead, as live mussels automatically close their shell when disturbed. Measurements of pH, DO, turbidity, NH_3, and

Fig. 1. *Anodonta* with attached zebra mussels.

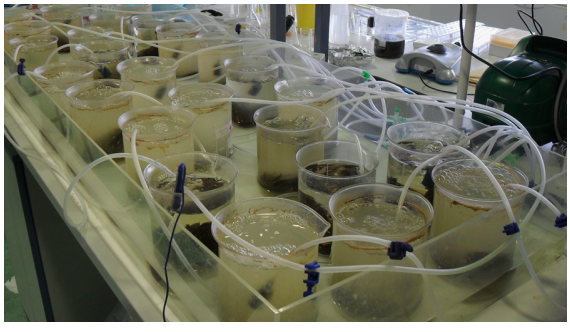

Fig. 2. Test chambers as used for *Anodonta*.

temperature were taken every 12 h. The ASTM guideline "Standard guide for conducting laboratory toxicity test with freshwater mussels" was referenced for the husbandry of the *Anodonta* (ASTM, 2006).

2.2. Test design (Chironomus plumosus)

C. plumosus were collected on February 20, 2012 from Lough Gill (54°15′16.91″ N, 8°19′22.81″W) by standard macroinvertebrate kick sampling (Stark et al., 2001). *C. plumosus* were selected from the samples gathered. All of the selected organisms were actively swimming (indicating health) when taken from the sample container and were placed in aerated 1000 ml test chambers (glass beakers) with 500 ml of test water. Lough Gill water was used as the dilution water throughout the testing period for optimal organism health and to mimic the conditions of an actual Zequanox treatment. The selected organism sizes ranged from approximately 2–10 mm.

The test water in the container was replaced with freshly collected water every 24 h for the duration of the test. Water quality parameters (pH, DO, turbidity, NH_3, and temperature) of the collected water were measured prior to its use.

C. plumosus testing was carried out during February 21–24, 2012. Zequanox spray dried powder (Zequanox SDP), the current formulation registered with the USEPA was used for this organism (Marrone Bio Innovations, 2012a). A small range finding test was carried out prior to testing and concentrations of 100, 200, 300, 400, and 500 mg a.i./L of Zequanox were evaluated in the main test along with controls. Each test concentration was replicated three times with five organisms per test chamber. Five control chambers were used.

Every 24 h mortality was determined by observing immobile *C. plumosus* under the microscope. Organisms that did not respond to gentle prodding were presumed dead. After observations, the test water and Zequanox were renewed to maintain the product concentration and ensure the quality of the water. Measurements of test water pH, DO, turbidity, NH_3, and temperature were taken every 24 h before and after product renewal to assess the effect of Zequanox on the water quality. The 72-h LC_{10}, LC_{50}, and LC_{100} were calculated. This test was carried out in accordance with Organization for Economic Cooperation and Development (OECD) Guideline 219 (OECD, 2004a).

2.3. Test design (Austropotamobius pallipes)

A. pallipes were collected from Lough Owel, Co. Westmeath (53°55′52″N, −7°36′36″W) via hand picking and traps on May 25, 2012 (Fig. 3). Collection and capture was performed according to the guidelines in "A technical manual for monitoring white-clawed crayfish, *A. pallipes*, in Irish lakes" (Reynolds et al., 2010). Removal of this species for experimental purposes requires a license, which was obtained from the Irish National Parks and Wildlife Service (NPWS). After collection, the crayfish were acclimatized for 48 h in the laboratory. Two crayfish were placed into each of the aerated 7 L test chambers (plastic tanks), which were filled with 2 L of water. The crayfish were separated in each tank during acclimatization. During testing, the crayfish were separated by a thin plastic divider with holes to allow for water circulation. The selected organisms all had their appendages intact and were reactive to touch when placed in the test chambers. The carapace lengths ranged from approximately 15–41 mm.

Lough Owel water was used as the dilution water throughout the testing period so that the effects on the crayfish and their habitat could be studied. The Lough Owel water was collected at the beginning of the test and was changed every 24 h for the duration of the test. Water quality parameters (pH, DO, turbidity, NH_3, and temperature) of the collected water were measured before use. Crayfish testing was carried out from May 17–20, 2012.

Fig. 3. Crayfish collected using traps.

No standards for toxicity testing on *A. pallipes* exist; however, "Studies on the white clawed crayfish (*Austropotamobius pallipes*) associated with muddy habitats," (Holdich et al., 2006) which demonstrates the mussels ability to withstand high turbidity, was used to help determine the concentrations of Zequanox to be tested. Concentrations of 350, 450, 550, 650, and 750 mg a.i./L of Zequanox SDP and controls were analyzed in the main test. The chambers for each concentration contained two organisms and were replicated three times. Three control chambers were used. Every 24 h observations of mortality were made and Zequanox was renewed. Mortality was determined by gently prodding any immobile crayfish during water changing; if no movement was noted the crayfish was presumed dead. Any removed individuals were put on a Petri dish for further inspection to confirm mortality and make any additional observations. Measurements of pH, DO, turbidity, NH_3, and temperature were taken every 24 h before and after product renewal. The 72-h LC_{50} was determined.

2.4. Statistical analysis

For each species, the LC_{10}, LC_{50}, and LC_{100} were calculated using linear regression to extrapolate the desired LC value. Additional statistical analyses were carried out using Minitab® 16 © 2010 Minitab Inc. (a software package for analyzing data). The effect of treatment concentration and the interaction of concentration with exposure time was analyzed using a general linear model (GLM). We simplify the reference of increasing treatment duration in the discussion to just "time". All water quality parameters measured every 12–24 h (pH, DO, turbidity, NH_3, and temperature) were then analyzed to determine whether the treatment concentration and treatment exposure or time had an effect (caused fluctuations) on water quality. This was carried out using separate two-way analyses of variance (ANOVAs).

2.5. Zequanox quality control

Prior to shipment of Zequanox product, the MBI quality control team confirmed the product met quality specifications, including; greater than 70 percent quagga mussel mortality at 200 mg a.i./L, less than 100 colony forming units (CFU) per gram (g) of *P. fluorescens*, less than 10\widehat5 CFU/g of aerobic bacteria, and absence of potential human pathogens (inclusive of total Coliform, *Escherichia coli*, and *Shigella*, *Vibrio*, and *Salmonella* species). Evaluation was completed according to standard methods or MBI's documented standard operating procedures for Zequanox.

3. Results and discussion

3.1. Anodonta

Using averages after a 72-h Zequanox treatment, mortality was zero in all of the concentrations tested (100, 200, 300, 400, and 500 mg a.i./L) and in the controls, indicating that the active substance in Zequanox is not harmful to *Anodonta*. Because no mortality was observed, it was determined that the LC_{50} of the active substance in Zequanox for A*nodonta* is $\geq$500 mg a.i/L. The optimal concentration of 150–200 mg a.i./L that has been shown to be effective at controlling zebra mussels in Ireland has no negative effect on *Anodonta* as determined by this assay (Meehan et al., 2013).

Separate two-way ANOVAs demonstrated that pH, DO, turbidity, and NH_3 changed due to treatment concentration ($P<0.05$), and temperature did not ($P>0.05$). Temperature, DO, and NH_3 were affected by exposure time. For the *Anodonta*, unlike the crayfish and *Chironomus*, an experimental formulation of Zequanox was used and could have caused the difference found in DO and NH_3. During the exposure period, pH and turbidity did not change whereas treatment concentration did. Zequanox biodegrades over time and therefore this result was expected.

Ecotoxicology tests on the *Anodonta* are important to ensure that this commonly co-occurring species is not negatively affected by Zequanox due to its already dwindling numbers. The future of the *Anodonta* population could depend on the success of Zequanox to control the zebra mussel, in that a decrease in zebra mussel populations has the potential to positively affect the *Anodonta* population allowing their numbers to increase.

Table 1
Average live *C. plumosus* counts after treatment with Zequanox at 24, 28, and 72 h and total % mortality ($N=6$).

Treatment	Mean no. of live *C. plumosus*			% Mortality (72 h)
	24 h	48 h	72 h	
Control	6	5.8	5.6	7
100 mg a.i./L	6	6	5.3	11
200 mg a.i./L	6	5.3	5.3	11
300 mg a.i./L	5.3	5	5	17
400 mg a.i./L	6	5.3	4.7	22
500 mg a.i./L	6	5	4.3	28

Table 2
Average live *A. Pallipes* counts after treatment with Zequanox at 24, 28, and 72 h and total % mortality ($N=2$).

Treatment	Mean no. of live *A. Pallipes*			% Mortality (72 h)
	24 h	48 h	72 h	
Control	2	2	2	0
350 mg a.i./L	2	1.7	1.7	15
450 mg a.i./L	2	1.7	1.7	15
550 mg a.i./L	2	2	2	0
650 mg a.i./L	2	2	2	0
750 mg a.i./L	2	2	2	0

3.2. *Chironomus plumosus*

Using averages, after 72 h exposure, mortality counts for 100, 200, 300, 400 and 500 mg a.i./L were 1(11percent), 1(11percent), 3 (17percent), 4(22percent) and 5(28percent) respectively (Table 1). The 72-h LC_{50} and LC_{100} could not be determined because mortality did not reach 50percent in any of the concentrations tested; therefore, an estimated LC_{50} and LC_{100} were calculated by extrapolating from a linear regression. The estimated 72-h LC_{50} was 1075 mg a.i./L and the estimated 72-h LC_{100} was 2286 mg a.i./L. The actual 72-h LC_{10} was 106 mg a.i./L.

The high level of turbidity encountered from high Zequanox concentration may have effected survival of the *C. plumosus*. Previous research has shown that Chironomidae numbers in the wild decrease in highly turbid environments. In the Colorado River, their density decreased from 0.42 species per kilometer (km) in clear water to 0.08 species/km in turbid water (Stevens et al., 1998).

As no significant mortalities occurred in *C. plumosus* at 200 mg a.i./L over the 72 h Zequanox testing period, the maximum allowable concentration for controlling zebra and quagga mussels, it is unlikely that Zequanox treatments will have a significant impact to *C. plumosus* (Meehan et al., 2013). In addition, no mortality was observed in the highest treatment concentrations, 400 and 500 mg a.i./L, in the first 24 h, therefore the impact to *C. plumosus* species is further minimized because the maximum allowable treatment time is 12 h (Marrone Bio Innovations, 2012a).

Through a GLM, treatment concentration was confirmed to have an effect on *C. plumosus* survival; this is evident from the linear regression showing an increase in mortality with treatment concentration. Time affected their mortality; as described below because most of the mortality occurred at 72 h.

Upon initial product application, there were no significant effects on pH, DO, turbidity, NH_3, and temperature due to the treatment concentration (two-way ANOVAs; $P>0.05$). Turbidity levels were affected ($P<0.05$), as expected, because Zequanox is composed of particulate matter and its concentration is positively correlated with turbidity. Over the duration of the study, pH, DO, turbidity, and temperature fluctuated, whereas NH_3 did not. The DO likely decreased as the product biodegraded over time. Water temperature was not controlled; therefore, the water temperature experienced slight fluctuations as a result of ambient laboratory conditions, which in turn caused the pH to fluctuate.

3.3. *Austropotamobius pallipes*

Using averages, after 72 h exposure, mortality counts for 350 and 450 mg a.i/L were 1(15percent) for each and zero mortality for 550, 650 and 750 mg a.i./L (Table 2). Due to lack of a significant treatment response (only two mortalities total, one due to cannibalism in 350 mg a.i./L and one juvenile crayfish in 450 mg a.i./L) an extrapolation was not possible; therefore, it was determined that the LC_{50} of Zequanox for crayfish was $\geq$750 mg a.i./L. No negative effects on crayfish occurred at higher concentrations tested. These results indicate that the maximum allowable Zequanox concentration of 200 mg a.i./L for controlling zebra mussels (Marrone Bio Innovations, 2012a) and concentrations, 150–200 mg a.i./L, that have been shown to be effective at controlling zebra mussels in Ireland (Meehan et al., 2013) would have minimal to no impact on *A. pallipes*. Crayfish naturally burrow and are able to withstand high turbidity, according to a number of case studies in Britain where white-clawed crayfish were found in mud anoxic conditions (Holdich et al., 2006). This ability was demonstrated in this non-target testing, as crayfish were exposed to highly turbid water without any negative impacts.

Separate two-way ANOVAs demonstrated that pH, DO, turbidity, NH_3, and temperature ($P>0.05$) did not change due to treatment concentration. Time had an effect on temperature and NH_3 ($P<0.05$) but had no effect on pH, DO and turbidity. The effects on temperature and NH_3 were expected, as they were reflective of daily 24 h measurements recorded before water change and product renewal. Also test chambers were kept in ambient laboratory temperature which can fluctuate.

3.4. *Water quality*

Table 3 displays the upper and lower limits of water quality parameters recorded in treated chambers and compares this to the limits set out by the European Communities (Quality of Salmonid Waters) Regulations, (1988), the Freshwater Fish Directive (78/659/EEC) (Cyprinid and Salmonid waters) and OECD guidelines 202, 203, 219. It is very important to note, that the water quality limits set out for Salmonid and Cyprinid waters are intended for the purpose of lakes and rivers and not small test chambers. As Zequanox is also intended for use in open water these regulations serve as an interesting proxy, however in most cases the NH_3 recorded will fall outside of these limits as testing was over a 72 h period and test chambers mostly contained only 500 ml of water. OECD guidelines are designed for ecotoxicology testing and give a better idea of appropriate water quality parameters in order to keep organisms healthy.

Water quality during treatment stayed for the most part stayed within the OECD water quality guidelines for test validity (OECD, 1992, 2004a, 2004b, 2011). For all three organisms tested, the pH stayed between 6 and 9. The DO concentration did not drop below 6 mg/L in the *Anodonta* and *C. plumosus* test, but it did for the *A. pallipes*. For *A. pallipes*, this DO drop occurred on only two occasions out of 25 measurements; therefore, this is considered only a minor deviation and had no effect on the scientific validity of the test. The temperature did not fluctuate by more than ± 1 °C, for *Anodonta* and *C. plumosus* but did for *A. pallipes*. The minimal crayfish mortalities indicate there were no negative impacts to the crayfish from temperature fluctuation. All the water quality parameters recorded, apart from the drop in DO for the crayfish, are within the European Communities Regulations (1998) and the Freshwater Fish Directive which demand the highest quality of water for the support of freshwater game fish.

Table 3
Upper and lower water quality parameters recorded, limits for Salmonid water quality and Cyprinid water quality are also given.

	DO (mg/L)	pH	Temperature (°C)	Unionized ammonia (NH_3) (mg/L)	Turbidity (NTU)
Salmonid water quality Limits (S.I. no. 293/1988)	**> 7**	**6–9**	**< 21.5**	**< 0.02**	**N/A**
Freshwater fish directive (78/659/EEC)					
Cyprinid water quality limits	**> 5**	**6–9**	**< 21.5**	**< 0.025**	**N/A**
Salmonid water quality limits	**> 7**	**6–9**	**< 21.5**	**< 0.025**	**N/A**
OECD validity criteria (202, 203, 219, 235)	**> 6**	**6–9**	**± 1 °C**	**N/A**	**N/A**
C. plumosus SDP	7.93–9.85	7.81–9.64	14.7–16.8	0.003–5.682	16.9–102
Anodonta FDP	7.63–9.4	8.0–8.66	16.3–18.1	0.23–7.25	17.8–148
A. pallipes SDP	4.01–9.92	7.15–8.0	14.7–17.4	0.168–3.06	79.8–231

No guidelines for turbidity and NH_3 exist for ecotoxicity testing, because they are not usually monitored in ecotoxicity trials. These parameters are, however, important to measure because turbidity increases with Zequanox concentration and NH_3 is a key parameter for aquatic organism health. Mortality was minimal for *Anodonta* and *A. pallipes*, indicating that there were no negative effects due to an increase in turbidity and NH_3 during testing. For *C. plumosus*, turbidity and mortality increased with Zequanox concentration (although the mortality was still low, at < 28percent). Considering other research demonstrating that *C. plumosus* are not common in turbid environments (Başçınar et al., 2009) and the data obtained in this study, it is possible that increases in turbidity coupled with treatment concentration caused an increase in mortality.

4. Conclusion

Overall the results showed Zequanox had minimal to no impact on the non-target organisms tested, with all three calculated median lethal concentrations being in excess of the treatment application concentrations. These non-target trials have helped to further confirm the specificity of Zequanox to zebra mussels, and have confirmed that Zequanox does not affect selected native Irish aquatic species, including one endangered species (*A. pallipes*). These results show that Zequanox will not further endanger depleting populations of *Anodonta* and *A. pallipes*, and even has potential to be used for the restoration of *Anodonta* populations.

This non-target testing has been fundamental in moving forward with the use of Zequanox in trial applications in Ireland (Meehan et al., 2013). The tests were requested by NPWS to be carried out before the in situ Zequanox demonstration trial at Tullamore Harbor could proceed (Meehan et al., 2014) to clearly demonstrate the effect of Zequanox on non-target organisms and their environment.

These non-target organism studies indicate that Zequanox is a suitable alternative to chlorine applications for facilities discharging to freshwater ecosystems when used at the maximum allowable concentration of 200 mg a.i./L, as currently prescribed by the USEPA (Marrone Bio Innovations 2012a). Furthermore, the majority of industrial applications will result in significant dilution upon discharge into the environment, further limiting the impact to non-target species.

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References

Apperson, C., Waldvogel, M., Bambara, S., 2006. Extension Entomology. ⟨http://www.ces.ncsu.edu/depts/ent/notes/Urban/midges.htm⟩ (last accessed 29.10.13).
Armitage, P.D., Cranston, P.S., Pinder, L.C.P., 1995. Substratum and chironomid distribution in lakes and rivers. In: Armitage, P.D., Cranston, P.S., Pinder, L.C.V. (Eds.), The Chironomidae: Biology and Ecology of Non-Biting Midges. Chapman and Hall, 2-6 Boundary Row, London SE1 8HN, pp. 121–126.
ASTM, 2006. Test Guideline E2455-06. Standard guide for conducting laboratory toxicity tests with freshwater mussels. In: Annual Book of ASTM Standards, vol. 11.06, Philadelphia, PA, pp. 1–53.
Başçınar, N.S., Düzgüneş, E., Mısır, S., Polat, H., Zengin, B., 2009. Growth and flesh yield of the swan/duck mussel [*Anodonta cygnea* (Linnaeus, 1758)] (Bivalvia: Unionidae) in Lake Çıldır (Kars, Turkey). Turk. J. Fish. Aquat. Sci. 9, 127–132.
European Communities (Quality of Salmonid Waters) Regulations, 1988. Irish Statue Book. S.I. No. 293/1988. ⟨http://www.irishstatutebook.ie/1988/en/si/0293.html⟩ (last accessed 04.12.13).
Great Lakes Restoration Initiative (GLRS). Evaluating the Safety and Efficacy of *Pseudomonas fluorescens* Strain CL145A to Control Dreissenid Mussels. ⟨http://cida.usgs.gov/glri/projects/invasive_species/zm_control.html⟩ (last accessed 07.03.13).
Heneghan, L., Murray, D.A., 1987. Additions to the inventory of Irish Chironomidae (Diptera). Ir. Naturalists J. 22 (5), 196–199.
Holdich, D.M., Peay, S., Foster, J., Hiley, P.D., Brickland, J.H., 2006. Studies on the white clawed crayfish (*Austropotamobius pallipes*) associated with muddy habitats. Bull. Fr. Pêche Piscic 380–381, 1055–1078.
Lucy, F., Sullivan, M., Minchin, D., 2005. Nutrient levels and the zebra mussel population in Lough Key. ERTDI Report Series No. 34. EPA, Wexford. ⟨http://www.epa.ie/pubs/reports/research/water/EPA_zebra_mussels_ERTDI34_synthesis.pdf⟩ (last accessed 27.05.13).
Lucy, F.E., Burlakova, L.E., Karatayev, A.Y., Mastitsky, S.E., Zanatta, D., 2014. Zebra mussel impacts on unionids: a synthesis of trends in North America and Europe. In: Schloesser, D.W., Nalepa, T.F. (Eds.), Quagga and zebra mussels: biology, impacts, and control, second edition. CRC Press, Taylor & Francis Group, Boca Raton (Chapter 42).
Mackie, G.L., Claudi, R., 2010. Monitoring and Control of Macrofouling Mollusks in Freshwater Systems. CRC Press, Boca Raton, FL p. 508.
Marrone Bio Innovations, 2012a. Product Label: MBI-401 SDP. EPA Reg. No. 84059-15. ⟨http://www.epa.gov/pesticides/chem_search/ppls/084059-00015-20120309.pdf⟩.
Marrone Bio Innovations, 2012b. Registration extension: MBI-401 SDP. EPA Reg. No. 84059-15. ⟨http://www.epa.gov/pesticides/chem_search/ppls/084059-00015-20120727.pdf⟩.
Meehan, S., Lucy, F.E., Gruber, B., Rackl, S., 2013. Comparing Zequanox® and chlorine as zebra mussel control strategies in an Irish drinking water treatment plant. Manag. Biol. Invasions 4 (2), 113–122.
Meehan, S., Gruber, B., Lucy, F.E., 2014. Zebra mussel control using Zequanox® in an Irish waterway. Manag. Biol. Invasions 5 (3). http://dx.doi.org/10.3391/mbi.2014.5.3.
Minchin, D., Moriarty, C., 1998. Zebra mussels in Ireland. Fisheries Leaflet 177. Marine Institute, Dublin p. 11.
Minchin, D., Lucy, F., Sullivan, M., 2002. Zebra Mussels: impacts and spread. In: Leppakowski, E., Gollasch, S., Olenin, S. (Eds.), Invasive Aquatic Species of Europe. Distribution, Impacts and Management. Kluwer Academic Publishers, Dordrecht, pp. 135–146.
Molloy, D.P., Mayer, D.A., Gaylo, M.J., Morse, J.T., Presti, K.T., Sawyko, P.M., Karatayev, A.Y., Burlakova, L.E., Laruelle, F., Nishikawa, K.C., Griffin, B.H., 2013a. A biopesticide for the

control of zebra and quagga mussels (Bivalvia: Dreissenidae). J. Invertebr. Pathol. 113 (1), 104–114.
Molloy, D.P., Mayer, D.A., Gaylo, M.J., Burlakova1, L.E., Karatayev, A.Y., Presti1, K.T., Sawyko, P.M., Morse, J.T., Paul, E.A., 2013b. Non-target trials with *Pseudomonas fluorescens* strain CL145A, a lethal control agent of dreissenid mussels (Bivalvia: Dreissenidae). Manag. Biol. Invasions 4 (1), 71–79.
OECD, 1992. Test guideline 203 Fish, Acute Toxicity Test. OECD Publishing, Paris, France.
OECD, 2004a. Test guideline 219 Sediment-Water Chironomid Toxicity Test Using Spiked Water. OECD Publishing, Paris, France.
OECD, 2004b. Test guideline 202 *Daphnia* sp., Acute Immobilisation Test. OECD Publishing, Paris, France.
OECD, 2011. Test Guideline 235 *Chironomus* sp., Acute Immobilisation Test. OECD Publishing, Paris, France.
Reynolds, J.D., 1998. Conservation Management of the White-Clawed Crayfish Austropotamobius pallipe. Irish Wildlife Manuals No.1.
Reynolds, J.D., O'Connor, W., O'Keeffe, C., Lynn, D., 2010. A technical manual for monitoring white-clawed crayfish Austropotamobius pallipes in Irish lakes, Irish Wildlife Manuals No. 45. National Parks and Wildlife Service, Department of the Environment, Heritage and Local Government, Dublin.
Stark, J.D., Boothroyd, I.K.G., Harding, J.S., Maxted, J.R., Scarsbrook, M.R., 2001. Protocols for sampling macroinvertebrates in wadeable streams. Macroinvertebrate Working Group Report no. 1.
Stephen, C.E., 1997. Methods for Calculating an LC50 Aquatic Toxicology and Hazard Evaluation. In: Mayer, F.L., Hamelink, J.L. (Eds.), ASTM STP 634. American Society for Testing Materials, Philadelphia, PA, pp. 65–84.
Stevens, L.E., Sublette, E.J., Shannon, J.P., 1998. Chironomidae (diptera) of the Colorado River, Grand Canyon, Arizona, USA, II: factors influencing distribution. Great Basin Nat. 58 (2), 147–155.

Appendix B: Water Quality Parameters of Collected Test Water

*Note temperature varies depending on when temperature was measured after water collection.

* Carbon filtered Shannon municipal water was used for the *Salmo trutta* test therefore water parameters are not presented

Table 1 *Chironomus:* water quality parameters of collected water for testing with MBI-401 FDP

Chironomus FDP	Day 1	Day 2	Day 3
Temperature (°C)	18.1	16	17.1
Dissolved oxygen (mg/l)	8.25	8.33	9.65
pH	9.42	10.15	8.4
Turbidity (NTU)	0.176	0.186	0.211
Unionized ammonia (mg/l)	0	0	0

Table 2 *Chironomus plumosus:* water quality parameters of collected water for testing with MBI-401 SDP

Chironomus SDP	
Temperature (°C)	18.4
Dissolved oxygen (mg/l)	9.66
pH	8.01
Turbidity (NTU)	1.67
Unionized ammonia (mg/l)	0.03

Table 3 *Asellus aquaticus:* water quality parameters of collected water for testing with MBI-401 FDP

Asellus Aquaticus FDP	Day 1	Day 2	Day 3
Temperature (°C)	20.3	20.3	16.9
Dissolved oxygen (mg/l)	9.06	9.63	9.71
pH	8.15	8.28	8.25
Turbidity (NTU)	0.854	0.732	0.741
Unionized ammonia (mg/l)	0	0	0

Table 4 *Ephemerella ignita*: Water quality parameters of collected water for testing with MBI-401 FDP

Ephemerella FDP	Day 1	Day 2	Day 3
Temperature (°C)	16.8	21.8	15.1
Dissolved oxygen (mg/l)	9.84	9.22	10.01
pH	8.28	8.25	8.26
Turbidity (NTU)	0.165	0.186	2.54
Unionized ammonia (mg/l)	0.0062	0.0013	0.001

Table 5 *Ephemerella ignita*: water quality parameters of collected water for testing with MBI-401 SDP

Ephemerella SDP	
Temperature (°C)	15.1
Dissolved oxygen (mg/l)	10.01
pH	8.26
Turbidity (NTU)	2.54
Unionized ammonia (mg/l)	0.001

Table 6 *Mytilus edulis*: water quality parameters of collected water for testing with MBI-401 FDP

Mytilus edulis FDP	
Temperature (°C)	14.1
Dissolved oxygen (mg/l)	10.25
pH	8.15
Turbidity (NTU)	3.55
Unionized ammonia (mg/l)	0.329

Table 7 *Anodonta*: water quality parameters of collected water for testing with MBI-401 FDP

Anodonta FDP	Day 1	Day 2	Day 3
Temperature (°C)	20.3	20.3	16.9
Dissolved oxygen (mg/l)	9.06	9.63	9.71
pH	8.15	8.28	8.25
Turbidity (NTU)	0.854	0.732	0.741
Unionized ammonia (mg/l)	0	0	0

Table 8 *Daphnia pulex*: water quality parameters of collected water for testing with MBI-401 SDP

Daphnia pulex SDP	
Temperature (°C)	15.9
Dissolved oxygen (mg/l)	10.11
pH	9.04
Turbidity (NTU)	1.53
Unionized ammonia (mg/l)	0.0032

Table 9 *Austropotamobius pallipes*: water quality parameters of collected water for testing with MBI-401 SDP

Austropotamobius pallipes SDP	
Temperature (°C)	15.4
Dissolved oxygen (mg/l)	10.08
pH	9.14
Turbidity (NTU)	1.41
Unionized ammonia (mg/l)	0.471

Table 10 *Lymnaea peregra:* water quality parameters of collected water for testing with MBI-401 SDP

Lymnaea peregra SDP	
Temperature (°C)	16.5
Dissolved oxygen (mg/l)	9.81
pH	8.26
Turbidity (NTU)	1.36
Unionized ammonia (mg/l)	0.001

Appendix C: Water Quality Parameters of Test Water Prior to and after Renewal

°C =temperature, DO=dissolved oxygen (mg/l), NTU=turbidity, NH3=unionized ammonia (mg/l)

Table 1 *Chironomus: w*ater quality parameters during testing with MBI-401 FDP (1-3 = test chambers)

***Chironomus* - Drumcliff river water**					
24 hours 6th-July-11 before exposure			**24 hours 6th-July-11 after exposure**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	18.7	19.5	Temp (°C)	18.1	19
DO (mg/l)	8.32	8.84	DO (mg/l)	8.68	9.29
pH	8.22	8.6	pH	8.17	8.55
NTU	18.9	0.711	NTU	37.8	5.33
NH_3(mg/l)	0	0	NH_3(mg/l)	0	0
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	18.5	19	Temp (°C)	18.4	18.8
DO (mg/l)	7.23	9.05	DO (mg/l)	6.91	9.26
pH	8.03	8.6	pH	7.91	8.52
NTU	39.8	0.306	NTU	62.5	1.1
NH_3(mg/l)	0	0	NH_3(mg/l)	0	0
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	18.5	21	Temp (°C)	19.3	18.7
DO (mg/l)	7.21	8.28	DO (mg/l)	7.12	9.42
pH	8	8.59	pH	7.92	8.53
NTU	58.1	0.401	NTU	130	1.41
NH_3(mg/l)	0-0.5	0	NH_3(mg/l)	0	0
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	18.5	22	Temp (°C)	19.2	19.1
DO (mg/l)	5.87	8.38	DO (mg/l)	6.94	9.4
pH	7.88	8.54	pH	7.87	8.47
NTU	129	0.369	NTU	167	1.63
NH_3(mg/l)	0-0.5	0	NH_3(mg/l)	0	0
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	18.1	20.1	Temp (°C)	18	19.2
DO (mg/l)	6.16	8.51	DO (mg/l)	6.86	9.23
pH	7.86	8.54	pH	7.85	8.55
NTU	124	0.585	NTU	229	2.61
NH_3	0-0.5	0	NH_3	0	0

***Chironomus* - Drumcliff river water**					
48 hours 7th-July-11 before exposure			**48 hours 7th-July-11 after exposure**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	19	19	Temp (°C)	19.6	18.7
DO (mg/l)	8.32	8.86	DO (mg/l)	7.72	9.18
pH	8.33	8.63	pH	8.75	8.55
NTU	17.8	0.331	NTU	36.9	0.977
NH_3(mg/l)	0	0	NH_3(mg/l)	0-0.5	0
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	18.8	19.1	Temp (°C)	19	18.8
DO (mg/l)	8.35	8.77	DO (mg/l)	6.85	9.17
pH	8.37	8.59	pH	7.99	8.54
NTU	34.1	0.328	NTU	84.7	1.28
NH_3(mg/l)	0	0	NH_3(mg/l)	0.5	0
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	19.5	19.3	Temp (°C)	19.3	18.8
DO (mg/l)	7.96	8.53	DO (mg/l)	6.85	9.25
pH	8.33	8.61	pH	7.99	8.47
NTU	49.9	0.351	NTU	84.7	1.11
NH_3(mg/l)	0-0.5	0	NH_3(mg/l)	0.5	0
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	18.7	20.7	Temp (°C)	18.8	18.8
DO (mg/l)	8.01	8.25	DO (mg/l)	4.56	9.17
pH	8.34	8.56	pH	7.75	8.56
NTU	77.8	0.304	NTU	183	1.56
NH_3(mg/l)	0-0.5	0	NH_3(mg/l)	0.5	0
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	19.6	19.6	Temp (°C)	19.2	18.8
DO (mg/l)	7.21	8.57	DO (mg/l)	4.52	9.15
pH	8.23	8.61	pH	7.72	8.54
NTU	104	0.536	NTU	234	2.81
NH_3(mg/l)	0-0.5	0	NH_3(mg/l)	0.5	0

***Chironomus* - Drumcliff river water**		
72 hours 8th- July-11		
100mg/l	1	Control
Temp (°C)	19.1	19.5
DO (mg/l)	7.77	8.55
pH	8.32	8.58
NTU	6.47	0.845
NH_3(mg/l)	0	0
200mg/l	1	Control
Temp (°C)	19.1	19.8
DO (mg/l)	7.79	8.33
pH	8.34	0.858
NTU	7.9	0.785
NH_3(mg/l)	0.5-1.0	0
300mg/l	1	Control
Temp (°C)	19.9	19.7
DO (mg/l)	7.09	8.49
pH	8.21	8.57
NTU	8.33	0.958
NH_3(mg/l)	3	0
400mg/l	1	Control
Temp (°C)	19.2	20.3
DO (mg/l)	8.56	8.33
pH	8.06	8.51
NTU	13.8	0.793
NH_3(mg/l)	3	0
500mg/l	1	Control
Temp (°C)	19.6	19.3
DO (mg/l)	6.43	8.58
pH	8.16	8.5
NTU	58.4	0.717
NH_3(mg/l)	3	0

Table 2 *Chironomus: w*ater quality parameters during testing with MBI-401 SDP (1-3 = test chambers)

***Chironomus plumosus* - Drumcliff River Water**					
24 hours 22th-Feb-12 before exposure			**24 hours 22th-Feb-12 after exposure**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	16	16.2	Temp (°C)	16.5	16.4
DO (mg/l)	9.52	9.64	DO (mg/l)	9.38	9.82
pH	8.22	8.32	pH	8.45	8.27
NTU	33.3	2.89	NTU	6.718	1.87
NH_3(mg/l)	0.433	0.458	NH_3(mg/l)	0.23	0.308
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	16.2	16.1	Temp (°C)	16.7	16.5
DO (mg/l)	9.39	9.75	DO (mg/l)	9.28	9.65
pH	8.18	8.32	pH	8.02	8.07
NTU	47.9	1.84	NTU	126	1.83
NH_3(mg/l)	4.21	0.557	NH_3(mg/l)	0.433	0.233
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	16.2	16	Temp (°C)	16.6	16.4
DO (mg/l)	9.39	9.64	DO (mg/l)	9.54	9.54
pH	8.17	8.26	pH	8.101	8.25
NTU	52.93	1.93	NTU	131	3.58
NH_3(mg/l)	5.682	0.589	NH_3(mg/l)	0.573	0.262
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	16.2	16.2	Temp (°C)	16.5	16.4
DO (mg/l)	9.66	9.52	DO (mg/l)	9.55	9.77
pH	8.22	8.27	pH	8.09	8.25
NTU	76.1	1.62	NTU	184	1.66
NH_3(mg/l)	0.833	0.341	NH_3(mg/l)	0.562	0.234
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	16.1	16.2	Temp (°C)	16.8	16.6
DO (mg/l)	9.34	9.4	DO (mg/l)	9.53	9.57
pH	8.11	8.22	pH	8.07	8.19
NTU	102	1.81	NTU	255	1.65
NH_3(mg/l)	0.546	0.543	NH_3(mg/l)	0.717	0.354

***Chironomus plumosus* - Drumcliff River Water**					
48 hours 23rd-Feb-12 before exposure			**48 hours 23rd-Feb-12 after exposure**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	16.8	16.9	Temp (°C)	16.5	16.4
DO (mg/l)	8.55	9.5	DO (mg/l)	8.45	9.46
pH	7.95	8.28	pH	7.98	8.11
NTU	28.4	1.56	NTU	46.8	1.66
NH_3(mg/l)	0.055	0.0151	NH_3(mg/l)	0.0356	0.395
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	16.6	16.6	Temp (°C)	16.7	16.5
DO (mg/l)	7.93	9.26	DO (mg/l)	7.89	9.14
pH	7.81	8.2	pH	7.65	8.09
NTU	56.5	1.76	NTU	81.9	2.44
NH_3(mg/l)	0.727	0.315	NH_3(mg/l)	0.682	0.095
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	16.5	16.5	Temp (°C)	16.6	16.2
DO (mg/l)	7.95	9.3	DO (mg/l)	8.38	9.41
pH	8.95	8.26	pH	7.84	8.16
NTU	46.6	1.41	NTU	106	3.44
NH_3(mg/l)	0.0299	0.002	NH_3(mg/l)	1.67	0.009
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	16.5	16.6	Temp (°C)	16.4	16.5
DO (mg/l)	8.54	8.94	DO (mg/l)	8.09	9.36
pH	7.86	8.41	pH	7.67	8.1
NTU	67.9	1.74	NTU	139	1.65
NH_3(mg/l)	0.003	0.00202	NH_3(mg/l)	0.527	0.0811
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	16.5	16.6	Temp (°C)	16.8	16.5
DO (mg/l)	8.37	9.18	DO (mg/l)	7.95	9.47
pH	7.83	8.24	pH	7.6	8.22
NTU	91.9	1.49	NTU	178	1.79
NH_3(mg/l)	0.0063	0.00137	NH_3(mg/l)	2.28	0.003

***Chironomus plumosus* - Drumcliff River Water**		
72 hours 24th-Feb-12		
100mg/l	1	Control
Temp (°C)	14.7	14.8
DO (mg/l)	9.85	10.3
pH	8.19	8.28
NTU	16.9	1.4
NH_3(mg/l)	1.02	0.0005
200mg/l	1	Control
Temp (°C)	15.1	14.9
DO (mg/l)	8.22	10.05
pH	9.64	8.31
NTU	64.6	1.47
NH_3(mg/l)	0.801	0.0101
300mg/l	1	Control
Temp (°C)	14.9	14.5
DO (mg/l)	9.67	10.03
pH	8.22	8.25
NTU	43.3	1.68
NH_3(mg/l)	1.32	0.0014
400mg/l	1	Control
Temp (°C)	14.7	14.9
DO (mg/l)	9.62	9.9
pH	8.14	8.32
NTU	55	1.48
NH_3(mg/l)	0.981	0.214
500mg/l	1	Control
Temp (°C)	15	14.8
DO (mg/l)	9.57	10.12
pH	8.22	8.17
NTU	79.7	1.32
NH_3(mg/l)	1.1	0.182

Table 3 *Asellus aquaticus:* water quality parameters during testing with MBI-401 FDP (1-3 = test chambers)

***Asellus aquaticus* - Drumcliff river water**							
24 hours 26th-July-11 before exposure					**24 hours 26th-July-11 after exposure**		
100mg/l	1	Control	Mixed	100mg/l	1	Control	Mixed
Temp (°C)	20.3	20.6	20.5	Temp (°C)	20.4	20.3	20.4
DO (mg/l)	8.54	8.73	7.63	DO (mg/l)	7.98	9.02	7.8
pH	8.31	8.43	8.1	pH	8.08	8.42	8.8
NTU	19.4	0.841	18	NTU	45.5	2.23	37.2
NH_3(mg/l)	0	0	0	NH_3(mg/l)	0	0	0
200mg/l	1	Control	Mixed	200mg/l	1	Control	Mixed
Temp (°C)	20.2	20.7	20.2	Temp (°C)	20.3	20.4	20.4
DO (mg/l)	5.87	8.83	7.18	DO (mg/l)	6.42	8.97	7.42
pH	7.85	8.46	7.97	pH	7.85	8.39	8.37
NTU	37.4	0.632	52.7	NTU	86.7	2.18	90.8
NH_3(mg/l)	0	0	0	NH_3(mg/l)	0	0	0
300mg/l	1	Control	Mixed	300mg/l	1	Control	Mixed
Temp (°C)	20.3	20.3	19.7	Temp (°C)	20.2	20.4	20.2
DO (mg/l)	7.07	8.85	3.56	DO (mg/l)	6.76	8.98	7.71
pH	7.93	8.45	7.66	pH	7.84	8.42	8.21
NTU	52.7	1.52	60.4	NTU	120	3.03	151
NH_3(mg/l)	0			NH_3(mg/l)	0	0	0
400mg/l	1	Control	Mixed	400mg/l	1	Control	Mixed
Temp (°C)	20	20	20	Temp (°C)	20.4	20.3	20.3
DO (mg/l)	5.63	8.89	6.47	DO (mg/l)	6.06	9.05	7.7
pH	7.97	8.51	7.86	pH	7.73	8.28	5.88
NTU	86.3	0.821	104	NTU	166	1.91	201
NH^3(mg/l)	0.6	0	0	NH^3(mg/l)	0	0	0
500mg/l	1	Control	Mixed	500mg/l	1	Control	Mixed
Temp (°C)	20.8	21	19.7	Temp (°C)	20.4	20.5	20.4
DO (mg/l)	5.56	9	4.36	DO (mg/l)	4.94	9.17	7.72
pH	7.55	8.49	7.72	pH	7.66	8.35	5.49
NTU	117	0.82	160	NTU	184	1.6	231
NH_3(mg/l)	0	0	0	NH_3(mg/l)	0	0	0

Asellus aquaticus - Drumcliff river water							
48 hours 27th-July-11 before exposure				**48 hours 27th-July-11 after exposure**			
100mg/l	1	Control	Mixed	100mg/l	1	Control	Mixed
Temp (°C)	20.3	20.4	20.6	Temp (°C)	19.2	19.3	19.4
DO (mg/l)	8.6	8.98	7.37	DO (mg/l)	8.51	9.58	8.36
pH	8.32	8.52	8	pH	8.15	8.52	8.17
NTU	10.2	0.207	21.3	NTU	36	1.46	37.4
NH_3(mg/l)	0	0	0	NH_3(mg/l)	0	0	0
200mg/l	1	Control	Mixed	200mg/l	1	Control	Mixed
Temp (°C)	20.2	20.6	20.3	Temp (°C)	19.2	19.1	19.5
DO (mg/l)	8.61	9.06	8.44	DO (mg/l)	7.77	9.74	7.45
pH	8.3	8.49	8.3	pH	8.02	8.52	7.98
NTU	33.9	0.809	42.2	NTU	79.2	2.05	76.8
NH_3(mg/l)	0.6	0	0.9	NH_3(mg/l)	0	0	0
300mg/l	1	Control	Mixed	300mg/l	1	Control	Mixed
Temp (°C)	20.8	20.4	20.5	Temp (°C)	19.5	19.5	19.3
DO (mg/l)	8.16	8.95	0.83	DO (mg/l)	7.65	9.54	7.52
pH	8.26	8.54	7.63	pH	7.84	8.5	7.92
NTU	48.7	0.681	73.2	NTU	118	3.51	133
NH_3(mg/l)	1.2		1.2	NH_3(mg/l)	0	0	0
400mg/l	1	Control	Mixed	400mg/l	1	Control	Mixed
Temp (°C)	20.1	20.4	20.7	Temp (°C)	19.2	19	19.1
DO (mg/l)	7.88	8.88	8.05	DO (mg/l)	5.36	19.56	3.6
pH	8.33	8.48	8.12	pH	7.71	8.51	7.38
NTU	58.4	0.787	789	NTU	167	4.39	180
NH_3(mg/l)	1.2	0	1.2	NH_3(mg/l)	0	0	0
500mg/l	1	Control	Mixed	500mg/l	1	Control	Mixed
Temp (°C)	20.6	20.9	20.4	Temp (°C)	19.2	19.4	19.2
DO (mg/l)	5.2	8.91	3.3	DO (mg/l)	3.28	9.84	5.15
pH	7.89	8.5	7.76	pH	7.53	8.55	7.68
NTU	85	1.32	115	NTU	243	2.7	224
NH_3(mg/l)	1.8	0	1.8	NH_3(mg/l)	0	0	0

***Asellus aquaticus* - Drumcliff river water**			
72 hours 28th-July-11			
100mg/l	1	4	Mixed
Temp (°C)	20.5	20.2	20.5
DO (mg/l)	8.48	9.05	8.46
pH	8.32	8.56	8.34
NTU	5.34	0.583	7.06
NH_3(mg/l)	0	0	0
200mg/l	1	Control	Mixed
Temp (°C)	20.2	20	20.4
DO (mg/l)	7.42	9.02	8.4
pH	8.12	8.63	8.29
NTU	7.59	0.854	21.5
NH_3(mg/l)	1.2	0	1.2
300mg/l	1	Control	Mixed
Temp (°C)	20.5	20.4	19.8
DO (mg/l)	7.44	9.23	8.65
pH	8.17	8.59	8.42
NTU	14.3	0.923	50.7
NH_3(mg/l)	1.8	0	1.2
400mg/l	1	Control	Mixed
Temp (°C)	19.8	19.8	19.9
DO (mg/l)	8.05	9.68	7.27
pH	8.29	8.5	8.15
NTU	55.9	1.14	81.6
NH_3(mg/l)	1.2	0	1.2
500mg/l	1	Control	Mixed
Temp (°C)	20.1	20	20.3
DO (mg/l)	6.18	1.26	7.94
pH	8.02	8.59	8.26
NTU	103	0.756	99.8
NH_3(mg/l)	2.4	0	2.4

Table 4 *Ephemerella:* water quality parameters during testing with MBI-401 FDP (1 = test chambers)

***Ephemerella ignita*- Drumcliff River water**					
12 Hours, 27th-Sept-11			**24 Hours, 28th-Sept-11**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	18	17.6	Temp (°C)	18.5	18.6
DO (mg/l)	8.32	9.06	DO (mg/l)	8.65	9.1
pH	8.31	8.47	pH	8.34	8.44
NTU	33.6	0.105	NTU	29.9	1.47
NH_3(mg/l)	0.421	0.435	NH_3(mg/l)	0.392	0.259
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	17.8	18	Temp (°C)	18.5	18.6
DO (mg/l)	8.58	8.65	DO (mg/l)	8.81	9.12
pH	8.43	8.5	pH	8.33	8.52
NTU	71.5	1.89	NTU	31.8	0.185
NH_3(mg/l)	0.381	0.232	NH_3(mg/l)	0.448	0.353
300mg/l	1	Control	300mg/l	1	18.6
Temp (°C)	18.2	17.9	Temp (°C)	18.6	9
DO (mg/l)	8.67	8.68	DO (mg/l)	8.37	8.5
pH	8.41	8.41	pH	8.74	1.41
NTU	76.3	1.32	NTU	28.2	0.574
NH_3(mg/l)	0.613	0.986	NH_3(mg/l)		
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	18.4	18.2	Temp (°C)	18.7	18.6
DO (mg/l)	8.55	8.61	DO (mg/l)	9.96	9.11
pH	8.36	8.51	pH	8	8.53
NTU	102	1.31	NTU	152	1.28
NH_3(mg/l)	0.481	0.542	NH_3(mg/l)	0.257	0.254
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	18.5	18.4	Temp (°C)	18.7	18.6
DO (mg/l)	8.41	8.7	DO (mg/l)	6.04	9.23
pH	8.35	8.49	pH	7.91	8.55
NTU	168	1.4	NTU	173	2
NH_3(mg/l)	0.803	0.374	NH_3(mg/l)	0.025	0.289

Ephemerella ignita - Drumcliff River water					
36 Hours, 28th-Sept-11			**48 Hours, 29th-Sept-11**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	19.2	19.3	Temp (°C)	19.9	19.8
DO (mg/l)	8.15	8.91	DO (mg/l)	8.53	8.58
pH	8.28	8.55	pH	8.34	8.43
NTU	28.2	1.16	NTU	30	1.24
NH_3(mg/l)	2.56	0.302	NH_3(mg/l)	0.0981	0.236
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	19.8	19.3	Temp (°C)	20	19.9
DO (mg/l)	7.34	8.96	DO (mg/l)	6.61	8.79
pH	8.14	8.55	pH	7.94	8.52
NTU	58	1.1	NTU	49.7	1.37
NH_3(mg/l)	3.18	0.201	NH_3(mg/l)	2.31	0.768
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	19.2	19.7	Temp (°C)	19.9	20
DO (mg/l)	7.78	8.89	DO (mg/l)	8.08	8.8
pH	8.13	8.59	pH	6.94	8.47
NTU	97.8	1.44	NTU	69.5	3.12
NH_3(mg/l)	2.81	0.266	NH_3(mg/l)	2.81	0.207
400mg/l	1	Control	400mg/l	1*	Control
Temp (°C)	19.9	19.8	Temp (°C)		19.8
DO (mg/l)	4.56	8.6	DO (mg/l)		8.92
pH	7.76	8.4	pH		8.5
NTU	154	0.876	NTU		1.89
NH_3(mg/l)	3.29	0.265	NH_3(mg/l)		0.455
500mg/l	1	Control	500mg/l	1*	Control
Temp (°C)	19.6	19.7	Temp (°C)		19.9
DO (mg/l)	5.52	7.02	DO (mg/l)		8.94
pH	7.85	7.95	pH		8.58
NTU	160	1.48	NTU		0.117
NH_3(mg/l)	3.21	0.183	NH_3(mg/l)		0.639

Ephemerella ignita - **Drumcliff River water**					
60 Hours, 29th-Sept-11			**72 Hours, 30th-Sept-11**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	19.7	19.8	Temp (°C)	19.7	19.7
DO (mg/l)	8.04	8.81	DO (mg/l)	8.44	8.79
pH	8.29	8.52	pH	8.42	8.45
NTU	32.3	0.203	NTU	18	0.197
NH_3(mg/l)	0.217	1.01	NH_3(mg/l)	2.373	0.472
200mg/l	1*	Control	200mg/l	1*	Control
Temp (°C)		19.8	Temp (°C)		19.7
DO (mg/l)		8.64	DO (mg/l)		8.74
pH		8.54	pH		8.45
NTU		0.199	NTU		1.43
NH_3(mg/l)		0.301	NH_3(mg/l)		0.706
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	19.6	19.6	Temp (°C)	19.8	19.7
DO (mg/l)	8.11	8.75	DO (mg/l)	7.68	8.94
pH	8.14	8.52	pH	8.16	8.56
NTU	87.9	1.42	NTU	53	3.7
NH_3(mg/l)	4.51	0.521	NH_3(mg/l)	6.37	0.436
400mg/l	1*	Control	400mg/l	1*	Control
Temp (°C)		19.8	Temp (°C)		19.7
DO (mg/l)		8.57	DO (mg/l)		8.94
pH		8.78	pH		8.56
NTU		0.194	NTU		3.7
NH_3(mg/l)		0.417	NH_3(mg/l)		0.436
500mg/l	1*	Control	500mg/l	1*	Control
Temp (°C)		19.8	Temp (°C)		19.7
DO (mg/l)		8.68	DO (mg/l)		8.71
pH		8.55	pH		8.53
NTU		1.16	NTU		0.127
NH_3(mg/l)		0.921	NH_3(mg/l)		0.56

* All mayfly deceased

Table 5 *Ephemerella:* water quality parameters during testing with MBI-401 SDP (1 = test chambers)

***Ephemerella ignita* - Drumcliff River water**					
24 hours, 14th-June-12 before exposure			**24 Hours 14th-June 12 after exposure**		
100mg/l	1	Control 1	100mg/l	1	Control 1
Temp (°C)	16.9	17	Temp (°C)	17.5	17.4
DO (mg/l)	9.19	9.4	DO (mg/l)	9.32	9.81
pH	8.24	8.35	pH	8.17	8.21
NTU	19.2	4.11	NTU	68.8	2.7
NH_3(mg/l)	0	0	NH_3(mg/l)	60.31	0.0061
200mg/l	1	Control 2	200mg/l	1	Control 2
Temp (°C)	17.1	17.1	Temp (°C)	17.7	17.6
DO (mg/l)	9.15	9.55	DO (mg/l)	9.07	9.31
pH	8.18	8.19	pH	8.09	8.29
NTU	31.8	2.61	NTU	144	3.17
NH_3(mg/l)		0	NH_3(mg/l)	0.219	0.0029
300mg/l	1	Control 3	300mg/l	1	Control 3
Temp (°C)	17.1	17.1	Temp (°C)	17.9	17.5
DO (mg/l)	9.15	9.56	DO (mg/l)	8.97	9.47
pH	8.17	8.23	pH	8.07	8.24
NTU	45.5	2.35	NTU	199	3.91
NH_3(mg/l)		0	NH_3(mg/l)	0.194	0.0131
400mg/l	1	Control 4	400mg/l	1	Control 4
Temp (°C)	17.1	17	Temp (°C)	17.8	17.5
DO (mg/l)	9.14	9.51	DO (mg/l)	0.02	9.39
pH	8.16	8.32	pH	8.06	8.1
NTU	60.8	2.56	NTU	244	3.28
NH_3(mg/l)	0.217	0.112	NH_3(mg/l)	0.0281	0.0861
500mg/l	1	Control 5	500mg/l	1	Control 5
Temp (°C)	17.2	17	Temp (°C)	17.9	17.5
DO (mg/l)	8.74	9.45	DO (mg/l)	8.57	9.4
pH	8.13	8.53	pH	7.9	8.18
NTU	126	2.98	NTU	458	7.96
NH_3(mg/l)	0.141	0.232	NH_3(mg/l)	0.016	0.0441

***Ephemerella ignita* - Drumcliff River water**					
48 hours 15th-June-12 before exposure			**48 Hours 15th-June-12 after exposure**		
100mg/l	1	Control 1	100mg/l	1	Control 1
Temp (°C)	16.7	16.9	Temp (°C)	17.6	17.8
DO (mg/l)	9.29	9.4	DO (mg/l)	8.96	9.31
pH	8.34	8.18	pH	8.11	8.16
NTU	31.8	2.33	NTU	61.5	2.31
NH_3(mg/l)	0.169	0.0003	NH_3(mg/l)	0.587	0
200mg/l	1	Control 2	200mg/l	1	Control 2
Temp (°C)	16.8	16.5	Temp (°C)	17.8	17.5
DO (mg/l)	9	9.34	DO (mg/l)	8.36	9.33
pH	8.27	8.27	pH	7.94	8.22
NTU	57.5	2.03	NTU	122	2.57
NH_3(mg/l)	0.0731	0*	NH_3(mg/l)	0.0811	0.001
300mg/l	1	Control 3	300mg/l	1	Control 3
Temp (°C)	17	16.7	Temp (°C)	17.8	17.6
DO (mg/l)	8.96	9.43	DO (mg/l)	8.97	9.31
pH	8.4	8.4	pH	7.94	8.26
NTU	63.5	2.39	NTU	191	2.48
NH_3(mg/l)	0.0726	0	NH_3(mg/l)	0.0919	0.0032
400mg/l	1	Control 4	400mg/l	1	Control 4
Temp (°C)	17	16.6	Temp (°C)	17.6	17.5
DO (mg/l)	7.91	9.45	DO (mg/l)	7.66	9.25
pH	7.9	8.31	pH	7.71	8.22
NTU	67.7	2.34	NTU	228	2.21
NH_3(mg/l)	0.0393	0*	NH_3(mg/l)	0.113	0.001
500mg/l	1	Control 5	500mg/l	1	Control 5
Temp (°C)	17	16.8	Temp (°C)	17.8	17.5
DO (mg/l)	4.69	9.46	DO (mg/l)	7.28	9.33
pH	7.55	8.4	pH	7.45	8.28
NTU	151	3.07	NTU	350	3.31
NH_3(mg/l)	0.45	0*	NH_3(mg/l)	0.0739	0.0004

Ephemerella ignita - Drumcliff River Water		
72 hours 16th-June-12		
100mg/l	1	Control 1
Temp (°C)	15.6	15.8
DO (mg/l)	9.54	9.67
pH	8.3	8.25
NTU	32	1.61
NH_3(mg/l)	0.719*	0.0081
200mg/l	1	Control 2
Temp (°C)	15.7	15.6
DO (mg/l)	9.42	9.74
pH	8.25	8.11
NTU	57	3.32
NH_3(mg/l)	0.455	0.0078
300mg/l	1	Control 3
Temp (°C)	15.9	15.6
DO (mg/l)	8.47	9.62
pH	8.08	8.3
NTU	97.8	3.46
NH_3(mg/l)	0.035*	0.0006*
400mg/l	1	Control 4
Temp (°C)	15.7	15.5
DO (mg/l)	8.66	9.74
pH	8.14	8.21
NTU	111	5.17
NH_3(mg/l)	0.0399	0.0005
500mg/l	1	Control 5
Temp (°C)	15.8	15.5
DO (mg/l)	8.33	9.51
pH	8.08	8.33
NTU	187	5.32
NH_3(mg/l)	0.0277*	0.0412*

* Double checked with ammonia test kit

Table 6 *Mytilus edulis:* water quality parameters during testing with MBI-401 FDP (1-3 = test chambers)

***Mytilus edulis* – Ross Seawater**					
12 Hours, 25-Oct-11			**24 Hours, 26th-Oct-11**		
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	17.6	17.6	Temp (°C)	16.4	15.8
DO (mg/l)	8.67	9	DO (mg/l)	8.89	8.96
pH	7.92	8.01	pH	8.01	8.05
NTU	47.6	0.345	NTU	134	0.277
NH_3(mg/l)	1.43	1.37	NH_3(mg/l)	5.61	0.392
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	17.6	17.2	Temp (°C)	15.8	16
DO (mg/l)	8.67	8.81	DO (mg/l)	8.64	8.7
pH	7.94	7.81	pH	7.92	7.86
NTU	85.7	0.358	NTU	230	0.394
NH_3(mg/l)	1.38	0.71	NH_3(mg/l)	5.55	0.538
400mg/l	1	Control	400mg/l	1	18.6
Temp (°C)	17.3	17.5	Temp (°C)	16.3	15.9
DO (mg/l)	8.83	9.05	DO (mg/l)	7.99	8.94
pH	7.96	8.01	pH	7.77	7.97
NTU	181	0.722	NTU	306	0.348
NH_3(mg/l)	1.54	0.655	NH_3(mg/l)	4.09	0.722
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	17.2	17.2	Temp (°C)	16.1	15.9
DO (mg/l)	8.82	8.88	DO (mg/l)	7.98	8.75
pH	7.96	7.94	pH	7.86	7.99
NTU	416	0.786	NTU	460	0.344
NH_3(mg/l)	1.43	1.82	NH_3(mg/l)	4.29	0.521
600mg/l	1	Control	600mg/l	1	Control
Temp (°C)	17.6	17.1	Temp (°C)	16.3	15.9
DO (mg/l)	7.49	8.83	DO (mg/l)	6.96	8.65
pH	7.64	7.86	pH	7.63	7.55
NTU	285	0.507	NTU	6.08	0.296
NH_3(mg/l)	1.7	0.795	NH_3(mg/l)	4.5	0.695

Mytilus edulis – Ross Seawater					
36 Hours, 26th-Oct-11			**48 Hours, 27th-Oct-11**		
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	16.4	16.5	Temp (°C)	16.7	16.3
DO (mg/l)	8.7	9.05	DO (mg/l)	8.61	8.27
pH	7.95	8	pH	8.09	7.93
NTU	134	1.48	NTU	129	0.191
NH_3(mg/l)	3.34	0.478	NH_3(mg/l)	4.56	0.59
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	16.1	16.5	Temp (°C)	16.3	16.5
DO (mg/l)	8.47	8.68	DO (mg/l)	8.55	8.79
pH	7.95	7.79	pH	8.06	7.97
NTU	197	0.543	NTU	168	0.527
NH_3(mg/l)	5.66	0.933	NH_3(mg/l)	8.37	0.834
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	16.3	16.2	Temp (°C)	16.5	16.2
DO (mg/l)	7.25	8.8	DO (mg/l)	7.98	9
pH	7.66	7.86	pH	7.92	8
NTU	308	0.103	NTU	279	0.076
NH_3(mg/l)	5.51	0.635	NH_3(mg/l)	10.3	0.659
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	16.5	16.5	Temp (°C)	16.6	16.2
DO (mg/l)	7.83	8.89	DO (mg/l)	8.36	8.57
pH	7.8	7.89	pH	7.97	7.96
NTU	7.45	0.895	NTU	395	0.216
NH_3(mg/l)	4.13	0.581	NH_3(mg/l)	16.2	0.782
600mg/l	1	Control	600mg/l	1	Control
Temp (°C)	16.4	16.1	Temp (°C)	16.4	16.3
DO (mg/l)	6.89	9	DO (mg/l)	8.08	8.79
pH	7.64	7.99	pH	8.43	7.84
NTU	597	0.817	NTU	647	1.38
NH_3(mg/l)	6.83	0.626	NH_3(mg/l)	13.4	0.997

Mytilus edulis – Ross Seawater					
60 Hours, 27th-Oct-11			**72 Hours, 28th-Oct-11**		
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	15.9	15.6	Temp (°C)	16.8	16.4
DO (mg/l)	8.96	9.24	DO (mg/l)	7.74	9.34
pH	7.94	8	pH	7.89	8.03
NTU	125	0.163	NTU	130	2.55
NH_3(mg/l)	4.52	1.2	NH_3(mg/l)	8.8	1.26
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	15.5	15.9	Temp (°C)	16.6	16.4
DO (mg/l)	8.88	9.09	DO (mg/l)	8.34	9
pH	7.97	7.88	pH	7.93	7.75
NTU	236	0.199	NTU	263	1.1
NH_3(mg/l)	8.36	1.76	NH_3(mg/l)	11.6	1.63
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	15.5	15.4	Temp (°C)	16.6	16.4
DO (mg/l)	7.15	9.3	DO (mg/l)	7.44	7.97
pH	7.63	8.07	pH	7.71	9.39
NTU	313	0.521	NTU	319	2.74
NH_3(mg/l)	7.7	0.732	NH_3(mg/l)	10.9	1.66
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	15.1	15.4	Temp (°C)	16.9	16.5
DO (mg/l)	7.32	9.3	DO (mg/l)	6.68	9.31
pH	7.7	8.07	pH	7.66	8.02
NTU	485	0.521	NTU	4.08	2.77
NH_3(mg/l)	5.77	0.732	NH_3(mg/l)	11.1	1.07
600mg/l	1	Control	600mg/l	1	Control
Temp (°C)	15.3	15.4	Temp (°C)	16.4	16.4
DO (mg/l)	8.77	9.09	DO (mg/l)	8	9.01
pH	7.97	7.95	pH	7.83	7.91
NTU	630	1.21	NTU	7.7	1.7
NH_3(mg/l)	11.7	0.905	NH_3(mg/l)	16.1	1.09

Table 7 *Anodonta*: water quality parameters during testing with MBI-401 FDP (1=test chambers)

***Anodonta* - Lough Gill water**					
12 Hours, 19th-Sept-11			**24 Hours, 19th-Sept-11**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	16.4	16.2	Temp (°C)	17.6	17.6
DO (mg/l)	8.81	9.23	DO (mg/l)	9.4	8.91
pH	8.21	8.3	pH	8.34	8.19
NTU	21.2	0.193	NTU	20.3	3.69
NH_3(mg/l)	0.23	0.39	NH_3(mg/l)	0.5	1.5
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	16.3	16.2	Temp (°C)	17.6	17.5
DO (mg/l)	8.84	9.22	DO (mg/l)	9.04	8.89
pH	8.36	8.34	pH	8.21	8.25
NTU	48.1	0.241	NTU	29.1	2.59
NH_3(mg/l)	0.51	0.63	NH_3(mg/l)	0.94	0.7
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	16.3	16.3	Temp (°C)	17.6	17.5
DO (mg/l)	8.96	7.01	DO (mg/l)	9.25	9.23
pH	8.35	7.96	pH	8.3	8.22
NTU	63.1	0.189	NTU	60	0.73
NH_3(mg/l)	0.45	0.048	NH_3(mg/l)	1.1	0.73
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	16.3	16.3	Temp (°C)	17.6	17.5
DO (mg/l)	9	9.08	DO (mg/l)	8.25	9.38
pH	8.15	8.42	pH	8.05	8.29
NTU	111	0.258	NTU	75.03	0.6
NH_3(mg/l)	1.3	0.034	NH_3(mg/l)	0.83	0.86
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	16.3	16.4	Temp (°C)	17.6	17.6
DO (mg/l)	9.06	8.27	DO (mg/l)	9.12	8.96
pH	8.3	8.14	pH	8.21	8.1
NTU	103	0.252	NTU	117	0.256
NH_3(mg/l)	0.61	0.081	NH_3(mg/l)	1.7	0.64

***Anodonta* - Lough Gill water**					
36 Hours, 20th-Sept-11			**48 Hours, 20th-Sept-11**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	17.6	17.6	Temp (°C)	17.3	17.5
DO (mg/l)	8.96	8.51	DO (mg/l)	8.99	9.03
pH	8.34	8.04	pH	8.25	8.17
NTU	19.4	0.158	NTU	21.4	1.75
NH_3(mg/l)	1.2	0.51	NH_3(mg/l)	2.33	0.587
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	17.8	17.9	Temp (°C)	17.6	17.2
DO (mg/l)	8.91	8.37	DO (mg/l)	8.28	9.28
pH	8.33	8.21	pH	8.66	8.19
NTU	38.6	0.104	NTU	44.5	1.08
NH_3(mg/l)	3.61	0.51	NH_3(mg/l)	3.11	0.623
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	17.7	17.7	Temp (°C)	17.5	17.5
DO (mg/l)	8.88	9.17	DO (mg/l)	8.62	9.08
pH	8.29	8.32	pH	8.17	8.22
NTU	67.1	0.139	NTU	76.6	1.93
NH_3(mg/l)	5.15	0.59	NH_3(mg/l)	4.28	0.813
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	17.9	17.7	Temp (°C)	17.9	17.3
DO (mg/l)	8.83	9.09	DO (mg/l)	8.08	9.18
pH	8.28	8.36	pH	8.06	8.24
NTU	81.3	0.162	NTU	93.9	0.101
NH_3(mg/l)	3.39	1.5	NH_3(mg/l)	4.35	0.591
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	17.9	17.8	Temp (°C)	17.4	17.7
DO (mg/l)	8.49	8.51	DO (mg/l)	8.04	8.66
pH	8.19	8.21	pH	8	8.13
NTU	112	0.185	NTU	148	1.52
NH_3(mg/l)	4.84	0.45	NH_3(mg/l)	5.23	0.707

***Anodonta* Collected - Lough Gill Water**					
60 Hours 21st-Sept-11			**72 Hours 21st-Sept-11**		
100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	17.7	17.8	Temp (°C)	16.4	16.8
DO (mg/l)	8.44	8.94	DO (mg/l)	8.27	8.97
pH	8.2	8.19	pH	8.18	8.06
NTU	17.8	0.112	NTU	23	0.128
NH_3(mg/l)	0.544	0.0543	NH_3(mg/l)	0.36	1.8
200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	17.8	17.9	Temp (°C)	16.5	16.6
DO (mg/l)	8.39	8.27	DO (mg/l)	8.49	9.26
pH	8.15	8.03	pH	8.16	8.21
NTU	42.2	2.28	NTU	50.6	1.55
NH_3(mg/l)	3.21	0.125	NH_3(mg/l)	1.92	1.105
300mg/l	1	Control	300mg/l	1	Control
Temp (°C)	18.1	18	Temp (°C)	16.7	16.7
DO (mg/l)	8.57	8.95	DO (mg/l)	8.44	9.29
pH	8.19	8.17	pH	8.24	8.21
NTU	64	0.84	NTU	82.9	0.139
NH_3(mg/l)	4.27	0.621	NH_3(mg/l)	4.79	0.9
400mg/l	1	Control	400mg/l	1	Control
Temp (°C)	18	17.6	Temp (°C)	16.6	16.7
DO (mg/l)	7.77	9.04	DO (mg/l)	7.63	9.27
pH	8.66	8.25	pH	8.02	9.27
NTU	83.7	0.166	NTU	93	0.132
NH_3(mg/l)	6.15	0.699	NH_3(mg/l)	5.41	0.218
500mg/l	1	Control	500mg/l	1	Control
Temp (°C)	17.8	17.6	Temp (°C)	16.5	16.7
DO (mg/l)	8.19	8.99	DO (mg/l)	8.23	9.04
pH	8.19	8.3	pH	8.12	8.26
NTU	114	0.972	NTU	117	0.157
NH_3(mg/l)	7.25	0.0311	NH_3(mg/l)	6.48	0.255

Table 8 *Daphnia pulex*: water quality parameters during testing with MBI-401 FDP (1 = test chambers)

***Daphnia pulex* - Lough Gill Water**								
24 hours 5th-April-12			**48 hours 6th-April-12**			**72 hours 7th-April-12**		
50mg/l	1	Control	50mg/l	1	Control	50mg/l	1	Control
Temp (°C)	18.8	19	Temp (°C)	19.2	19	Temp (°C)	19	19.6
DO (mg/l)	7.09	9.05	DO (mg/l)	7.02	9.21	DO (mg/l)	6.76	8.59
pH	7.74	7.79	pH	7.92	8.22	pH	7.84	8.28
NTU	33.6	3.27	NTU	31.7	1.23	NTU	28.9	1.01
NH_3(mg/l)	0.005	0.0272	NH^3(mg/l)	0.0022	0.108	NH_3(mg/l)	0.0898	0.0108
100mg/l	1	Control	100mg/l	1	Control	100mg/l	1	Control
Temp (°C)	18.4	18.5	Temp (°C)	19.4	19.2	Temp (°C)	19.1	19.1
DO (mg/l)	8.39	9.47	DO (mg/l)	6.06	9.14	DO (mg/l)	6.41	8.49
pH	7.93	8.18	pH	7.63	8.37	pH	7.4	8.32
NTU	58.7	1.28	NTU	54.1	2.42	NTU	78.3	0.134
NH_3(mg/l)	0.0034	0.128	NH^3(mg/l)	0.006	0.0048	NH_3(mg/l)	0.115	0.0094
150mg/l	1	Control	150mg/l	1	Control	150mg/l	1	Control
Temp (°C)	18.8	18.5	Temp (°C)	19.2	19.6	Temp (°C)	19.2	19.3
DO (mg/l)	6.53	9.46	DO (mg/l)	6.13	9.02	DO (mg/l)	7.32	8.31
pH	7.62	8.27	pH	7.42	8.42	pH	7.58	8.3
NTU	82.4	2.05	NTU	133	0.761	NTU	100	1.14
NH_3(mg/l)	0.0029	0.0451	NH^3(mg/l)	0.022	0.135	NH_3(mg/l)	0.113	0.142
200mg/l	1	Control	200mg/l	1	Control	200mg/l	1	Control
Temp (°C)	19.4	18.4	Temp (°C)	19.7	19.3	Temp (°C)	19.1	19.2
DO (mg/l)	5.76	9.17	DO (mg/l)	6.12	9.1	DO (mg/l)	3.77	8.45
pH	7.53	8.06	pH	7.17	8.45	pH	7.35	8.32
NTU	147	2.73	NTU	294	0.964	NTU	128	2.04
NH_3(mg/l)	0.0216	0.0021	NH^3(mg/l)	0.0039	0.0151	NH_3(mg/l)	0.111	0.0741
250mg/l	1	Control	250mg/l	1	Control	250mg/l	1	Control
Temp (°C)	18.9	18.9	Temp (°C)	19.6	19.4	Temp (°C)	19	19.1
DO (mg/l)	6.84	9.61	DO (mg/l)	6.41	9.09	DO (mg/l)	2.12	8.88
pH	7.7	8.3	pH	6.63	8.32	pH	6.84	8.29
NTU	156	2.59	NTU	247	1.64	NTU	632	1.04
NH_3(mg/l)	0.012	0.086	NH^3(mg/l)	0.138	0.0002	NH_3(mg/l)	0.102	0.006

Table 9 *Austropotamobius pallipes:* water quality parameters during testing with MBI-401 FDP (1 = test chambers)

***Austropotamobius pallipes* - Lough Gill Water**					
24 hours 18th-May-12 before treatment			**24 Hours 18th-May-12 after treatment**		
350mg/l	1	Control 1	350mg/l	1	Control
Temp (°C)	16.9	16.9	Temp (°C)	17.4	17
DO (mg/l)	8.98	9.84	DO (mg/l)	9.52	10.31
pH	7.15	8.09	pH	7.87	8.15
NTU	117	1.43	NTU	249	1.54
NH_3(mg/l)	2.74	1.64	NH_3(mg/l)	0.753	0.558
450mg/l	1	Control 2	450mg/l	1	Control
Temp (°C)	17.4	16.4	Temp (°C)	17.2	16.9
DO (mg/l)	7.7	10.15	DO (mg/l)	9.68	10.15
pH	7.76	8.13	pH	7.9	8.1
NTU	130	1.04	NTU	285	1
NH_3(mg/l)	2.73	2.9	NH_3(mg/l)	0.957	0.473
550mg/l	1	Control 3	550mg/l	1	Control
Temp (°C)	16.9	16.5	Temp (°C)	16.9	17
DO (mg/l)	9.92	9.05	DO (mg/l)	9.58	9.71
pH	8	8.11	pH	7.95	8.14
NTU	134	1.66	NTU	374	1.66
NH^3(mg/l)	3.06	2.51	NH_3(mg/l)	1.07	0.605
650mg/l	1		650mg/l	1	
Temp (°C)	17		Temp (°C)	16.9	
DO (mg/l)	8.93		DO (mg/l)	9.49	
pH	7.84		pH	7.91	
NTU	123		NTU	430	
NH_3(mg/l)	2.02		NH_3(mg/l)	1.12	
750mg/l	1		750mg/l	1	
Temp (°C)	16.7		Temp (°C)	16.7	
DO (mg/l)	9.45		DO (mg/l)	10.09	
pH	7.9		pH	8.05	
NTU	136		NTU	452	
NH_3(mg/l)	2.58		NH_3(mg/l)	1.58	

***Austropotamobius pallipes* - Lough Gill Water**					
48 hours 19th-May-12 before treatment			**48 Hours 19th-May-12 after treatment**		
350mg/l	1	Control 1	350mg/l	1	Control 1
Temp (°C)	14.7	14.9	Temp (°C)	16.2	15.4
DO (mg/l)	8.17	9.84	DO (mg/l)	8.42	9.71
pH	7.59	8.24	pH	7.73	7.71
NTU	130	1.08	NTU	204	1.14
NH_3(mg/l)	0.964	0.256	NH_3(mg/l)	0.674	1
450mg/l	1	Control 2	450mg/l	1	Control 2
Temp (°C)	15	14.6	Temp (°C)	15.8	15.9
DO (mg/l)	5.58	9.97	DO (mg/l)	9.4	9.69
pH	7.49	8.16	pH	7.91	7.98
NTU	151	1.27	NTU	284	0.878
NH_3(mg/l)	0.203	0.953	NH_3(mg/l)	0.435	0.891
550mg/l	1	Control 3	550mg/l	1	Control 3
Temp (°C)	14.9	14.8	Temp (°C)	15.9	15.7
DO (mg/l)	6.91	9.76	DO (mg/l)	9.05	9.36
pH	7.58	8.2	pH	7.83	7.99
NTU	175	1.32	NTU	289	1.57
NH_3(mg/l)	0.907	0.806	NH_3(mg/l)	0.557	1
650mg/l	1		650mg/l	1	
Temp (°C)	15.1		Temp (°C)	16	
DO (mg/l)	7.12		DO (mg/l)	8.94	
pH	7.78		pH	7.91	
NTU	117		NTU	472	
NH_3(mg/l)	0.182		NH_3(mg/l)	0.333	
750mg/l	1		750mg/l	1	
Temp (°C)	15		Temp (°C)	15.8	
DO (mg/l)	7.99		DO (mg/l)	9.52	
pH	7.8		pH	7.94	
NTU	126		NTU	525	
NH_3(mg/l)	0.907		NH_3(mg/l)	0.289	

***Austropotamobius pallipes* - Lough Gill Water**		
72 hours 20th-May-12		
350mg/l	1	Control 1
Temp (°C)	15.7	15.6
DO (mg/l)	7.58	9.67
pH	7.63	8.01
NTU	79.8	0.926
NH_3(mg/l)	0.168	0.441
450mg/l	1	Control 2
Temp (°C)	15.9	15.1
DO (mg/l)	8.9	9.79
pH	7.85	8.21
NTU	87.6	1.31
NH_3(mg/l)	0.18	0.793
550mg/l	1	Control 3
Temp (°C)	15.7	15.4
DO (mg/l)	8.56	9.35
pH	7.8	8.34
NTU	104	1.13
NH_3(mg/l)	0.221	0.426
650mg/l	1	
Temp (°C)	15.7	
DO (mg/l)	4.01	
pH	7.37	
NTU	231	
NH_3(mg/l)	0.519	
750mg/l	1	
Temp (°C)	15.6	
DO (mg/l)	8.02	
pH	7.64	
NTU	162	
NH_3(mg/l)	1.08	

Table 10 *Lymnaea peregra:* water quality parameters during testing with MBI-401 SDP (1 = test chambers)

***Lymnaea peregra* - Lough Gill Water**					
24 hours 24th July 12 before exposure			**24 Hours 24th July12 after exposure**		
500 mg/L	Chamber 1	Control	500 mg/L	Chamber	Control
Temp (°C)	22.9	22.3	Temp (°C)	93.3	23.4
DO (mg/l)	8.79	9.52	DO (mg/l)	8.78	8.64
pH	7.97	8.27	pH	7.88	8.59
NTU	55.6	1.315	NTU	277	1.22
NH_3(mg/l)	0.0587	0.0757	NH_3(mg/l)	0.0731	0.0612
600 mg/L	Chamber 1	Control	600 mg/L	Chamber	Control
Temp (°C)	22.2	22.9	Temp (°C)	23.5	23.5
DO (mg/l)	9.05	9.44	DO (mg/l)	8.12	8.8
pH	8.02	8.2	pH	7.71	8.73
NTU	55.2	1.57	NTU	387	1.18
NH_3(mg/l)	0.0648	0.015	NH_3(mg/l)	0.0669	0.101
700 mg/L	Chamber 1	Control	700mg/l	Chamber	Control
Temp (°C)	22.7	22.7	Temp (°C)	23.7	23.3
DO (mg/l)	8.2	9.53	DO (mg/l)	7.38	9.53
pH	7.81	8.26	pH	7.52	8.1
NTU	128	1.67	NTU	457	1.5
NH_3(mg/l)	0.0809	0.0598	NH_3(mg/l)	0.0754	0.116
800 mg/L	Chamber 1	Control 4	800mg/l	Chamber 1	Control 4
Temp (°C)	23	23	Temp (°C)	23.6	23.5
DO (mg/l)	7.64	9.39	DO (mg/l)	8.18	8.06
pH	7.7	8.16	pH	7.66	9.42
NTU	117	1.46	NTU	585	1.77
NH_3(mg/l)	0.107	0.0087	NH_3(mg/l)	0.0719	0.0088
900 mg/L	Chamber	Control	900mg/l	Chamber	Control
Temp (°C)	23.2	23	Temp (°C)	23.6	23.5
DO (mg/l)	7.39	9.42	DO (mg/l)	7.49	9.5
pH	7.67	8.11	pH	7.47	7.72
NTU	136	1.36	NTU	683	1.29
NH_3(mg/l)	0.0868	0.0069	NH_3(mg/l)	0.0719	0.0563

***Lymnaea peregra* - Lough Gill Water**					
48 hours 25th July 12 before exposure			**48 Hours 25th July 12 after exposure**		
500 mg/L	Chamber 1	Control 1	500mg/l	Chamber 1	Control 1
Temp (°C)	22.8	22.6	Temp (°C)	19.8	19.5
DO (mg/l)	8.43	9.29	DO (mg/l)	7.86	9.12
pH	8.02	8.25	pH	7.82	8.26
NTU	53.9	1.12	NTU	361	1.98
NH_3(mg/l)	0.0517	0.0807	NH_3(mg/l)	0.108	0.169
600 mg/L	Chamber 1	Control 2	600mg/l	Chamber 1	Control 2
Temp (°C)	22.9	22.7	Temp (°C)	19.5	19.7
DO (mg/l)	8.03	9.36	DO (mg/l)	7.08	9.24
pH	7.17	8.18	pH	7.6	8.21
NTU	92.4	1.32	NTU	528	1.58
NH_3(mg/l)	0.0457	0.0906	NH_3(mg/l)	0.979	0.0321
700 mg/L	Chamber 1	Control 3	700mg/l	Chamber 1	Control 3
Temp (°C)	23	22.7	Temp (°C)	19.6	19.6
DO (mg/l)	8.27	9.24	DO (mg/l)	7.09	9.14
pH	8.01	8.22	pH	7.61	8.24
NTU	73.4	1.41	NTU	616	1.33
NH_3(mg/l)	0.063	0.0814	NH_3(mg/l)	0.0975	0.0221
800 mg/L	Chamber 1	Control 4	800mg/l	Chamber 1	Control 4
Temp (°C)	22.8	22.9	Temp (°C)	19.8	19.6
DO (mg/l)	7.66	9.35	DO (mg/l)	7.01	9.18
pH	7.87	8.12	pH	7.44	8.24
NTU	121	1.19	NTU	836	1.79
NH_3(mg/l)	0.0486	0.0414	NH_3(mg/l)	0.0832	0.0192
900 mg/L	Chamber 1	Control 5	900mg/l	Chamber 1	Control 5
Temp (°C)	23	22.8	Temp (°C)	19.9	19.6
DO (mg/l)	6.28	9.34	DO (mg/l)	6.74	9.97
pH	7.77	8.07	pH	7.48	8.15
NTU	142	1.37	NTU	939	1.67
NH_3(mg/l)	0.0328	0.0186	NH_3(mg/l)	0.0826	0.135

***Lymnaea peregra* - Lough Gill Water**		
72 hours 26th July 12 before exposure		
500 mg/L	Chamber 1	Control 1
Temp (°C)	18.6	18.2
DO (mg/l)	8.18	9.34
pH	8.02	8.17
NTU	67.6	1.79
NH_3(mg/l)	0.0425	0.0449
600 mg/L	Chamber 1	Control 2
Temp (°C)	18.8	18.3
DO (mg/l)	7.01	9.3
pH	7.66	8.29
NTU	114	1.49
NH_3(mg/l)	0.0437	0.167
700 mg/L	Chamber 1	Control 3
Temp (°C)	18.3	18.1
DO (mg/l)	7.26	9.28
pH	7.77	8.21
NTU	136	1.54
NH_3(mg/l)	0.0398	0.0265
800 mg/L	1	Control 4
Temp (°C)	18.6	18.4
DO (mg/l)	6.53	9.36
pH	7.71	8.23
NTU	185	1.56
NH_3(mg/l)	0.047	0.0339
900 mg/L	1	Control 5
Temp (°C)	18.5	18.4
DO (mg/l)	7.41	9.24
pH	7.8	8.28
NTU	168	2.76
NH_3(mg/l)	0.0355	0.0498

Table 11 *Salmo trutta:* water quality parameters during testing with MBI-401 SDP (1 = test chambers)

Salmo trutta				
11th-15th June 12 before exposure				
Concentration	**°C**	**DO (mg/l)**	**pH**	**NTU**
24 Hours				
Control	16.2	91	7.9	1
18mg/L	15.4	8.4	7.5	10
32mg/L	15.2	7.9	7.4	21
56mg/L	15.3	8.4	7.5	39
100mg/L	15.5	8.3	7.2	62
180mg/L	15.5	8.5	7.1	107
48 Hours				
Control	15.6	10.6	7.8	<1
18mg/L	15.3	9.7	7.6	8
32mg/L	15.4	9.8	7.4	20
56mg/L	15.4	9.9	7.3	34
100mg/L	15.6	9.6	7.4	61
180mg/L	15.6	10.3	7.5	110
72 Hours				
Control	15	10.5	7.5	1
18mg/L	15.3	10.1	7.4	12
32mg/L	15.3	9.8	7.7	21
56mg/L	15.2	9.8	7.7	36
100mg/L	15.2	10	7.7	51
180mg/L*				

*100% parr mortality

11th-15th June 12 after exposure					
Concentration	**Temp**	**DO mg/l**	**pH**	**NTU**	**NH_4*(mg/l)**
24 Hours					
Control	14.9	8.8	7.8	2	
18mg/L	14.4	8.4	7.9	11	
32mg/L	14.2	8.7	8	15	
56mg/L	14.7	8.8	8	30	
100mg/L	14.8	8.9	8	48	
180mg/L	14.8	8.8	8.1	68	0.027
48 Hours					
Control	14.8	9.4	8	<1	
18mg/L	14.5	7.8	7.6	10	
32mg/L	14.6	8.7	7.9	14	
56mg/L	14.9	8.8	8	22	
100mg/L	14.8	8.7	7.7	34	0.018
180mg/L	14.7	9.1	7.9	42	
72 Hours					
Control	14.6	10.5	7.9	3	
18mg/L	14.5	10.1	7.8	6	
32mg/L	14.5	9.8	7.7	11	
56mg/L	14.7	9.8	7.7	18	0.003
100mg/L	14.7	10	7.8	31	
180mg/L					
96 Hours					
Control	14.6	9.7	7.8	2	
18mg/L	14.5	9.5	7.8	6	
32mg/L	14.7	9.5	7.8	10	0.003
56mg/L	14.8	9.3	7.7	18	
100mg/L					
180mg/L					

*Ammonium

Appendix D: Statistical analysis in Minitab 16

Table 1 *Chironomus:* General Linear model of species survival V concentration (conc), time and replication.

FDP

Factor	Type	Levels	Values
Conc	fixed	5	100mg/l, 200mg/l, 300mg/l, 400mg/l, 500mg/l
Time	fixed	3	24, 48, 72
Replicate	fixed	3	1, 2, 3

Analysis of Variance for Organisms 4, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Conc	4	52.8000	52.8000	13.2000	24.00	0.000
Time	2	39.5111	39.5111	19.7556	35.92	0.000
Replicate	2	2.7111	2.7111	1.3556	2.46	0.117
Conc*Time	8	38.9333	38.9333	4.8667	8.85	0.000
Error	16	8.8000	8.8000	0.5500		
Total	44	149.9111				

S = 0.741620 R-Sq = 94.13% R-Sq(adj) = 83.86%

Unusual Observations for Organisms 4

Obs	Organisms 4	Fit	SE Fit	Residual	St Resid
38	6.00000	4.97778	0.59535	1.02222	2.31 R

R denotes an observation with a large standardized residual.

*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
-Accept Ha

Ho There is no difference in mortality between replicates
Ha There is a difference in mortality between replication
-Accept Ho

Ho There is no effect on response due to time
Ha There is an effect on response due to time
-Accept Ha

Table 2 *Chironomus:* Two way Anova for water quality parameters V time and concentration (conc)

FDP

Two-way ANOVA: Temp versus time, concentration

Source	DF	SS	MS	F	P
time_2	2	0.21102	0.105510	1.30	0.325
dose_2	4	0.66585	0.166463	2.05	0.180
Error	8	0.65043	0.081303		
Total	14	1.52730			

S = 0.2851 R-Sq = 57.41% R-Sq(adj) = 25.47%

Two-way ANOVA: DO versus time, concentration

Source	DF	SS	MS	F	P
time_2	2	16.714	8.3571	0.79	0.484
dose_2	4	19.969	4.9922	0.47	0.754
Error	8	84.106	10.5132		
Total	14	120.789			

S = 3.242 R-Sq = 30.37% R-Sq(adj) = 0.00%

Two-way ANOVA: pH versus time, concentration

Source	DF	SS	MS	F	P
time_2	2	0.31402	0.157010	2.96	0.109
dose_2	4	0.34890	0.087224	1.65	0.254
Error	8	0.42365	0.052957		
Total	14	1.08657			

S = 0.2301 R-Sq = 61.01% R-Sq(adj) = 31.77%

Two-way ANOVA: NH_3 versus time, concentration

Source	DF	SS	MS	F	P
time_2	2	10.8	5.4000	9.19	0.008
dose_2	4	4.0	1.0000	1.70	0.242
Error	8	4.7	0.5875		
Total	14	19.5			

S = 0.7665 R-Sq = 75.90% R-Sq(adj) = 57.82%

Two-way ANOVA: NTU versus time, concentration

Source	DF	SS	MS	F	P
time_2	2	796.1	398.0	0.09	0.911
dose_2	4	58716.5	14679.1	3.48	0.063
Error	8	33749.0	4218.6		
Total	14	93261.5			

S = 64.95 R-Sq = 63.81% R-Sq(adj) = 36.67%

*If p >0.05 accept Ho
*If p < 0.05 accept Ha

Ho There is no effect on temperature due to concentration
Ha There is an effect on temperature due to concentration
-Accept Ho

Ho There is no effect on temperature due to time
Ha There is an effect on temperature due to time
-Accept Ho

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
-Accept Ho

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
-Accept Ho

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
-Accept Ho

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
Accept Ho

Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
-Accept Ho

Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
-Accept Ho

Ho There is no effect on NH_3 due to concentration
Ha there is an effect on NH_3 due to concentration
-Accept Ho

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
-Accept Ha

Table 3 *Chironomus plumosus:* General Linear model of species survival V concentration (conc), time and replication.

SDP

Factor	Type	Levels	Values
Time	fixed	3	24, 48, 72
Replication	fixed	3	1, 2, 3
Conc	fixed	5	100mg/l, 200mg/l, 300mg/l, 400mg/l, 500mg/l

Analysis of Variance for Organisms 1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	2	6.5778	6.5778	3.2889	12.87	0.000
Replication	2	1.6444	1.6444	0.8222	3.22	0.067
Conc	4	3.0222	3.0222	0.7556	2.96	0.053
Time*Conc	8	2.3111	2.3111	0.2889	1.13	0.395
Error	16	4.0889	4.0889	0.2556		
Total	44	28.5778				

S = 0.505525 R-Sq = 85.69% R-Sq(adj) = 60.65%

Unusual Observations for Organisms

Obs	Organisms	Fit	SE Fit	Residual	St Resid
4	6.00000	5.22222	0.40582	0.77778	2.58 R
10	6.00000	6.77778	0.40582	-0.77778	-2.58 R
40	6.00000	5.37778	0.40582	0.62222	2.06 R

R denotes an observation with a large standardized residual.
*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
-Accept Ho

Ho There is no difference in mortality between replicates
Ha There is a difference in mortality between replication
-Accept Ho

Ho There is no effect on response due to time
Ha There is an effect on response due to time
-Accept Ha

Ho There is no effect due to interaction between time and concentration
Ha There is an effect due to interaction between time and concentration
-Accept Ho

Table 4 *Chironomus plumosus:* Two way Anova for water quality parameters V time and concentration

SDP
Two-way ANOVA: Temp versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	8.22533	4.11267	181.44	0.000
Concentration	4	0.09067	0.02267	1.00	0.461
Error	8	0.18133	0.02267		
Total	14	8.49733			

S = 0.1506 R-Sq = 97.87% R-Sq(adj) = 96.27%

Two-way ANOVA: DO versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	5.24857	2.62429	20.50	0.001
Concentration	4	1.08347	0.27087	2.12	0.170
Error	8	1.02429	0.12804		
Total	14	7.35633			

S = 0.3578 R-Sq = 86.08% R-Sq(adj) = 75.63%

Two-way ANOVA: pH versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	1.28249	0.641247	4.57	0.047
Concentration	4	0.62369	0.155923	1.11	0.415
Error	8	1.12291	0.140363		
Total	14	3.02909			

S = 0.3747 R-Sq = 62.93% R-Sq(adj) = 35.13%

Two-way ANOVA: NTU versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	8166.2	4083.11	9.02	0.009
Concentration	4	16128.8	4032.20	8.91	0.005
Error	8	3620.8	452.60		
Total	14	27915.8			

S = 21.27 R-Sq = 87.03% R-Sq(adj) = 77.30%

Two-way ANOVA: NH_3 versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	4.8375	2.41874	1.04	0.398
Concentration	4	9.2723	2.31808	0.99	0.464
Error	8	18.6821	2.33526		
Total	14	32.7919			

S = 1.528 R-Sq = 43.03% R-Sq(adj) = 0.30%

*If p >0.05 accept Ho
*If p < 0.05 accept Ha

Ho There is no effect on temperature due to concentration
Ha There is an effect on temperature due to concentration
Accept Ho

Ho There is no effect on temperature due to time
Ha There is an effect on temperature due to time
Accept Ha

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
Accept Ho

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
Accept Ha

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
Accept Ho

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
Accept Ha

Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
Accept Ha

Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
Accept Ha

Ho There is no effect on NH_3 due to concentration
Ha There is an effect on NH_3 due to concentration
Accept Ho

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
Accept Ho

Table 5 *Asellus aquaticus:* General Linear model of species survival V concentration (conc), time and replication.

Factor	Type	Levels	Values
Time	fixed	3	24, 48, 72
Dose	fixed	5	100mg/l, 200mg/l, 300mg/l, 400mg/l, 500mg/l
Replicate	fixed	3	1, 2, 3

Analysis of Variance for Organisms, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	2	0.5778	0.5778	0.2889	1.25	0.312
conc	4	3.0222	3.0222	0.7556	3.28	0.038
Replicate	2	1.3778	1.3778	0.6889	2.99	0.079
Time* conc	8	1.6444	1.6444	0.2056	0.89	0.545
Error	16	3.6889	3.6889	0.2306		
Total	44	17.9111				

S = 0.480162 R-Sq = 79.40% R-Sq(adj) = 43.36%

Unusual Observations for Organisms

Obs	Organisms	Fit	SE Fit	Residual	St Resid
1	5.00000	3.91111	0.38546	1.08889	3.80 R
2	5.00000	5.57778	0.38546	-0.57778	-2.02 R
31	2.00000	2.57778	0.38546	-0.57778	-2.02 R

R denotes an observation with a large standardized residual.

*If p >0.05 accept Ho
*If p < 0.05 accept Ha

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
-Accept Ha

Ho There is no difference in mortality between replicates
Ha There is a difference in mortality between replication
-Accept Ho

Ho There is no effect on response due to time
Ha There is an effect on response due to time
-Accept Ho

Ho There is no effect due to interaction between time and concentration
Ha There is an effect due to interaction between time and concentration
-Accept Ho

Table 6 *Asellus aquaticus:* Two Way ANOVA for Water Quality Parameters V time and concentration (conc)

Two-way ANOVA: Temperature versus time, concentration

Source	DF	SS	MS	F	P
Time	2	0.018815	0.0094074	0.21	0.815
Conc	4	0.245185	0.0612963	1.37	0.325
Error	8	0.357481	0.0446852		
Total	14	0.621481			

S = 0.2114 R-Sq = 42.48% R-Sq(adj) = 0.00%

Two-way ANOVA: DO versus time, concentration

Source	DF	SS	MS	F	P
Time	2	8.7410	4.37052	8.68	0.010
Conc	4	11.1621	2.79053	5.54	0.019
Error	8	4.0282	0.50353		
Total	14	23.9314			

S = 0.7096 R-Sq = 83.17% R-Sq(adj) = 70.54%

Two-way ANOVA: pH versus time, concentration

Source	DF	SS	MS	F	P
Time	2	0.333019	0.166510	11.20	0.005
Conc	4	0.214585	0.053646	3.61	0.058
Error	8	0.118966	0.014871		
Total	14	0.666570			

S = 0.1219 R-Sq = 82.15% R-Sq(adj) = 68.77%

Two-way ANOVA: NTU versus time, concentration

Source	DF	SS	MS	F	P
Time	2	2881.4	1440.72	11.18	0.005
Conc	4	15338.4	3834.60	29.77	0.000
Error	8	1030.6	128.83		
Total	14	19250.4			

S = 11.35 R-Sq = 94.65% R-Sq(adj) = 90.63%

Two-way ANOVA: NH_3 versus time, concentration

Source	DF	SS	MS	F	P
Time	2	4.35733	2.17867	9.53	0.008
Conc	4	3.24267	0.81067	3.55	0.060
Error	8	1.82933	0.22867		
Total	14	9.42933			

S = 0.4782 R-Sq = 80.60% R-Sq(adj) = 66.05%

*If p >0.05 accept Ho
*If p < 0.05 accept Ha

Ho There is no effect on temp due to concentration
Ha There is an effect on temp due to concentration
-Accept Ho

Ho There is no effect on temp due to time
Ha There is an effect on temp due to time
-Accept Ho

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
-Accept Ha

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
-Accept Ha

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
-Accept Ho

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
-Accept Ha
Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
-Accept Ha

Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
-Accept Ha

Ho There is no effect on NH_3 due to concentration
Ha there is an effect on NH_3 due to concentration
-Accept Ho

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
-Accept Ha

Table 7 *Ephemerella ignita:* General Linear model of species survival V concentration (conc), time and replication.
FDP

Factor	Type	Levels	Values
Time	fixed	6	12, 24, 36, 48, 60, 72
Replication	fixed	3	1, 2, 3
Conc	fixed	5	100mg/l, 200mg/l, 300mg/l, 400mg/l, 500mg/l

Analysis of Variance for Organisms, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	5	178.4556	178.4556	35.6911	51.89	0.000
Replication	2	9.7556	9.7556	4.8778	7.09	0.002
Conc	4	143.3778	143.3778	35.8444	52.12	0.000
Time* Conc	20	63.1556	63.1556	3.1578	4.59	0.000
Error	40	27.5111	27.5111	0.6878		
Total	89	444.3222				

S = 0.829324 R-Sq = 93.81% R-Sq(adj) = 86.22%

Unusual Observations for Organisms

Obs	Organisms	Fit	SE Fit	Residual	St Resid
26	5.00000	3.83333	0.61814	1.16667	2.11 R
28	5.00000	3.83333	0.61814	1.16667	2.11 R
29	0.00000	2.16667	0.61814	-2.16667	-3.92 R
37	4.00000	2.85556	0.61814	1.14444	2.07 R
77	1.00000	2.64444	0.61814	-1.64444	-2.97 R

R denotes an observation with a large standardized residual.

*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
-Accept Ha

Ho There is no difference in mortality between replicates
Ha There is a difference in mortality between replication
-Accept Ha

Ho There is no effect on response due to time
Ha There is an effect on response due to time
-Accept Ha

Ho There is no effect due to interaction between time and concentration
Ha There is an effect due to interaction between time and concentration
-Accept Ha

Table 8 *Ephemerella ignita:* Two way Anova for water quality parameters V time and concentration (conc)

FDP

Two-way ANOVA: DO versus time, concentration

Source	DF	SS	MS	F	P
Time	5	21.6991	4.33982	3.87	0.013
Conc	4	26.1839	6.54597	5.84	0.003
Error	20	22.4303	1.12151		
Total	29	70.3133			

S = 1.059 R-Sq = 68.10% R-Sq(adj) = 53.74%

Two-way ANOVA: pH versus time, concentration

Source	DF	SS	MS	F	P
Time	5	1.07723	0.215445	4.25	0.009
Conc	4	0.72035	0.180088	3.55	0.024
Error	20	1.01481	0.050740		
Total	29	2.81239			

S = 0.2253 R-Sq = 63.92% R-Sq(adj) = 47.68%

Two-way ANOVA: Temp versus time, concentration

Source	DF	SS	MS	F	P
Time	5	12.7977	2.55953	76.33	0.000
Conc	4	0.2333	0.05833	1.74	0.181
Error	20	0.6707	0.03353		
Total	29	13.7017			

S = 0.1831 R-Sq = 95.11% R-Sq(adj) = 92.90%

Two-way ANOVA: NTU versus time, concentration

Source	DF	SS	MS	F	P
Time	5	3510.5	702.1	3.13	0.030
Conc	4	59466.0	14866.5	66.24	0.000
Error	20	4488.4	224.4		
Total	29	67464.9			

S = 14.98 R-Sq = 93.35% R-Sq(adj) = 90.35%

Two-way ANOVA: NH_3 versus time, concentration

Source	DF	SS	MS	F	P
Time	5	165.389	33.0778	16.19	0.000
Conc	4	29.827	7.4567	3.65	0.022
Error	20	40.856	2.0428		
Total	29	236.072			

S = 1.429 R-Sq = 82.69% R-Sq(adj) = 74.91%

*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on temperature due to concentration
Ha There is an effect on temperature due to concentration
-Accept Ho

Ho There is no effect on temperature due to time
Ha There is an effect on temperature due to time
-Accept Ha

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
-Accept Ha

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
-Accept Ha

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
-Accept Ha

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
-Accept Ha

Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
-Accept Ha

Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
-Accept Ha

Ho There is no effect on NH_3 due to concentration
Ha There is an effect on NH_3 due to concentration
-Accept Ha

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
-Accept Ha

Table 9 *Ephemerella ignita:* General Linear model of species survival V concentration (conc), time and replication.

SDP

Factor	Type	Levels	Values
Conc	fixed	5	100mg/l, 200mg/l, 300mg/l, 400mg/l, 500mg/l
Replication	fixed	3	1, 2, 3
Time	fixed	3	24, 48, 72

Analysis of Variance for Organisms, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Conc	4	50.3111	50.3111	12.5778	87.08	0.000
Replication	2	0.5333	0.5333	0.2667	1.85	0.190
Time	2	16.5333	16.5333	8.2667	57.23	0.000
Conc*Time	8	25.0222	25.0222	3.1278	21.65	0.000
Error	16	2.3111	2.3111	0.1444		
Total	44	97.2000				

S = 0.380058 R-Sq = 97.62% R-Sq(adj) = 93.46%

Unusual Observations for Organisms

Obs	Organisms	Fit	SE Fit	Residual	St Resid
12	5.00000	5.48889	0.30510	-0.48889	-2.16 R
35	4.00000	4.51111	0.30510	-0.51111	-2.26 R
40	3.00000	3.48889	0.30510	-0.48889	-2.16 R

R denotes an observation with a large standardized residual.

*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
-Accept Ha

Ho There is no difference in mortality between replicates
Ha There is a difference in mortality between replication
-Accept Ho

Ho There is no effect on response due to time
Ha There is an effect on response due to time
-Accept Ha

Ho There is no effect due to interaction between time and concentration
Ha There is an effect due to interaction between time and concentration
-Accept Ha

Table 10 *Ephemerella ignita:* Two way Anova for water quality parameters V time and concentration

SDP
Two-way ANOVA: Temp versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	5.72933	2.86467	99.35	0.000
Concentration	4	0.35733	0.08933	3.10	0.081
Error	8	0.23067	0.02883		
Total	14	6.31733			

S = 0.1698 R-Sq = 96.35% R-Sq(adj) = 93.61%

Two-way ANOVA: DO versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	3.4839	1.74193	1.83	0.222
Concentration	4	8.3058	2.07646	2.18	0.162
Error	8	7.6266	0.95333		
Total	14	19.4163			

S = 0.9764 R-Sq = 60.72% R-Sq(adj) = 31.26%

Two-way ANOVA: pH versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	0.021960	0.0109800	0.30	0.747
Concentration	4	0.275093	0.0687733	1.89	0.205
Error	8	0.290507	0.0363133		
Total	14	0.587560			

S = 0.1906 R-Sq = 50.56% R-Sq(adj) = 13.47%

Two-way ANOVA: NTU versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	4081.2	2040.61	13.25	0.003
Concentration	4	27992.5	6998.13	45.45	0.000
Error	8	1231.7	153.96		
Total	4	33305.4			

S = 12.41 R-Sq = 96.30% R-Sq(adj) = 93.53%

Two-way ANOVA: NH_3 versus Time, Concentration

Source	DF	SS	MS	F	P
Time 2	2	0.0035177	0.0017589	0.32	0.738
Concentration	4	0.0087243	0.0021811	0.39	0.810
Error	8	0.0446617	0.0055827		
Total	14	0.0569038			

S = 0.07472 R-Sq = 21.51% R-Sq(adj) = 0.00%

*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on temperature due to concentration
Ha There is an effect on temperature due to concentration
-Accept Ho

Ho There is no effect on temperature due to time
Ha There is an effect on temperature due to time
-Accept Ha

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
-Accept Ho

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
-Accept Ho

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
-Accept Ho

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
-Accept Ho
Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
-Accept Ha

Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
-Accept Ha

Ho There is no effect on NH_3 due to concentration
Ha There is an effect on NH_3 due to concentration
-Accept Ho

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
-Accept Ho

Table 11 *Mytilus edulis:* General Linear model of species survival V concentration (conc), time and replication.

Factor	Type	Levels	Values
Time	fixed	6	12, 24, 36, 48, 60, 72
Replication	fixed	3	1, 2, 3
Conc	fixed	5	200mg/l, 300mg/l, 400mg/l, 500mg/l, 600mg/l

Analysis of Variance for Organisms, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	5	5.20000	5.20000	1.04000	18.72	0.000
Replication	2	0.06667	0.06667	0.03333	0.60	0.554
Conc	4	2.28889	2.28889	0.57222	10.30	0.000
Time*Conc	20	5.57778	5.57778	0.27889	5.02	0.000
Error	40	2.22222	2.22222	0.05556		
Total	89	16.40000				

S = 0.235702 R-Sq = 86.45% R-Sq(adj) = 69.85%

Unusual Observations for Organisms

Obs	Organisms	Fit	SE Fit	Residual	St Resid	
58	7.00000	7.34444	0.17568	-0.34444	-2.19	R
73	7.00000	7.34444	0.17568	-0.34444	-2.19	R
78	7.00000	7.42222	0.17568	-0.42222	-2.69	R
82	7.00000	7.65556	0.17568	-0.65556	-4.17	R
84	8.00000	7.58889	0.17568	0.41111	2.62	R
85	8.00000	7.54444	0.17568	0.45556	2.90	R

R denotes an observation with a large standardized residual.

*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
Accept Ha

Ho There is no difference in mortality between replicates
Ha There is a difference in mortality between replication
Accept Ho

Ho There is no effect on response due to time
Ha There is an effect on response due to time
Accept Ha

Ho There is no effect due to interaction between time and concentration
Ha There is an effect due to interaction between time and concentration
Accept Ha

Table 12 *Mytilus edulis:* Two Way ANOVA for Water Quality Parameters V time and concentration

Two-way ANOVA: Temp versus time, concentration

Source	DF	SS	MS	F	P
time	5	10.6507	2.13013	57.62	0.000
Concentration	4	0.3367	0.08417	2.28	0.097
Error	20	0.7393	0.03697		
Total	29	11.7267			

S = 0.1923 R-Sq = 93.70% R-Sq(adj) = 90.86%

Two-way ANOVA: DO versus time, concentration

Source	DF	SS	MS	F	P
time	5	2.5128	0.50255	1.65	0.193
Concentration	4	4.9607	1.24019	4.07	0.014
Error	20	6.0998	0.30499		
Total	29	13.5733			

S = 0.5523 R-Sq = 55.06% R-Sq(adj) = 34.84%

Two-way ANOVA: pH versus time, concentration

Source	DF	SS	MS	F	P
Time	5	0.30571	0.061142	3.07	0.033
Concentration	4	0.17238	0.043095	2.16	0.111
Error	20	0.39874	0.019937		
Total	29	0.87683			

S = 0.1412 R-Sq = 54.52% R-Sq(adj) = 34.06%

Two-way ANOVA: NTU versus time, concentration

Source	DF	SS	MS	F	P
Time	5	154170	30833.9	1.02	0.435
Concentration	4	218290	54572.6	1.80	0.169
Error	20	607249	30362.4		
Total	29	979709			

S = 174.2 R-Sq = 38.02% R-Sq(adj) = 10.13%

Two-way ANOVA: NH_3 versus time, concentration

Source	DF	SS	MS	F	P
Time	5	369.112	73.8223	15.97	0.000
Concentration	4	56.930	14.2325	3.08	0.040
Error	20	92.472	4.6236		
Total	29	518.514			

S = 2.150 R-Sq = 82.17% R-Sq(adj) = 74.14

*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on temperature due to concentration
Ha There is an effect on temperature due to concentration
Accept Ho

Ho There is no effect on temperature due to time
Ha There is an effect on temperature due to time
Accept Ha

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
Accept Ha

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
Accept Ho

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
Accept Ho

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
Accept Ha

Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
Accept Ho

Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
Accept Ho

Ho There is no effect on NH_3 due to concentration
Ha There is an effect on NH_3 due to concentration
Accept Ha

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
Accept Ha

Table 13 *Anodonta:* Two Way ANOVA for Water Quality Parameters V time and concentration (conc)

Two-way ANOVA: Temp versus time, concentration

Source	DF	SS	MS	F	P
Time	5	11.2457	2.24913	153.70	0.000
Concentration	4	0.1553	0.03883	2.65	0.063
Error	20	0.2927	0.01463		
Total	29	11.6937			

S = 0.1210 R-Sq = 97.50% R-Sq(adj) = 96.37%

Two-way ANOVA: DO versus time, concentration

Source	DF	SS	MS	F	P
Time	5	3.12038	0.624075	9.88	0.000
Concentration	4	1.22695	0.306738	4.86	0.007
Error	20	1.26281	0.063140		
Total	29	5.61014			

S = 0.2513 R-Sq = 77.49% R-Sq(adj) = 67.36%

Two-way ANOVA: pH versus time, concentration

Source	DF	SS	MS	F	P
Time	5	0.071947	0.0143893	0.57	0.723
Concentration	4	0.072380	0.0180950	0.71	0.592
Error	20	0.506220	0.0253110		
Total	29	0.650547			

S = 0.1591 R-Sq = 22.19% R-Sq(adj) = 0.00%

Two-way ANOVA: NTU versus time, concentration

Source	DF	SS	MS	F	P
Time	5	1000.7	200.14	2.37	0.076
Concentration	4	35647.5	8911.88	105.62	0.000
Error	20	1687.5	84.37		
Total	29	38335.7			

S = 9.186 R-Sq = 95.60% R-Sq(adj) = 93.62%

Two-way ANOVA: NH_3 versus time, concentration

Source	DF	SS	MS	F	P
Time	5	69.252	13.8504	9.72	0.000
Concentration	4	40.921	10.2304	7.18	0.001
Error	20	28.487	1.4244		
Total	29	138.661			

S = 1.193 R-Sq = 79.46% R-Sq(adj) = 70.21%

If p >0.05 accept ho
If p < 0.05 accept Ha

Ho There is no effect on temperature due to concentration
Ha There is an effect on temperature due to concentration
Accept Ho

Ho There is no effect on temperature due to time
Ha There is an effect on temperature due to time
Accept Ha

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
Accept Ha

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
Accept Ha

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
Accept Ho

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
Accept Ho

Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
Accept Ha
Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
Accept Ho

Ho There is no effect on NH_3 due to concentration
Ha There is an effect on NH_3 due to concentration
Accept Ha

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
Accept Ha

Table 14 *Daphnia pulex:* General Linear model of species survival V concentration, time and replication

Factor	Type	Levels	Values
Concentration	fixed	5	100mg/l, 150mg/l, 200mg/l, 250mg/l, 50mg/l
Replication	fixed	3	1, 2, 3
Time	fixed	3	24, 48, 72

Analysis of Variance for Organisms, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Concentration	4	48.4444	48.4444	12.1111	34.33	0.000
Replication	2	1.3778	1.3778	0.6889	1.95	0.174
Time	2	10.9778	10.9778	5.4889	15.56	0.000
Concentration*Time	8	6.3556	6.3556	0.7944	2.25	0.079
Error	16	5.6444	5.6444	0.3528		
Total	44	102.4444				

S = 0.593951 R-Sq = 94.49% R-Sq(adj) = 84.85%

Unusual Observations for Organisms

Obs	Organisms 1	Fit	SE Fit	Residual	St Resid
31	5.00000	4.24444	0.47681	0.75556	2.13 R
34	2.00000	2.91111	0.47681	-0.91111	-2.57 R

R denotes an observation with a large standardized residual.

*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
-Accept Ha

Ho There is no difference in mortality between replicates
Ha There is a difference in mortality between replication
-Accept Ho

Ho There is no effect on response due to time
Ha There is an effect on response due to time
-Accept Ha

Ho There is no effect due to interaction between time and concentration
Ha There is an effect due to interaction between time and concentration
-Accept Ho

Table 15 *Daphnia pulex:* Two Way ANOVA for Water Quality Parameters V time and concentration

Two-way ANOVA: Temp versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	0.796	0.398	8.29	0.011
Concentration	4	0.364	0.091	1.90	0.205
Error	8	0.384	0.048		
Total	14	1.544			

S = 0.2191 R-Sq = 75.13% R-Sq(adj) = 56.48%

Two-way ANOVA: DO versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	6.9800	3.48998	2.02	0.194
Concentration	4	10.4286	2.60714	1.51	0.286
Error	8	13.7977	1.72471		
Total	14	31.2062			

S = 1.313 R-Sq = 55.79% R-Sq(adj) = 22.62%

Two-way ANOVA: pH versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	0.36001	0.180007	2.73	0.125
Concentration	4	1.06313	0.265783	4.03	0.044
Error	8	0.52759	0.065948		
Total	14	1.95073			

S = 0.2568 R-Sq = 72.95% R-Sq(adj) = 52.67%

Two-way ANOVA: NTU versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	15703	7851.3	0.45	0.650
Concentration	4	206573	51643.3	2.99	0.088
Error	8	138237	17279.7		
Total	14	360513			

S = 131.5 R-Sq = 61.66% R-Sq(adj) = 32.90%

Two-way ANOVA: NH_3 versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	0.0165229	0.0082614	5.69	0.029
Concentration	4	0.0093321	0.0023330	1.61	0.263
Error	8	0.0116253	0.0014532		
Total	14	0.0374803			

S = 0.03812 R-Sq = 68.98% R-Sq(adj) = 45.72%

*If p >0.05 accept ho
*If p < 0.05 accept Ha

Ho There is no effect on temperature due to concentration
Ha There is an effect on temperature due to concentration
-Accept Ho

Ho There is no effect on temperature due to time
Ha There is an effect on temperature due to time
-Accept Ha

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
-Accept Ho

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
-Accept Ho

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
-Accept Ha

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
-Accept Ho

Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
-Accept Ho

Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
-Accept Ho

Ho There is no effect on NH_3 due to concentration
Ha There is an effect on NH_3 due to concentration
-Accept Ho

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
-Accept Ha

Table 16 *Austropotamobius pallipes:* General Linear model of species survival V concentration, time and replication

Factor	Type	Levels	Values
Time 1_1	fixed	3	24, 48, 72
Replication	fixed	3	1, 2, 3
concentration 1	fixed	5	350mg/l, 450mg/l, 550mg/l, 650mg/l, 750mg/l

Analysis of Variance for Organisms 1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time 1_1	2	0.17778	0.17778	0.08889	1.00	0.381
Replication	2	0.17778	0.17778	0.08889	1.00	0.381
concentration 1	4	0.53333	0.53333	0.13333	1.50	0.229
Time 1_1*concentration	8	0.26667	0.26667	0.03333	0.37	0.925
Error	28	2.48889	2.48889	0.08889		
Total	44	3.64444				

S = 0.298142 R-Sq = 31.71% R-Sq(adj) = 0.00%

Unusual Observations for Organisms 1

Obs	Organisms 1	Fit	SE Fit	Residual	St Resid
18	1.00000	1.62222	0.18325	-0.62222	-2.65 R
19	1.00000	1.62222	0.18325	-0.62222	-2.65 R
33	1.00000	1.62222	0.18325	-0.62222	-2.65 R
34	1.00000	1.62222	0.18325	-0.62222	-2.65 R

R denotes an observation with a large standardized residual.

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
-Accept Ha

Ho There is no difference in mortality between replicates
Ha There is a difference in mortality between replication
-Accept Ho

Ho There is no effect on response due to time
Ha There is an effect on response due to time
-Accept Ha

Ho There is no effect due to interaction between time and concentration
Ha There is an effect due to interaction between time and concentration
-Accept Ho

Table 17 *Austropotamobius pallipes:* Two Way ANOVA for Water Quality Parameters V time and concentration

Two-way ANOVA: Temp versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	10.5960	5.29800	248.34	0.000
Concentration	4	0.2373	0.05933	2.78	0.102
Error	8	0.1707	0.02133		
Total	14	11.0040			

S = 0.1461 R-Sq = 98.45% R-Sq(adj) = 97.29%

Two-way ANOVA: DO versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	9.9845	4.99226	2.65	0.131
Concentration	4	7.4474	1.86186	0.99	0.466
Error	8	15.0672	1.88340		
Total	14	32.4992			

S = 1.372 R-Sq = 53.64% R-Sq(adj) = 18.87%

Two-way ANOVA: pH versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	0.115773	0.0578867	1.87	0.216
Concentration	4	0.053533	0.0133833	0.43	0.783
Error	8	0.248227	0.0310283		
Total	14	0.417533			

S = 0.1761 R-Sq = 40.55% R-Sq(adj) = 0.00%

Two-way ANOVA: NTU versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	351.6	175.78	0.10	0.909
Concentration	4	4042.6	1010.66	0.56	0.701
Error	8	14555.5	1819.44		
Total	14	18949.7			

S = 42.65 R-Sq = 23.19% R-Sq(adj) = 0.00%

Two-way ANOVA: NH_3 versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	14.7277	7.36384	55.84	0.000
Concentration	4	0.7676	0.19190	1.46	0.301
Error	8	1.0551	0.13189		
Total	14	16.5504			

S = 0.3632 R-Sq = 93.63% R-Sq(adj) = 88.84%

If p >0.05 accept ho
If p < 0.05 accept Ha

Ho There is no effect on temperature due to concentration
Ha There is an effect on temperature due to concentration
-Accept Ho

Ho There is no effect on temperature due to time
Ha There is an effect on temperature due to time
-Accept Ha

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
-Accept Ho

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
-Accept Ho

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
-Accept Ho

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
-Accept Ho

Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
-Accept Ho

Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
-Accept Ho

Ho There is no effect on NH_3 due to concentration
Ha There is an effect on NH_3 due to concentration
-Accept Ho

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
-Accept Ha

Table 18 *Lymnaea peregra:* General Linear model of species survival V concentration, time and replication

Factor	Type	Levels	Values
Concentration	fixed	5	500mg/l, 600mg/l, 700mg/l, 800mg/l, 900mg/l
Time	fixed	3	24, 48, 72
Replication	fixed	3	1, 2, 3

Analysis of Variance for Organisms 1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Concentration	4	0.53333	0.53333	0.13333	2.67	0.071
Time	2	0.31111	0.31111	0.15556	3.11	0.072
Replication	2	0.04444	0.04444	0.02222	0.44	0.649
Concentration*Time	8	0.80000	0.80000	0.10000	2.00	0.113
Error	16	0.80000	0.80000	0.05000		
Total	44	3.64444				

S = 0.223607 R-Sq = 78.05% R-Sq(adj) = 39.63%

Unusual Observations for Organisms

Obs	Organisms	Fit	SE Fit	Residual	St Resid
10	5.00000	4.60000	0.17951	0.40000	3.00 R
13	5.00000	5.26667	0.17951	-0.26667	-2.00 R
40	4.00000	4.26667	0.17951	-0.26667	-2.00 R
43	5.00000	4.60000	0.17951	0.40000	3.00 R

R denotes an observation with a large standardized residual.

If p >0.05 accept ho
If p < 0.05 accept Ha

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
Accept Ho

Ho There is no difference in mortality between replicates
Ha There is a difference in mortality between replication
Accept Ho

Ho There is no effect on response due to time
Ha There is an effect on response due to time
Accept Ho

Ho There is no effect due to interaction between time and concentration
Ha There is an effect due to interaction between time and concentration
Accept Ho

Table 19 *Lymnaea peregra* : Two Way ANOVA for Water Quality Parameters V time and concentration

Two-way ANOVA: Temp versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	61.3720	30.6860	399.38	0.000
Concentration	4	0.1373	0.0343	0.45	0.772
Error	8	0.6147	0.0768		
Total	14	62.1240			

S = 0.2772 R-Sq = 99.01% R-Sq(adj) = 98.27%

Two-way ANOVA: DO versus Time , Concentration

Source	DF	SS	MS	F	P
Time	2	2.19072	1.09536	3.67	0.074
Concentration	4	4.09364	1.02341	3.43	0.065
Error	8	2.38728	0.29841		
Total	14	8.67164			

S = 0.5463 R-Sq = 72.47% R-Sq(adj) = 51.82%

Two-way ANOVA: pH versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	0.011160	0.0055800	0.11	0.899
Concentration	4	0.250173	0.0625433	1.21	0.380
Error	8	0.415107	0.0518883		
Total	14	0.676440			

S = 0.2278 R-Sq = 38.63% R-Sq(adj) = 0.00%

Two-way ANOVA: NTU versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	4490.6	2245.29	5.61	0.030
Concentration	4	16737.3	4184.33	10.45	0.003
Error	8	3202.2	400.28		
Total	14	24430.1			

S = 20.01 R-Sq = 86.89% R-Sq(adj) = 77.06%

Two-way ANOVA: NH_3 versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	0.0041037	0.0020519	12.27	0.004
Concentration	4	0.0006714	0.0001678	1.00	0.459
Error	8	0.0013383	0.0001673		
Total	14	0.0061134			

S = 0.01293 R-Sq = 78.11% R-Sq(adj) = 61.69%

If p >0.05 accept ho
If p < 0.05 accept Ha

Ho There is no effect on temp due to concentration
Ha There is an effect on temp due to concentration
Accept Ho

Ho There is no effect on temp due to time
Ha There is an effect on temp due to time
Accept Ha

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
Accept Ho

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
Accept Ho

Ho There is no effect on pH due to concentration
Ha There is an effect on pH due to concentration
Accept Ho

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
Accept Ho

Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
Accept Ha
Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
Accept Ha

Ho There is no effect on NH_3 due to concentration
Ha There is an effect on NH_3 due to concentration
Accept Ho

Ho There is no effect on NH_3 due to time
Ha There is an effect on NH_3 due to time
Accept Ha

Table 20 *Salmo trutta:* Two way Anova of species survival V concentration and time

Two-way ANOVA: Organisms versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	12.133	6.0667	6.17	0.024
Concentration	4	164.933	41.2333	41.93	0.000
Error	8	7.867	0.9833		
Total	14	184.933			

S = 0.9916 R-Sq = 95.75% R-Sq(adj) = 92.56%

Ho There is no effect on response due to concentration
Ha There is an effect on response due to concentration
Accept Ha

Ho There is no effect on response due to time
Ha There is an effect on response due to time
Accept Ha

Table 21 *Salmo trutta* Two Way ANOVA for Water Quality Parameters V time and concentration

Two-way ANOVA: Temp versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	0.037333	0.0186667	1.56	0.269
Concentration	4	0.340000	0.0850000	7.08	0.010
Error	8	0.096000	0.0120000		
Total	14	0.473333			

S = 0.1095 R-Sq = 79.72% R-Sq(adj) = 64.51%

Two-way ANOVA: DO versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	3.98533	1.99267	11.60	0.004
Concentration	4	0.33333	0.08333	0.48	0.747
Error	8	1.37467	0.17183		
Total	14	5.69333			

S = 0.4145 R-Sq = 75.85% R-Sq(adj) = 57.75%

Two-way ANOVA: pH versus Time, Concentration

Source	DF	SS	MS	F	P
Time	2	0.137333	0.0686667	6.15	0.024
Concentration	4	0.066667	0.0166667	1.49	0.291
Error	8	0.089333	0.0111667		
Total	14	0.293333			

S = 0.1057 R-Sq = 69.55% R-Sq(adj) = 46.70%

Two-way ANOVA: NTU V Time, Concentration

Source	DF	SS	MS	F	P
Time	2	452.80	226.400	6.97	0.018
Concentration	4	3593.73	898.433	27.66	0.000
Error	8	259.87	32.483		
Total	14	4306.40			

S = 5.699 R-Sq = 93.97% R-Sq(adj) = 89.44%

If p >0.05 accept ho
If p < 0.05 accept Ha

Ho There is no effect on temp due to concentration
Ha There is an effect on temp due to concentration
Accept Ha

Ho There is no effect on temp due to time
Ha There is an effect on temp due to time
Accept Ho

Ho There is no effect on DO due to concentration
Ha There is an effect on DO due to concentration
Accept Ho

Ho There is no effect on DO due to time
Ha There is an effect on DO due to time
Accept Ha
Ho There is no effect on pH due to concentration

Ha There is an effect on pH due to concentration
Accept Ho

Ho There is no effect on pH due to time
Ha There is an effect on pH due to time
Accept Ha

Ho There is no effect on NTU due to concentration
Ha There is an effect on NTU due to concentration
Accept Ha

Ho There is no effect on NTU due to time
Ha There is an effect on NTU due to time
Accept Ha

Appendix E: 'Comparing a microbial biocide and chlorine as zebra mussel control strategies in an Irish drinking water treatment plant'. This paper is presented as it appears in the 'Management of Biological Invasions', 2014, Volume 4, Issue 2, Pages 113-122.

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Research Article

Comparing a microbial biocide and chlorine as zebra mussel control strategies in an Irish drinking water treatment plant

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Abstract

A need exists for an environmentally friendly mussel control method to replace chlorine and other traditional control methods currently utilised in drinking water plants and other infested facilities. Zequanox® is a newly commercialised microbial biocide for zebra and quagga mussels comprised of killed *Pseudomonas fluorescens* CL145A cells. The objective of this study was to compare the efficacy of a developmental formulation of Zequanox (referred to as MBI 401 FDP) and chlorine treatments on adult and juvenile zebra mussels by running a biobox trial in conjunction with chlorine treatments at an infested Irish drinking water treatment plant. Since 2009, the plant management has used a residual chlorine concentration of 2 mg/L in autumn to control both adult zebra mussels and juvenile settlement in their three concrete raw water chambers. Juvenile mussel settlement was monitored in three bioboxes as well as in three treatment chambers in the plant for three months prior to treatment. Adult mussels were seeded into the chambers and bioboxes four days before treatment. In October 2011, the bioboxes were treated with MBI 401 FDP at 200 mg active substance/L, while chlorine treatment took place in the water chambers. The MBI 401 FDP treatment lasted only 8 hours while chlorine treatment lasted seven days. Juvenile numbers were reduced to zero in both the bioboxes and treated chambers within seven days. Adult mussel mortality reached 80% for both the chlorine and MBI 401 FDP treatment; however, mortality was achieved faster in the chlorine treatment. These results provided important insights into zebra mussel control alternatives to chlorine and supported further development of the now commercial product, Zequanox.

Key words: invasive mussel control; juveniles; adults; water quality

Introduction

The zebra mussel *Dreissena polymorpha* (Pallas, 1771), is an invasive, exotic aquatic bivalve, which has greatly affected lakes, canals and other aquatic ecosystems in Ireland (Minchin et al. 2002; Lucy 2010; Lucy et al. in press) since first invading in the early 1990's (Minchin and Moriarty 1998). The control methods currently used in Ireland, Europe and North America are necessary in industries requiring water abstraction, for example in drinking water treatment plants and land-based fish hatcheries where juvenile zebra mussels settle in water pipe networks and ancillary plants, developing into fully grown zebra mussels (Mackie and Claudi 2010). In such cases, either physical removal and/or chlorine dosed at approximately 2 mg/L is frequently used to control the mussels (Mackie and Claudi 2010) as is the case in the drinking water treatment plant in Sligo, used in this study. At 2 mg/L chlorine treatments can take up to 21 days to be effective (Mackie and Claudi 2010). At the Sligo drinking water treatment plant, flow through raw water chambers receiving chlorine treatment are bypassed for the chlorination period and the treated water is released back to the discharged water body. Trihalomethanes can be formed in drinking water as a result of the chlorination of organic matter in the raw water supplies (Coffin et al. 2000) and according to Wright et al (2007) THM formation is enhanced when dead mussels are present. The use of chlorine also presents

more risks to the user; oversaturation of the air can cause the mucous membrane to become irritated and severe coughing can occur (West Virginia Department of Health and Human Resources 2010). With drinking water plants in particular, high chlorine concentrations in the water may impact the taste and odour (Roche and Benanou 2007) . In the USA, chlorine discharge limits permissible in receiving water should not exceed 19 µg/L more than once every three years on average under the acute toxicity criterion. Under the chronic toxicity criterion, the 4 day average concentration should not exceed 11 µg/L more than once every three years on average (Tikkanen et al 2001).

Marrone Bio Innovations (MBI), a company specialising in the development and commercealisation of natural biocides in Davis, CA, USA, is the commercial license holder of *Pseudomonas fluorescens* strain CL145A; a microbe used to control invasive zebra and quagga (dreissenid) mussels. In 2012, MBI registered and commercialised Zequanox, a spray dried powder comprised of killed *Pseudomonas fluorescens* CL145A cells, in the United States and Canada. *Pseudomonas fluorescens* CL145A cells have been shown to be lethal to dreissenid mussels (Molloy et al 2013a), but pose minimal to no risk to other aquatic organisms (Molloy et al 2013b). This bacterial species is present worldwide and commonly found in food. In nature, it is a harmless bacterial species that is known to protect the roots of plants from disease (Marrone Bio Innovations 2012). It has been shown that killed *Pseudomonas fluorescens* CL145A cells have no negative impacts to aquatic organisms in Irish waters at treatment concentrations required to achieve >80% zebra mussel mortality (ecotoxicology trials Sara Meehan unpublished).

The main objective of this study was to demonstrate the efficacy of MBI 401 FDP (a developmental formulation of Zequanox) at controlling zebra mussels in Ireland. This was done in a biobox trial at a drinking water treatment plant by comparing juvenile settlement pre and post treatment with MBI 401 FDP as well as adult mussel survival after treatment. In addition, these results were compared to juvenile settlement and adult mussel survival after chlorine treatment in the plant's raw water chambers. Water quality, before, during and after treatment with MBI 401 FDP, was also monitored to determine the impact from treatment to source water quality and to the environment.

Sligo drinking water treatment plant, Ireland

This research study was carried out at a drinking water treatment plant, located on the perimeter of Sligo city in the north-west of Ireland (54°25'07"N, 08°45'22"W). This plant extracts between 6000 to 7500 m^3 of raw water per day for treatment from a nearby lake, Lough Gill (14.3 km^2). The raw water chambers in the plant house are infested with zebra mussels (Figure 1). During summer reproduction, the free floating zebra mussel larvae (veligers) are able to pass through the first stage of mesh filtration at the lake abstraction point. The veligers are then pumped 1 km with the influent water, via the intake pipe, and then enter the water chambers in the treatment plant where they settle on the walls and begin to grow. Lough Gill has been infested with zebra mussels since approximately 2004 and high densities were present in the raw water chambers by 2009.

Sligo drinking water treatment plant began using chlorine to treat the zebra mussel infestation in the raw water chambers in 2009 and have been treating once a year, in autumn following the reproductive season. During treatment, the plant is forced to shut down the chambers being treated; this process delays operations for the duration of the treatment (typically seven days) as well as the additional time for the set up and break down of the treatment.

Materials and methods

Biobox and chamber set up

Bioboxes are used to monitor mussel settlement in power plants or other similar facilities by mimicking the flow in industrial piping and demonstrating the resulting zebra mussel settlement in piping and water chambers (Mackie and Claudi 2010). The biobox is connected to the main inflow of raw water to the plant.

Three 200 L bioboxes were placed on a flow through system in the Sligo drinking water treatment plant on the 13th of July 2011 (Figure 2). These tanks received water from the water treatment plant's main chambers via gravity flow, with a total flow of 287,000 L over 13 weeks until the 11th of October 2011. Of these three tanks, one was established to serve as the experimental control (tank 1) and the other two (tanks 2 and 3) were to receive MBI 401 FDP treatments. The tanks were covered with heavy plastic with weights on each side to protect from any harsh weather exposure or interference.

Figure 1. Zebra mussel infestation in raw water chambers at Sligo drinking water plant (photograph by Eamon Fox).

Figure 2. Bioboxes outside of Sligo drinking water treatment plant (photograph by Sara Meehan).

Three PVC plates (15 cm × 15 cm) were placed in each of the three tanks to allow for natural zebra mussel settlement (Marsden 1992; Lucy 2006). These plates were suspended in the tanks from a metal rod inserted lengthways across the top of the tank. Every week, either the middle or bottom plate was removed (in rotation) and replaced by a new plate so biweekly juvenile settlement rates could be estimated (Marsden 1992; Lucy 2006). The top plate was maintained throughout in order to monitor seasonal settlement. Water temperature, dissolved oxygen, and pH were recorded every week in each tank using a handheld Orion 5-star meter.

Three PVC plates (15 cm × 15 cm) were also placed in each of the plant's three raw water chambers on the 13th of July 2011. These plates were suspended lengthways from the top of each chamber and were held in place by a rope hung from a ladder (Figure 3). Of these three chambers, one was established to serve as the experimental

Figure 3. Bags with adult mussels and PVC juvenile settlement plates attached to the suspension rope, deployed in the drinking water treatment plant chambers (photograph by Sara Meehan).

control (chamber B) and the other two (chambers A and C) were to receive chlorine treatment. Weekly removal of plates and recording of water quality parameters was the same as for the bioboxes.

Preparation of bioboxes and chambers for MBI 401 FDP and chlorine treatment

In addition to measuring and treating juvenile settlement, adult zebra mussels from a wild population in Lough Conn, Co. Mayo were seeded into each of the bioboxes to test whether treatment is effective on all life stages (Mackie and Claudi 2010). Three mesh bags containing 50 mussels each were suspended in each biobox on the 7th of October 2011; this was 3 days in advance of treatment to allow the mussels to acclimatise (Figure 4).

Prior to treatment on the 10th of October 2011, the bioboxes were moved from the water treatment plant to the research facility at IT Sligo (Figure 5). The bioboxes were then no longer on a flow-through system. Twenty-four hours prior to MBI 401 FDP treatment, the seeded mussels were checked for mortality and any dead mussels were replaced with healthy, live mussels.

Pre-treatment juvenile settlement on the PVC plates was assessed. The middle and bottom plates in the treated tanks (tanks 2 and 3) were removed prior to treatment due to the low numbers of established mussels. The top plate (which was the plate that accumulated settlement over the duration of the settlement season) was left in the bioboxes for treatment. Treatment was carried out after the Irish seasonal reproductive period (Lucy 2006).

One week after treatment of the bioboxes with MBI 401 FDP, the treatment of the raw water chambers at the drinking water treatment plant took place on the 17th of October 2011. The same methods for assessing adult mortality and juvenile settlement were applied here as with the bioboxes - adult mussels were seeded into the chambers and the top plate was assessed for settlement before repositioning in the chambers.

Application in bioboxes

MBI 401 FDP (a dry powder) was a 100% active substance (or active ingredient). The powder was mixed on-site with Lough Gill water to create the following stock solution concentration:

$C_1V_1 = C_2V_2$ where

C_1 = target treatment concentration (mg active substance (a.s.)/L)

Figure 4. Bags with adult mussels used to assess mortality were suspended in the bioboxes and chambers (photograph by Sara Meehan).

Figure 5. Bioboxes set up outside of IT Sligo (photograph by Sara Meehan).

V_1 = volume of bioboxes (200 L)

C_2 = stock concentration (g a.s./L)

V_2 = volume of stock concentration to be injected (ml).

The target concentration was 200 mg active substance (a.s.)/L. These preliminary tests were carried out with the maximum allowable concentration in the U.S. in order to show efficacy and potential impact to water quality.

For each tank treated, 42 g (a.s.) of product was mixed with 0.93 L of water on a stir plate to achieve a stock concentration of 45 g (a.s.)/L. This stock concentration was injected into each

tank at a rate of 50 mL/min for 19 minutes to achieve the target concentration of 200 mg a.s./L. The product was fed to the tanks using a peristaltic pump. A mixer was placed in the chambers to keep the product in suspension for the duration of the treatment.

As MBI 401 FDP is comprised of organic material, it is known that turbidity and MBI 401 FDP concentrations are strongly correlated. To confirm that the target concentration of MBI 401 FDP in each treatment tank was reached and maintained, a site specific linear regression was developed to determine the linear relationship between product concentration and turbidity (Figure 6). This was done according to MBI standard operating procedure, Turbidity and MOI-401 Active Ingredient Correlation and Application Monitoring (MBI personal communication). Turbidity was monitored throughout the application and post-treatment period with a Hach 2100N turbidimeter.

Once the target concentration was reached, the treated water was held for 8 hours. The application time was based on previous trials carried out by MBI at Davis Dam, Lower Colorado River, and Bullhead City, Arizona, USA. After the 8 hour treatment time, the tanks were rinsed three times and replaced with fresh Lough Gill water that was transported to IT Sligo in 1000 L containers. All MBI 401 FDP treated water was discharged to the sewer.

After all rinses were completed, bioboxes were transported back to the drinking water plant and hooked back up to the flow through system. Adult and juvenile mussels were then checked for mortality, initially daily and eventually once a week until juvenile survival reached zero and adult mussel mortality reached a plateau.

Water quality in bioboxes treated with MBI 401 FDP

Water quality samples were taken before treatment, during treatment at 4 and 8 hours, and for each of the three rinses in treated tank 3 and the control tank. Water quality measurements included: temperature, dissolved oxygen (DO), pH, turbidity, biological oxygen demand (BOD), and total organic carbon (TOC). DO, pH and temperature were measured with an Orion 5 star meter. The analysis of BOD and TOC were subcontracted out to Alcontrol Laboratories. BOD was analysed following MEWAM BOD5 2nd Ed.HMSO 1988/ Method 5210B, AWWA/ APHA, 20th Ed., 1999; SCA Blue Book 130 and TOC was determined using US EPA Method 415.1 & 9060.

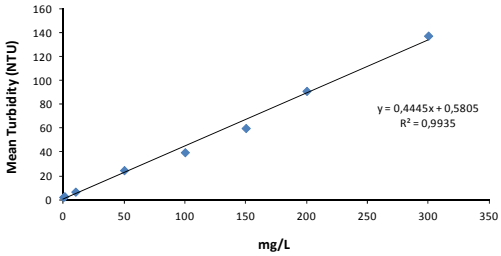

Figure 6. Site specific linear regression of MBI 401 FDP concentration and turbidity.

Application in chambers

On the 17th of October 2011, the raw water chambers were treated with chlorine. The chambers receiving treatment were bypassed meaning that the raw water goes directly to the pre ozone chamber bypassing micro straining. The treatment was carried out by the plant manager where drums of chlorine were slowly poured into the receiving chambers. The chlorine concentration was monitored via a hand held meter to ensure that the concentration of 2 mg/L residual chlorine was maintained in the treated chambers; when the concentration dropped below 2 mg/L more chlorine was added. This treatment was carried out over a total of seven days; adult and juvenile mussels were then checked for mortality, initially daily and eventually once a week until juvenile survival reached zero and adult mussel mortality reached a plateau.

Results and discussion

Several long-standing and accepted chemical treatment methods exist for controlling zebra mussels, including chlorination. Chorine however, carries potential impacts for the surrounding environment and potential hazards to the user during its application, all previously stated. A need exists for a control method that has a quick application time and does not pose risks to the receiving water and the user. The results presented below demonstrate the efficacy of MBI 401 FDP in controlling zebra mussels and compares MBI 401 FDP treatment to chlorine treatment.

MBI 401 FDP treatment - juvenile mussels

Juvenile settlement counted biweekly prior to treatment was relatively low reaching a peak of 5,000 juveniles/m^2 in the control biobox on the 10th of August 2011. As the number of settled juveniles is determined by the number of planktonic larva in the water, which in turn is determined by the water temperature (Lucy 2006; Garton and Haag 1993), relatively low summer water temperatures in 2011 (reaching < 10°C in August in the bioboxes) may have contributed to low settlement rates. In another Irish study, settlement reached a peak of 170,000 juveniles/m^2 where temperatures where higher and the same methodologies for gathering settlement was used (Lucy et al. 2005). Seasonal plates are also known to underestimate total natural settlement but are considered a good proxy (Lucy et al. 2005).

For the seasonal settlement plates, the control tank had the highest settlement with 4,670 juveniles/m^2, treated tank 2 had 3,670 juveniles/m^2, and treated tank 3 had 2,000 juveniles/m^2 (Figure 7). Treated juvenile survival declined rapidly between treatment and day 3; treated tank 2 reached 18% survival by day three and 0% survival seven days after treatment and treated tank 3 reached 16% survival by day three and 0% survival 6 days after treatment. The juvenile survival in the control began to decline between day 3 and 6. It is hypothesised that this decline in the control tank occurred from natural causes, as by day three, juvenile settlement was nearly depleted in treated tanks 2 and 3, whereas in the control tank, juvenile numbers did not begin to decline until after day three. The decline in the control and treated plates after day 3 could be attributed to the regular removal of the plates from the biobox for monitoring settlement and other natural causes. Additionally, according to Nichols (1996), 20% up to 100% natural mortality can occur pre and post settlement. It is hypothesised that the decline in juvenile survival prior to day 3 in treated tanks 2 and 3 was due to MBI 401 FDP treatment.

Chlorine treatment – juvenile mussels

Juvenile settlement measured biweekly in the chambers, prior to treatment, was relatively high in comparison to the biweekly biobox settlement reaching a peak of 14,670 juveniles/m^2 in chamber A on the 4th of August. Although this count is higher than that of the bioboxes it is still relatively low in comparison to the juvenile settlement measured in the study by Lucy et al (2005) for the 1st week of August between 2001 to 2003.

Treated chamber C had the highest seasonal settlement with 31,000 juveniles/m^2, treated chamber B had 18,330 juveniles/m^2, and control chamber A had 10,670 juveniles/m^2. Figure 8 displays mean juvenile counts in the water chambers before and after treatment with chlorine. Treated juvenile survival declined rapidly between treatment and day 2; treated chamber A reached 12% survival by day two and 0% survival six days after treatment and treated chamber C reached 35% survival by day two and 0% survival 6 days after treatment. The juvenile survival in the control began to decline between treatment and day 2. Although control survival initially declined more rapidly than treated chamber C, overall survival reached 0% more rapidly in the treated chambers, therefore we can attribute this decline in survival to treatment with chlorine, with decline in juvenile survival on the control plate resulting from its removal from the chambers during examination.

MBI 401 FDP treatment - adult mussels

After treatment, adult mussel mortality was monitored every 2–3 days for 16 days and then weekly for four weeks. At the end of the monitoring period on day 48 the control tank had 1.3% mortality, treated tank 2 had 80% mortality, and treated tank 3 had 81% mortality (Figure 9). Most of the adult mortality in the bioboxes occurred within the first 16 days after treatment; in treated tank 2 mortality was at 71% and in treated tank 3 mortality was at 76% by day 16. In similar biobox studies conducted in North America and Canada, >90% adult mussel mortality was observed (Figure 10). The water temperature during the Irish treatment was 13.8°C and for the post treatment monitoring period the min and max temperature was 13–15°C, in trials conducted in the USA the average water temperature was > 16°C.

Chlorine treatment - adult mussels

Adult zebra mussel mortality after treatment with chlorine was monitored every 2–3 days for ten days and then weekly for five weeks until 80% mortality was reached (the plant's treatment goal). In treated chamber A, by day 16, the adult mortality was at 76.5% reaching 87% by day 49, and in treated chamber C, at day 16, mortality was 79% reaching 83% by day 49 (Figure 11).

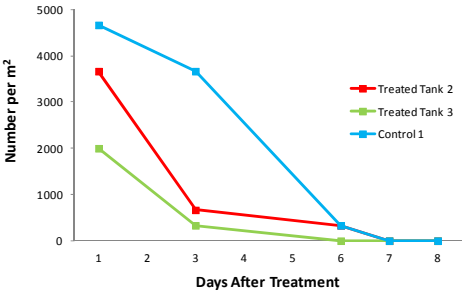

Figure 7. Mean number of juvenile mussels in the bioboxes after treatment with MBI 401 FDP.

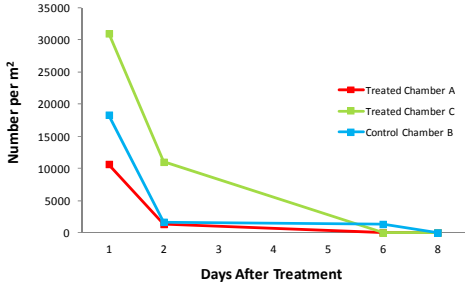

Figure 8. Mean number of juveniles in the water chambers after treatment with chlorine.

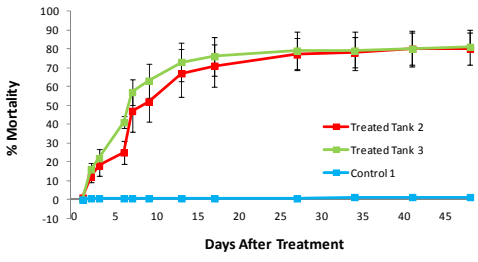

Figure 9. Mean mortality (± SD) of adult mussels in bioboxes after treatment with MBI 401 FDP.

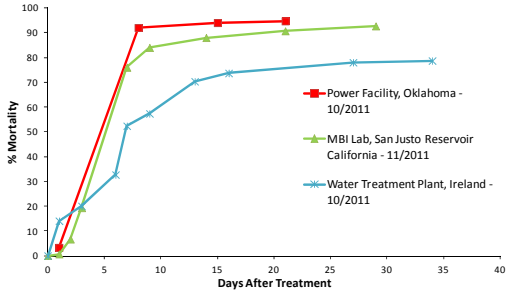

Figure 10. 2011 biobox trials with MBI 401 FDP in North America and Ireland.

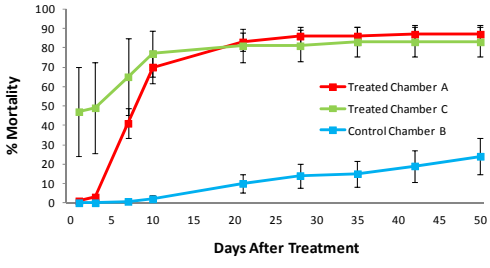

Figure 11. Mean mortality (± SD) of adult mussels in chambers after treatment with chlorine.

The low water temperature during chlorination (< 10°C) directly affects the length of time chlorination is required (Rajagopal et al. 2002) and the length of time it takes for mortality to reach > 70%. At the end of the monitoring period mortality in control chamber B was 24%. It is unknown why control mortality reached 24%; nevertheless, the high mortality attained in both the treated chambers indicates the treatment was effective. The rate of adult mussel mortality after chlorine treatment is on par with the mortality after MBI 401 FDP treatment.

Table 1. Water quality results before, during (4 and 8 hours) and after (3 rinses) biobox treatment with MBI 401 FDP. R1 = rinse 1, R2 = rinse 2, R3 = rinse 3.

Sample Date	Location	Turbidity (NTU)	Temp (°C)	BOD	TOC	pH	DO
Before Treatment							
	Control Tank 1	3.27	14.6	1.21	15	7.84	9.08
11-Oct	Treated Tank 3	3.18	14.5	2.94	10.6	7.82	9.23
4hr							
	Control Tank 1	2.79	14.5	1.48	15.2	7.76	8.93
11-Oct	Treated Tank 3	80.1	14.5	9	56.2	7.59	9.07
8hr							
	Control Tank 1	2.34	14.7	1.58	10	7.71	8.7
11-Oct	Treated Tank 3	79.3	14.9	8.81	54.6	7.59	8.81
R1							
	Control Tank 1	5.81	15	1.04	9.73	8.04	9.63
11-Oct	Treated Tank 3	7.36	15	2.14	10.3	7.83	9.68
R2							
	Control Tank 1	4.99	16	3.59	9.9	7.86	10.7
12-Oct	Treated Tank 3	4.48	15.9	3.22	9.91	7.89	10.6
R3							
	Control Tank 1	3.99	14.8	2.61	9.59	7.86	9.4
13-Oct	Treated Tank 3	4.35	14.6	1.17	9.41	7.96	9.22

MBI 401 FDP treatment - water quality

Results of water quality parameters taken before, during and after MBI 401 FDP treatment are presented in Table 1. These results, though gathered from samples in the static bioboxes, give an indication of the effects MBI 401 FDP would have on water quality if used in a similar static treatment in the raw water chambers of the Sligo drinking water treatment plant. However, if used in the plant the treated water would be discharged gradually back to the receiving lake, Lough Gill and would eventually be heavily diluted upon discharge. In treated tank 3, the temperature ranged from 14.5–15.9°C, and pH varied between 7.59 and 7.96. The turbidity ranged between 3.18 and 80.1 NTU. Dissolved oxygen varied between 8.81 and 10.61 mg/L. Biological oxygen demand (BOD) ranged between 1.17 and 9 mg/L and the TOC ranged between 9.42 and 56.2 mg/L.

Measurements of temperature, DO and pH did not differ by more than ± 1 unit before during and after treatment in the bioboxes; therefore, the treatment had little effect on these parameters. Turbidity did increase substantially; however, since turbidity and MBI 401 FDP concentration are strongly correlated, this increase was expected. After the three rinses, turbidity returned to background levels. An increase in turbidity is due to the nature of the product which is primarily composed of particulate organic matter.

A similar trend occurred with the BOD, which also increased to a peak of 9.00 mg/L during treatment at 4 hours and went down to 8.81 mg/L at 8 hours. Over time, it is expected that the BOD measurements would have continued to decrease as the dissolved organic matter degraded (Graham and Gilbert 2012). TOC followed the same pattern as BOD; at 4 hours it increased to 56.2 mg/L and then decreased to 54.6 mg/L at 8 hours. The TOC increased over the 8 hour treatment duration but decreased to background levels after the first rinse. This increase in TOC was expected as the product is primarily particulate organic matter.

Conclusions

Adult mortality reached 80% after treatment with both chlorine and MBI 401 FDP. The mortality of adults after chlorine treatment reached 80% by day 20. After MBI 401 FDP treatment, mortality was at 76% by day 20 and reached 80% by day 27. Mayer (2011) demonstrated that at lower water temperatures following treatment with *Pseudomonas fluorescens* mortality is slower. This was apparent in this trial when compared to

those carried out in the USA (Figure 10) mortality at Cairns Hill was slower to occur as the water temperature was lower.

It must be remembered that MBI 401 FDP treatment duration was 8 hours and chlorine treatment duration was 7 days. MBI 401 FDP treatment can begin and end within the working day whereas chlorine treatment is a continuous 24 hours a day treatment, and in this instance, 7 days long. This does not include the set up and breakdown. Chlorine treatments require this longer application time because the zebra mussels recognise chlorine as a harmful substance and shut their valves and cease feeding (Rajagopal et al 2003). Formulated *Pseudomonas fluorescens* CL145A cells (like those in MBI 401 FDP), however, are not recognised as harmful and the zebra mussels feed readily on them (Marrone Bio Innovations 2012).

Studies indicate *Pseudomonas fluorescens* CL145A cells specifically target zebra and quagga mussels (Molloy et al 2013b). In addition to many non-target studies carried out in the USA, (Molloy et al 2013b) Canada, and Europe, non-target trials carried out at IT Sligo in accordance with OECD and ASTM guidelines on 12 Irish aquatic organisms (some of which were collected from Lough Gill) show that calculated median effective concentration or median lethal concentration values were noted to be in excess of the treatment rates.

Chlorine is a general biocide; with its original purpose being a bleaching agent, chlorine gas was also used as a chemical warfare agent (Winder 2001). Airborne chlorine gas at a concentration of 3 mg/L causes mild irritation of the mucous membrane (the concentration used in this study fits within this category), above 5 mg/L causes eye irritation, 15–30 mg/L causes a cough, choking and burning, and finally 430 mg/L causes death after just 30 seconds exposure (Winder 2001). *Pseudomonas fluorescens* CL145A cells are designated as "Biosafety Level 1" by the American Type Culture Collection, and are defined as "having no known potential to cause disease in humans or animals" by American Biological Safety Association.

This study shows that MBI 401 FDP was an effective alternative zebra mussel control method and could be used in place of chlorine treatments, or, in conjunction with chlorine treatments in an Integrated Pest Management program (IPM). As an example, for this Sligo water treatment plant, a final chlorine treatment or an MBI 401 FDP treatment at 100–150 mg a.s./L at the end of the season could be performed to control zebra mussels in the system.

Moving forward, this trial has offered a suitable alternative to chlorine and has shown MBI 401 FDP's effectiveness as a zebra mussel control option.

Acknowledgements

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References

Coffin JC, Ge R, Yang S, Kramer PM, Tao L, Pereira MA (2000) Effect of trihalomethanes on cell proliferation and DNA methylation in female B6C3F1 mouse liver. *Toxicologicol Sciences* 58(2): 243–52, http://dx.doi.org/10.1093/toxsci/58.2.243

Garton DW, Haag WR (1993) Seasonal reproductive cycles and settlement patterns of *Dreissena polymorpha* in western Lake Erie. In: Nalepa TF, Schloesser DW (eds), Zebra mussels, Biology, Impacts and Control. Lewis Publishers, Florida, pp 111–128

Graham R, Gilbert J (2012) MBI-401 SDP: Assessment of Ready Biodegradable by Measurement of CO2 Evolution. Unpublished Report

Lucy F (2006) Early life stages of *Dreissena polymorpha* (zebra mussel): the importance of long-term datasets in invasion ecology. *Aquatic Invasions* 1: 171–182, http://dx.doi.org/10.3391/ai.2006.1.3.12

Lucy FE (2010) Zebra Mussels: Review of ecology and impacts since invasion in Ireland. In: Mackie G, Claudi R (eds), Monitoring and control of macrofouling molluscs in freshwater systems. 2nd Edition, CRC Press, Boca Raton, FL., pp 386–389

Lucy F, Sullivan M, Minchin D (2005) Nutrient levels and the zebra mussel population in Lough Key. ERTDI Report Series No. 34. EPA, Wexford. Available at: http://www.epa.ie/downloads/pubs/research/water/epa_zebra_mussels_ertdi34_final.pdf (Last accessed 11 December 2012)

Lucy FE, Burlakova LE, Karatayev AY, Mastitsky SE, Zanatta DT (in press) Zebra mussel impacts on unionids. In: Schloesser D, Nalepa T (eds), Quagga and Zebra Mussels. Taylor and Francis

Mackie GL, Claudi R (2010) Monitoring and control of macrofouling mollusks in freshwater systems. 2nd Edition, CRC Press, Boca Raton, FL, 508 pp

Marrone Bio Innovations (2012) http://www.marronebioinnovations.com/products/brand/zequanox/ (Last accessed 20th December 2012)

Marsden JE (1992) Standard protocols for monitoring and sampling zebra mussels. Illinois Natural History Survey, Biological Notes 138 https://www.ideals.illinois.edu/bitstream/handle/2142/17305/standardprotocol138mars.pdf?sequence=1 (Last accessed 8 January 2013)

Mayer DA (2011) Evaluation of a biopesticide against invasive species for native species restoration. A dissertation submitted to the University at Albany, State University of New York http://udini.proquest.com/view/evaluation-of-a-biopesticide-pqid:2557999051/ (Last accessed 21 May 2013)

Minchin D, Moriarty C (1998) Zebra mussels in Ireland. Fisheries Leaflet 177, Marine Institute, Dublin, 11 pp

Minchin D, Lucy F, Sullivan M (2002) Zebra mussel: Impacts and Spread. In: Leppakoski E, Gollasch S, Olenin S (eds), Invasive Aquatic Species of Europe: Distribution, Impacts and Spread. Kluwer Press, Dordrecht, pp 135–146, http://dx.doi.org/10.1007/978-94-015-9956-6_15

Molloy DP, Mayer DA, Gaylo MJ, Morse JT, Presti KT, Sawyko PM, Karatayev AY, Burlakova LE, Laruelle F, Nishikawa KC, Griffin BH (2013a) A biopesticide for the control of zebra and quagga mussels (Bivalvia: Dreissenidae) *Journal of Invertebrate Pathology* 113(1): 104–114, http://dx.doi.org/10.1016/j.jip.2012.12.012

Molloy DP, Mayer DA, Gaylo MJ, Burlakova1 LE, Karatayev AY, Presti1 KT, Sawyko PM, Morse JT, Paul EA (2013b) Non-target trials with *Pseudomonas fluorescens* strain CL145A, a lethal control agent of dreissenid mussels (Bivalvia: Dreissenidae) *Management of Biological Invasions* 4(1): 71–79, http://dx.doi.org/10.3391/mbi.2013.4.1.09

Nichols SJ (1996) Variations in the reproductive cycle of *Dreissena polymorpha* in Europe, Russia and North America. *American Zoologist* 36: 311–325

Rajagopal S, Van der Veldea G, Jennerb HA (2002) Effects of low-level chlorination on zebra mussel, *Dreissena polymorpha*. *Water Research* 36: 3029–3034, http://dx.doi.org/10.1016/S0043-1354(01)00516-4

Rajagopal S, Van der Veldea G, Van der Gaaga M, Jennerb HA (2003) How effective is intermittent chlorination to control adult mussel fouling in cooling water systems? *Water Research* 37: 329–338, http://dx.doi.org/10.1016/S0043-1354(02)00270-1

Roche P, Benanou D (2007) Impact of chlorination on the formation of odour compounds and their precursors in the treatment of drinking water. TECHNEAU. Available at http://www.techneau.org/fileadmin/files/Publications/Publications/Deliverables/D5.3.8.pdf (Last accessed 8 January 2013)

Tikkanen MW, Schroeter JH, Leong LYC, Ganesh R (2001) Guidance Manual for the Disposal of Chlorinated Water. Awwa Research Foundation and American Water Works Association. Available at: http://www.vita-d-chlor.com/specs/AWWARFDechlorGuides.htm (Last accessed 8 January 2012)

West Virginia Department of Health and Human Resources (2010) Class 1 Water Operator Course Manual. Available at: http://www.wvdhhr.org/oehs/eed/swap/training&certification/drinking_water/documents/ClassIManual.pdf (Last accessed 17 December 2012)

Wright DA, Magee JA, Setzler-Hamilton EM, Chalker-Scott L, Morgan GL (1997) Use of high energy monochromatic UV light to kill dreissenid larvae. In: D'Itri FM (ed), Zebra Mussels and Aquatic Nuisance Species, Ann Arbor Press Inc., Chelsea, MI, pp 467–475

Winder C (2001) The Toxicology of Chlorine. *Environmental Research* 85: 105–114, http://dx.doi.org/10.1006/enrs.2000.4110

Appendix F: 'Zebra mussel control using Zequanox® in an Irish waterway'. This paper is presented as it appears in the 'Management of Biological Invasions', 2014, Volume 5, Issue 3.

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Research Article CORRECTED PROOF

Zebra mussel control using Zequanox® in an Irish waterway

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Abstract

Due to the invasion of zebra and quagga mussels in European and North American waters, there is a need for an environmentally benign mussel control method to replace chlorine and other currently used control products. Zequanox® is a natural product comprised of *Pseudomonas fluorescens* strain CL145A, which effectively controls zebra and quagga mussels. The objective of this study was to demonstrate an effective method of zebra mussel control in inland waterways using Zequanox. Water quality was monitored to determine any negative impacts and to observe product dispersion. A curtain made of an impermeable material was placed in the Grand Canal at Tullamore Harbour sealing off two 8 x 0.5 m sections of canal wall under the bridge, and a control site was chosen further down the docking area. Both sections were treated with Zequanox at a concentration of 150 mg active substance/L for an 8 hour treatment period. Water quality was monitored in the treatment area and in the selected control area before, during, and after treatment. Naturally settled and seeded adult zebra mussels were observed for mortality in the treatment and control areas and juveniles were monitored for survival in both the treatment and control areas. Naturally settled adult mussel numbers were reduced by approximately 46% in treatment side 1, and 65% in treatment side 2, seeded adult mussel mortality reached 75% in treatment side 1 and 56% in treatment side 2. These results demonstrate that under the optimum conditions Zequanox effectively controls zebra mussels in open water.

Key words: Grand canal, invasive mussel control, water quality

Introduction

The zebra mussel, *Dreissena polymorpha* (Pallas, 1771), is an invasive, aquatic bivalve shellfish, which has impacted freshwater ecosystems and water abstraction in all invaded countries including Ireland (Minchin et al. 2002; Lucy 2010; Lucy et al. 2013). The zebra mussel arrived in Ireland in the early 1990's (Minchin and Moriarty 1998) in the lower River Shannon on the hulls of boats, most likely attached to used leisure crafts from Britain (Pollux et al. 2003). Inland waterway systems (canals) in Ireland have allowed for movement of the zebra mussel both of its own accord and by accidental movement, largely attributed to boaters and recreational anglers (Minchin et al. 2005). Not only is the zebra mussel causing problems for Ireland's rivers and lakes through their role as ecosystem engineers (Karatayev et al. 2002), but industries are also suffering from the high costs of controlling these mussels (Aldridge et al. 2004). Currently chlorine is the most commonly used control method (Mackie and Claudi 2010); however, its use is limited and is only suitable in enclosed systems (intake pipes) as it is a non selective general biocide and is lethal to all living organisms. Presently the only control method for zebra mussels in inland waterways is physical removal, and therefore, there is a need for a more efficient management option.

Marrone Bio Innovations (MBI), a company specialising in the development and commercialisation of natural biocides in Davis, CA, USA, is the commercial license holder for the invasive zebra and quagga mussel (dreissenid) control product Zequanox®. The active ingredient in Zequanox is killed *Pseudomonas fluorescens* strain CL145A cells, which is lethal to dreissenid

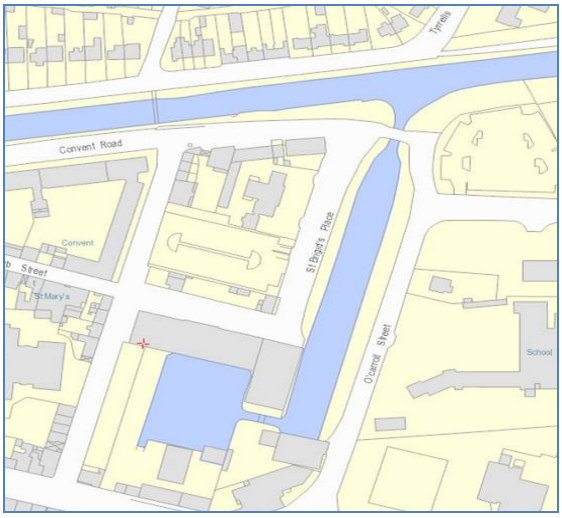

Figure 1. Tullamore Harbour, Co. Offaly, Ireland.

Mussels, but studies show it has minimal to no impact on other aquatic organisms (Molloy et al. 2013a). *Pseudomonas fluorescens* is present world-wide and commonly found in food. In nature, it is a harmless bacterial species that is known to protect the roots of plants from disease (Marrone Bio Innovations 2012). Ecotoxicology studies were carried out in the Institute of Technology Sligo and in the USA, where Zequanox was tested on a number of aquatic species. No negative effects were observed at concentrations required to sufficiently control zebra mussels (150 mg active ingredient/L) (Marrone Bio Innovations Ecotoxicology Studies 2012). Additionally, Molloy et al. (2013b) carried out a number of non target trials using the active ingredient in Zequanox (*Pseudomonas fluorescens* CL145A) and again found no negative impacts to the organisms tested at concentrations required to control zebra mussels.

In March, 2012 the United States Environmental Protection Agency registered Zequanox for use in the USA in enclosed or semi-enclosed systems. In 2011, successful Zequanox trials were conducted within the cooling water system of Davis Dam in Bullhead City, Arizona in the USA, and in 2012 within the cooling water system of DeCew II Generating Station of Ontario Power Generation in St. Catharine's, Ontario, Canada. MBI also conducted a successful open water trial in Deep Quarry in DuPage County, Illinois, USA in 2012; this open water trial was similar to the canal trial described in this report.

Tullamore Harbour is part of the Grand Canal, connecting the east of Ireland to the Shannon River navigation in central Ireland. It was traditionally used for transporting goods via barge boats, and now is solely used for leisure purposes (Byrne 2007). The Grand Canal at Tullamore Harbour has a zebra mussel infestation spanning from under the bridge, along the harbour branch of the canal, and into a harbour and dock area (Figure 1).

A pilot demonstration trial using Zequanox was conducted under the bridge in the Grand Canal at Tullamore Harbour. The objectives of this trial were to firstly demonstrate an effective method of zebra mussel control in inland waterways and secondly trial a method which could be used for zebra mussel fouled jetties, pontoons and navigational structures.

Materials and methods

Experimental set-up

This trial was conducted under the bridge at Tullamore Harbour (53º27'82"N, -7º48'86"W) where dreissenid infested canal walls on both banks were treated with Zequanox to test its effect on settled juveniles, seeded adult mussels and naturally settled adult mussels. The areas of the canal were labeled treatment side 1, treatment side 2 (treated areas under the bridge) and control. Two impermeable curtains were set up to enclose the treatment area (canal wall). These curtains were comprised of an impermeable material (scaffband), which was weighted down with stainless steel chains at the bottom and attached to aluminum at the sides, with foam used to seal in the containment area (Figure 2). The curtains were on average 7.70 m in length, 0.45 m in width and 1.31 m in depth, so that approximately 4.5 m^3 (4500 L) of water was enclosed along each concrete wall. The curtains were set up one day in advance of treatment to allow the mussels to acclimatise and resume normal feeding behavior prior to treatment.

The infested canal walls under the bridge at Tullamore Harbour were treated with Zequanox at a target concentration of 150 mg active substance (a.s.)/L (active substance is synonymous with active ingredient). The target concentration was maintained for 8 hours. This treatment concentration and duration was based on the results of trials carried out in North America and in Ireland (Meehan et al. 2013).

Figure 2. Impermeable curtains used to hold treated water within treatment area along canal walls. Photograph by Sara Meehan.

Figure 3. PVC plates used to monitor juvenile survival. Photograph by Sara Meehan.

Figure 4. Mesh cages to hold seeded adult mussels (26 cm in length). Photograph by Bridget Gruber.

Juvenile mussel collection

PVC plates were deployed in Lough Key (53°593'04"N, 08°16'46"W) on July 23rd, 2012 to gather juvenile zebra mussel settlement, as this lake is known for high settlement (Lucy 2005). These plates were removed from Lough Key on September 2nd, 2012 and an initial baseline count was made. These plates were then transported to the Grand Canal at Tullamore Harbour and placed in the two treatment areas and the control area on weighted rope (Figure 3). Juvenile plates were counted 24 hours after treatment then daily followed by weekly until juvenile settlement reached zero.

Adult mussel collection

Adult zebra mussels were collected from the Grand Canal at Tullamore via a long-handled scraper (Minchin 2007; Minchin et al. 2002) and by hand removal from the wall while wading. Healthy mussels were then seeded into three mesh cages (mesh size 3mm), each containing three compartments housing 50 mussels each (Figure 4). These mesh cages were attached to bricks via cable ties. Floating rope was then tied to the bricks so the cages could be easily removed from the canal using a boat hook; this method was developed so the cages would not be visible to the public as they were to remain in the canal for an extended period of time. Once the mesh cages were ready, they were left to acclimatise overnight in the canal. One cage was placed in the control area, and one in each treatment area. Mussels were checked for mortality before treatment and any dead ones were removed and replaced with live healthy ones. Mussels were presumed dead if shells were open and did not close after being gently prodded. After treatment seeded adult mussels were counted first daily then weekly for seven weeks.

Naturally settled adult mussels

The number of naturally settled adult mussels in the two treated areas and the control area was estimated prior to treatment using 25 cm x 25 cm quadrats. Three quadrats per defined area were used to estimate mussel settlement/m^2. Quadrats were placed at random and at different depths by divers. Divers counted the number of live mussels within each quadrat. A record of the exact spot the quadrats were placed was kept by measuring its distance from a pre-determined point along the bank and the depth at which the quadrat was placed. Photographs were also taken so that the same quadrats could be counted again after treatment. Quadrats were re-counted seven weeks after treatment.

Zequanox application

The curtains were placed in the canal 24 hours prior to treatment to allow the naturally settled mussels to resume normal behavior after the disturbance of the curtain placement. Twenty four hours after the curtains were placed in the canal (before treatment), dissolved oxygen (DO) inside the curtained areas had significantly reduced and was approximately 3 mg/L lower than the DO outside of the curtains. This was likely due to the natural diurnal cycle and flow restriction. Therefore, treatment side 1 was aerated with bubblers until the curtains were removed to ensure DO stayed at background levels, whilst on treatment side 2, DO was not controlled and no aeration occurred. This experimental design allowed us to quantitatively determine if observed mortality could be attributed to Zequanox, or whether the observed mortality could be attributed to low DO levels. It also allowed us to infer if water quality conditions impacted zebra mussel ingestion of Zequanox.

Zequanox, a dry powder formulation (as registered in the US), was used to treat the canal walls. The powder was mixed on-site with canal water to create the following stock solution concentration:

$C_1V_1 = C_2V_2$ where
C_1 = target treatment concentration (mg a.s./L)
V_1 = volume of treatment area (4500 L)
C_2 = stock concentration (100 g a.s./L)
V_2 = volume of stock concentration to be applied (L)

For each curtained off area a total of 675 g a.s. of Zequanox was mixed with 6.75 L of canal water using a small hand blender to achieve a concentrated product solution of 100 g a.s./L. This solution was slowly poured into the curtained off area so as to evenly distribute the product. Once all of the product was in the water, a wooden paddle was used to gently mix the treated water to achieve an even distribution of product within the treated area. As turbidity and treatment concentration have a linear relationship (Meehan et al. 2013), turbidity inside the curtains was monitored throughout the application process using a Hach 2100Q portable turbidimeter to ensure the target concentration was reached and maintained.

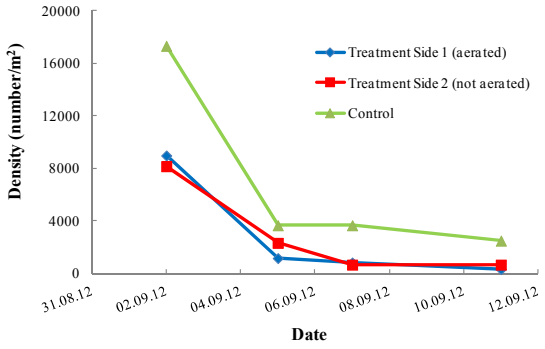

Figure 5. Mean density of juveniles before and after Zequanox treatment.

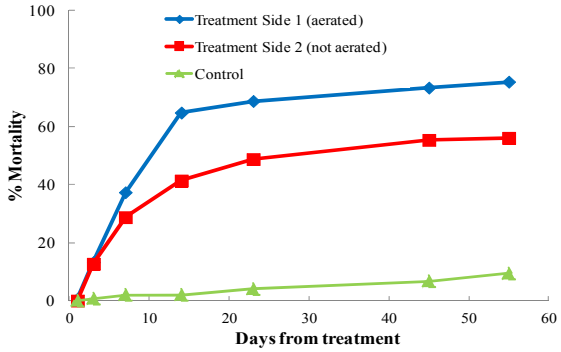

Figure 6. Seeded adult mussel mortality after treatment with Zequanox.

As flow in the canal increased, nominal leakage of product from within the curtain occurred and concentrations within the treatment area decreased. This leakage likely occurred due to an increase in wind speed or the passing of a barge along the canal. In order to maintain a target concentration of 150 mg a.s./L, additional product was mixed in two stages and added.

After the 8 hour treatment period in which Zequanox concentrations were maintained at 150 mg a.s./L, the curtains were then held in place for a further 16 hours (but no additional product was added) making the hold time 24 hours in total. This additional hold time allowed for natural degradation of the product. Studies indicate that, once Zequanox is wetted, it biodegrades rapidly and the efficacy significantly decreases after 8 hours in water, and after 24 hours in water it is no longer efficacious. After the 24 hour hold time, the curtains were removed and, based on water quality measurements, the product dispersed to non-detectable levels within the canal system.

Water quality measurements

Turbidity inside the treatment area was monitored throughout the application and post-treatment period with a Hach 2100Q portable turbidimeter; as turbidity and concentration are correlated this ensures that the target concentration was reached and maintained throughout the application period, and that Zequanox had dispersed to non-detectable levels after the curtains were removed.

Additional water quality measurements were taken before treatment, during treatment (at 4 and 8 hours), 24 hours after treatment before the curtain was removed, and 24 hours after the curtain was removed. These water quality measurements included: temperature, dissolved oxygen (DO), pH, turbidity, biological oxygen demand (BOD), and total organic carbon (TOC).

Dissolved oxygen, pH and temperature were measured with an Orion 5 star meter. The analysis of BOD and TOC was subcontracted out to Alcontrol Laboratories. Method 5210B, AWWA/ APHA, 20th Ed., 1999; SCA Blue Book 130 was used to determine BOD. US EPA Method 415.1 and 9060 was used to determine TOC.

Results

Juvenile mussels

Figure 5 and Table 1 show the mean juvenile counts for the treatment and the control areas. Juvenile numbers were high (over 8,000/m^2) 48 hours in advance of the trial. Between 48 hours and the first count carried out after treatment, survival dropped considerably for both the treated juveniles and the control juveniles. After this initial drop, juvenile survival in the treated areas continued to decrease, while juvenile survival in the control area stayed approximately the same between 05/09/12 and 07/09/12.

Adult mussels

Seeded adult mussels

After 55 days, treatment side 1 had 75% seeded adult mussel mortality and treatment side 2 had 56% mortality. The mortality in the control was 9% (Figure 6).

Table 1. Mean density of juveniles before and after Zequanox treatment with standard deviation (juveniles/m^2).

Date	Treatment Side 1 (aerated)	SD	Treatment Side 2 (aerated)	SD	Control	SD
02/09/2012	8983	4820	8167	2593	17333	3300
05/09/2012	1167	236	2333	943	3666	2828
07/09/2012	833	236	667	0	3667	0
11/09/2012	333	471	667	471	2500	1179
% Survival	4		8		14	

Table 2. Mean density of naturally settled adult mussels (live adult mussels/m^2) before and after Zequanox treatment with standard deviation (SD).

Date	Treatment Side 1 (aerated)	SD	Treatment Side 2 (aerated)	SD	Control	SD
03/09/2012	1000	662	272	136	69	37
22/10/2012	539	272	96	34	59	24
% Mortality	46		65		15	

Table 3. Water quality measurements before, during (4 and 8 hours) and after treatment (before and after curtain removal).

Sample Date & Time	Location	Turbidity (NTU)	Temp (°C)	BOD (mg/l)	TOC (mg/l)	pH	DO (mg/l)
Before treatment							
4-Sep, 09:30	Control	4.97	17.1	<2	22.8	7.76	7.22
04-Sep, 09:30	Treated 1	2.72	17.8	<2	21.7	8.03	5.6
04-Sep, 09:30	Treated 2	2.97	17.9	<2	20.7	7.82	3.84
4 hrs into treatment							
04-Sep, 14:00	Control	4.26	19.3	<2	21.6	7.85	8.2
04-Sep, 15:00	Treated 1	109	18.4	91.5	49.5	7.59	7.15
04-Sep, 14:10	Treated 2	125	18.2	71	43.3	7.68	4.29
8 hrs into treatment							
04-Sep, 18:09	Control	3.74	19.6	<3	21.1	7.83	8.44
04-Sep, 18:56	Treated 1	127	18.5	103	31.9	7.93	7.63
04-Sep, 18:20	Treated 2	59.9	18.6	28.6	23.1	7.85	5.08
24 hrs after treatment; before curtain removal							
05-Sep, 07:45	Control	8.78	17.9	<2	20.8	7.87	6.85
05-Sep, 08:00	Treated 1	26.5	18.3	13.1	22.3	7.84	7.68
05-Sep, 07:55	Treated 2	32.3	18.5	17.1	25.3	7.58	2.38
24 hrs after curtain removal							
06-Sep, 12:00	Control	5.12	17.9	3.2	21.6	7.88	7.18
06-Sep, 12:00	Treated 1	9.31	18	3.65	22.7	7.63	7.42
06-Sep, 12:00	Treated 2	8.19	18.1	<2	22.3	7.85	7.58

Naturally settled adult mussels

Table 2 shows the mean number of naturally settled mussels before and after treatment within the treatment areas and the control. The mean number of live adult mussels decreased by approximately 46% in treatment side 1, and by approximately 65% in treatment side 2. The mean number of live mussels decreased by 15% in the control area (there was one less mussel observed in the control area after treatment).

Water quality

In treatment areas 1 and 2 the temperature ranged from 17.8 to 18.6°C; in the control area the temperature ranged from 17.1 to 19.6°C (Table 3). In treated areas, pH varied between 7.58 and 8.03, similar to the range seen in the control area (7.76–7.88). Dissolved oxygen in treatment side 1 (aerated side) ranged from 5.6 to 7.68 mg/L. Dissolved oxygen levels in treatment side 2 (not aerated) ranged from 2.38 to 7.58 mg/L. In treat-

ment side 2, 24 hours after treatment, DO dropped to 2.38 mg/L. Once the curtain was removed DO levels increased to 7.58 mg/L (background levels). Biological oxygen demand in the treated areas ranged between < 2 and 103 mg/L. Total organic carbon ranged from 20.7 to 49.5 mg/L. The turbidity in the treated areas before treatment was < 3 NTUs. During treatment, the turbidity in the treated areas increased and ranged between 59.9 and 127 NTUs. Approximately 24 hours after treatment, prior to curtain removal, turbidity decreased to 26.5 and 32.3 in the treated areas. Once the curtains were removed, within 24 hours, turbidity decreased to 9.31 and 8.19 NTUs. The turbidity of the control throughout the 48 hour monitoring period ranged from 3.74 to 8.78 NTUs.

Discussion

Juvenile mussel survival

Juvenile survival on the treated plates and the control plates initially declined after treatment. After this decline, control survival leveled out and survival on the treated plates continued to drop. There is no way to determine if any of the mortality during the initial decline in survival is due to Zequanox treatment therefore it must be assumed that it is due to outside influences namely the transportation of the plates to the treatment site. However the continued decline of settlement on the treated plates was due to Zequanox as the control survival was maintained. These results parallel studies conducted by MBI at Davis Dam (Arizona, US) where a decline in juvenile survival on settlement plates treated with Zequanox was observed, and a study carried out in Sligo, Ireland (a demonstration trial for a water treatment plant) where juvenile survival after treatment with Zequanox decreased (Meehan et al. 2013). It is also important to note that seasonal plates are known to underestimate total natural settlement but are considered a good proxy (Lucy et al. 2005). The initial high mortality in both the treated and control plates is not representative of what would happen in a real time application as there would be no movement of settlement plates from one site to the other. Therefore further research is necessary to examine the effects of Zequanox on settled juveniles in situ.

Adult mussel mortality

Seeded mussel mortality was observed in treatment side 1 (aerated) and 2 (not aerated); however, mortality was greater on treatment side 1 (75%) than side 2 (56%). Several factors may have contributed to this difference. The lower DO levels on treatment side 2 may have disturbed the mussel's feeding, by causing them to shut their valves as a response to unfavourable conditions, as is the case with intermittent chlorination (Rajagopal et al. 2003). Zequanox must be ingested by the mussels to have an effect. Mixing and aeration may also have contributed to the difference in mortality, making Zequanox more bioavailable throughout the treatment area. On treatment side 2 only hand mixing aided in the distribution of the product whereas aeration on treatment side 1 may have helped to more evenly distribute Zequanox.

A decrease in naturally settled mussels after treatment with Zequanox was observed; however, in contrast to the seeded mussel mortality, more mortality occurred in treatment side 2 (65%) than in treatment side 1 (46%). This may have been due to the aeration bubblers and air tubing on treatment side 1 being located close to the wall thus disturbing the mussel's causing them to shut their valves and cease feeding. The seeded adult mussels on aerated side 1 were located at the bottom of the canal away from the direct interference of the aeration system and this could account for the difference in mortality between the seeded and naturally settled mussels.

Water quality

No negative impacts from Zequanox treatment to temperature or pH was observed. The temperature range seen in the treated and control areas was consistent with the natural diurnal and seasonal cycles in Ireland. The slightly higher temperatures in the control area was likely due to that area being in direct sunlight while the treated areas were under the bridge and therefore had less sun exposure. The difference in sunlight had no apparent impact on pH levels. The zebra mussels in this study (seeded and naturally settled) at all sites were present at depths of between 1.0–1.5 m and due to low water transparency were at naturally low light levels. In fact the divers required torch light to take samples on both sampling dates. Therefore sunlight is not considered a varying environmental factor in this study.

During treatment, the turbidity in the treated areas increased (since Zequanox is made up of organic material, turbidity was expected to increase significantly) and ranged between 59.9 and 127 NTUs. After treatment was terminated, but prior to curtain removal, turbidity, as expected, began to decrease due to natural degradation of the product. Once the curtains were removed, within 24 hours, turbidity dropped to control levels.

Aeration sufficiently controlled DO levels in treatment side 1. In treatment side 2, 24 hours after treatment, DO dropped to 2.38 mg/L. This was expected as Zequanox is comprised of dead bacterial cells that degrade in the natural environment causing a decrease in DO, particularly in low flow environments. However, once the curtain was removed and flow was restored, DO increased to background levels.

TOC increased in treated areas four hours into the treatment; however, by eight hours TOC levels were decreasing to background levels. This increase again was expected because Zequanox is primarily made up of particulate organic matter. TOC levels decreased as degradation of the product took place. Since Zequanox is organic in nature, biochemical oxygen demand also followed a similar pattern, increasing at 4 hours into treatment and then decreasing as time passed and Zequanox degraded.

Environmental monitoring before, during, and after treatment indicated there was minimal impact to water quality in the canal. Though TOC, BOD, and turbidity temporarily increased during treatment in the enclosed treatment areas, by 8 hours, measurements were decreasing and returned to background levels 24 hours after treatment once Zequanox had naturally biodegraded.

Conclusion

Presently the only zebra mussel control option for canals in Ireland is mechanical removal. This study shows that Zequanox effectively controlled up to 75% of zebra mussels in an Irish canal. Though Zequanox is not yet registered in the EU, it has potential as an alternative control option for Irish waterways; the results of the study show that when Zequanox is applied under the correct conditions (sufficient DO levels and minimal disturbance to the mussels) it can be an effective zebra mussel control method for inland waterways and structures.

Future recommendations for a similar trial would include aeration in all enclosures ensuring that the aeration occurs a sufficient distance from settled mussels so as to cause minimal disturbance. Also, settlement plates should be removed less frequently and allowed more time to acclimatise after plate transportation so as to avoid high levels of control mortality. This trial was the first canal treatment with Zequanox and the methods used here support further development of similar application techniques for static, contained, and open water treatments.

Acknowledgements

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References

Aldridge DC, Elliott P, Moggridge DG (2004) The recent and rapid spread of the zebra mussel (*Dreissena polymorpha*). *Biological Conservation* 119: 253–261, http://dx.doi.org/10.1016/j. biocon.2003.11.008

Byrne M (2007) The grand Canal and Tullamore. Offaly Historical and Archaeological society. http://www.offaly-history.com/articles/78/1/The-Grand-Canal-amp-Tullamore/Page1.html (Accessed 27 August 2013)

Karatayev AY, Burlakova LB, Padilla DK (2002) The impact of zebra mussels on aquatic communities and their role as ecosystem engineers. In: Leppäkoski E, Olenin S, Gollasch S (eds), Invasive aquatic species of Europe: Distribution, Impacts and Spread. Kluwer Scientific Publishers, Dordrecht, pp 433–446, http://dx.doi.org/10.1007/978-94-015-9956-6_43

Lucy FE, Burlakova LE, Karatayev AY, Mastitsky SE, Zanatta DT (2013) Zebra mussel impacts on unionids: A Synthesis of Trends in North America and Europe. In: Nalepa T, Schloesser D (eds), Quagga and Zebra Mussels. Taylor and Francis, pp 623–646

Lucy FE (2010) Zebra Mussels: Review of ecology and impacts since invasion in Ireland. In: Mackie G, Claudi R (eds), Monitoring and control of macrofouling molluscs in freshwater systems 2nd Edition. CRC Press, Boca Raton, FL., pp 386–389

Lucy F, Sullivan M, Minchin D (2005) Nutrient levels and the zebra mussel population in Lough Key. ERTDI Report Series No. 34. EPA, Wexford. Available at: http://www.epa.ie/downloads/pubs/research/water/epa_zebra_mussels_ertdi34_final.pdf (Accessed 11 December 2012)

Mackie GL, Claudi R (2010) Monitoring and control of macrofouling mollusks in freshwater systems, 2nd Edition. CRC Press, Boca Raton, FL, 508 pp

Marrone Bio Innovations (2012) http://www.marronebioinnovations.com/products/brand/zequanox/ (Accessed 16 Sept 2013)

Marrone Bio Innovations Ecotoxicology Studies (2012) http://www.marronebioinnovations.com/lightray/site/wp-content/uploads/2012/09/ZQ_EcotoxStudies_0413_web.pdf (Accessed 16 Sept 2013)

Minhin D (2007) Rapid coastal survey for targeted alien species associated with floating pontoons in Ireland. *Aquatic Invasions* 2: 63–70, http://dx.doi.org/10.3391/ai.2007.2.1.8

Minchin D, Lucy F, Sullivan M (2005) Ireland a new frontier for the zebra mussel *Dreissena polymorpha* (Pallas). *Oceanological and Hydrobiological Studies* 34 (Suppl. 1): 19–30

Minchin D, Lucy F, Sullivan M (2002) Zebra mussel: Impacts and Spread. In: Leppakoski E, Gollasch S, Olenin S (eds), Invasive Aquatic Species of Europe: Distribution, Impacts and Spread. Kluwer Scientific Publishers, Dordrecht, pp 135–146, http://dx.doi.org/10.1007/978-94-015-9956-6_15

Minchin D, Moriarty C (1998) Zebra mussels in Ireland. Fisheries Leaflet 177, Marine Institute, Dublin, 11 pp

Meehan S, Lucy FE, Gruber B, Rackl S (2013) Comparing Zequanox® and chlorine as zebra mussel control strategies in an Irish drinking water treatment plant. *Management of Biological Invasions* 4: 113–122, http://dx.doi.org/10.3391/mbi.2013.4.2.03

Molloy DP, Mayer DA, Giamberini L, Gaylo MJ (2013a) Mode of action of *Pseudomonas fluorescens* strain CL145A, a lethal control agent of dreissenid mussels (Bivalvia: Dreissenidae) *Journal of Invertebrate Pathology* 113(1): 113–115

Molloy DP, Mayer DA, Gaylo MJ, Burlakova1 LE, Karatayev AY, Presti1 KT, Sawyko PM, Morse JT, Paul EA (2013b) Non-target trials with *Pseudomonas fluorescens* strain CL145A, a lethal control agent of dreissenid mussels (Bivalvia: Dreissenidae) *Management of Biological Invasions* 4: 71–79, http://dx.doi.org/10.3391/mbi.2013.4.1.09

Pollux B, Minchin C, Van Der Velde G, Van Alen T, Van Der Staay SYM, Hackstein J (2003) Zebra mussels (*Dreissena polymorpha*) in Ireland, AFLP-fingerprinting and boat traffic both indicate an origin from Britain. *Freshwater Biology* 48: 1127–1139, http://dx.doi.org/10.1046/j.1365-2427.2003.01063.x

Rajagopal S, Van der Velde G, Van der Gaag M, Jenner HA (2003) How effective is intermittent chlorination to control adult mussel fouling in cooling water systems? *Water Research* 37: 329–338, http://dx.doi.org/10.1016/S0043-1354(02)00270-1

Appendix G: Risk Assessments

Off Campus Activities
Hazard Identification and Risk Assessment

Version No: Rev 0 August 2010

Off Campus Activity	
An **"Off Campus Activity"** is defined as any external teaching, field work or other sanctioned activity carried out by IT Sligo staff or students to fulfil the teaching requirements of any Full time Programme, in places or premises which are not rented or owned by IT Sligo and which the college does not exert direct control.	
Category	**Type of Off campus activity**
Category 1	Students working off campus, unsupervised, to conduct research etc.
Category 2	Students working off campus with direct supervision by an academic e.g. classes in a local pool, beach, archaeology dig, cultural visits, visits to business premises, river sampling, site surveys etc and returning to campus the same day.
Category 3	Students working off campus with direct supervision by an academic and requiring an overnight stay in Ireland e.g. archaeology dig, cultural visits, visits to business premises, river sampling, site surveys, adventure sports etc
Category 4	Students working off campus with or without direct supervision by an academic and requiring foreign travel e.g. archaeology dig, cultural visits, visits to business premises, river sampling, site surveys, adventure sports etc
Please Consult IT Sligo Policy on Off Campus Activities prior to undertaking any off campus activity	

Hazard Identification and Risk Assessment Please complete Section 1, 2 and 3	
Name of activity	Postgraduate, deployment of settlement plates.
Name of Programme for which the activity applies	Postgraduate project.
Location (s) of where the Activity takes place	Lough Key
Give a bullet point overview of what the activity entails	• Using a boat • Working on water
Assessment Date	N/A
Assessment Undertaken By	N/A
Reviewed by Head of Department (Sign and Date) Supervisor	

Off Campus Activities
Hazard Identification and Risk Assessment

Version No: Rev 0

August 2010

Potential Hazards	Who May be Harmed		Control Measures and Precautions that staff agree to adopt as their normal practice. (Tick X all that apply and delete those that do not)	Is the Overall Risk Acceptable (Yes or No)	Person in Charge Sign and Date
Section 1	**General Hazards** Which may be expected on any Off Campus Activity				
Inadequate planning and organisation	Students, staff and members of the Public		All appropriate gear to be organised prior to the trip	Yes	
			All appropriate members of the public will be notified	Yes	
Insufficient staff training, qualifications and supervision	Students, staff and members of the Public		All staff and postgraduate students are appropriately trained, experienced and qualified to competently fulfil their roles and responsibilities	Yes	
Transport Own Car Other, Transport Accidents	Students, staff and members of the Public		All drivers will have a full license	Yes	
Exposure to adverse effects of weather	Students and staff		plan appropriately do not go out on the boat if the weather is not suitable , bring appropriate clothing and equipment required	Yes	
			Obtain daily weather forecast and adjust plans accordingly	Yes	
			Wear life jackets at all times when on or near the water	Yes	
Emergencies, illness, injury	Students, staff and members of the Public		Make sure staff and students are familiar with the IT Sligo Accident Investigation and reporting procedure.	Yes	
			Carry a charged phone with credit.	Yes	
			Make sure if you have any underlying conditions or illness the appropriate people know	Yes	
			Have all emergency contact numbers to hand	Yes	

Off Campus Activities
Hazard Identification and Risk Assessment

IT Sligo
An Institiúid Teicneolaíochta, Sligeach
Health & Safety

Version No: Rev 0 August 2010

Section 2	Who May be Harmed	Control Measures and Precautions that staff agree to adopt as their normal practice.	Is the Overall Risk Acceptable (Yes or No)	Person in Charge Sign and Date
Activity Specific Hazards	Activity Specific Hazards			
	The Person in Charge must identify the Hazards specific to the activity they have organised and the necessary control measures they will implement to control that hazard. Examples of activities which require a specific Risk Assessment include water based activities, river sampling, travel in foreign countries, caving, archaeology digs, coastal visits, mountain climbing etc. Please contact the Health and Safety Officer for assistance if required			
		Water. Work will be carried out on Lough Key. Life vest worn at all times	Yes	
		Weather. An accurate forecast from will be obtained	Yes	

Section 3	Mandatory Requirements					
What is the Staff to Student Ratio	N/A					
Is a Certificate of Medical Fitness required	N/A					
Is parental consent Required for young persons under 18	N/A					
Category Of Activity	1					
Date(s) of Activity	23-07-13					
Location of Activity	Lough Key					
Person in Charge Sign and Date	Sara Meehan 9/9/13					
	Have you left the list of all staff and students who will be on the trip with emergency contact numbers with the School Secretary N/A					
	A Copy of this document forwarded to the School Secretary for record keeping purposes					

RESEARCH RISK ASSESSMENT FORM		IT Sligo An Institiúid Teicneolaíochta, Shligeach Health & Safety	
Version No	Rev 1	**Date**	December 2010

Name of Research Supervisor	Dr. Frances Lucy
Name of Research Worker	Sara Meehan
Where will the work be undertaken (Lab numbers etc)	G2013 and some field work
Date Research will commence	February 2011
Date Research is due to finish	February 2014

Is the Research being undertaken by a

Under graduate		Post graduate	yes	Other	

Project Title

Assessment and Utilisation of Zequanox® for Zebra Mussels (*Dreissena polymorpha) control in Irish waters*

Please give a brief Description of the work to be undertaken including the nature of the materials and techniques to be used

Scientific Trial for zebra mussel control using Zequanox®, a specific biologically friendly bio control agent. The work will be undertaken in the research lab G2013 and field work will also be undertaken, the specific site location will be dependent on the species to be collected, namely Drumcliff river and Lough Gill. The final stage will involve and open water trial in Tullamore Harbour. No chemical necessary, lone working will be carried out in the lab in IT Sligo only.

Hazard Identification and Risk Assessment
Form the Categories below please tick the hazards which apply to the Research Work being undertaken

Hazard (Tick all those that Apply)		Risk Score (H, M, L)	List the Control Measures which will be put in place to manage the Hazard
Chemicals		N/A	
Fire or Explosion Risk		N/A	
Gas Release/high pressure		N/A	
Highly Flammable Liquid		N/A	
Oxidiser		N/A	
Corrosive		N/A	
Endo-exothermic		N/A	
Known carcinogen/mutagen		N/A	
Reproductive Hazard		N/A	
Highly Toxic		N/A	
Biological		N/A	
Radioactive		N/A	
Cryogenic		N/A	
Large quantities of dust e.g. from wood or		N/A	

RESEARCH RISK ASSESSMENT FORM		IT Sligo An Institiúid Teicneolaíochta, Sligeach Health & Safety	
Version No	Rev 1	**Date**	December 2010

Hazard Identification and Risk Assessment
Form the Categories below please tick the hazards which apply to the Research Work being undertaken

Hazard (Tick all those that Apply)		Risk Score (H, M, L)	List the Control Measures which will be put in place to manage the Hazard
concrete			
Hazardous to the environment		N/A	
SDS for any chemicals being used in the Research have been read and understood by the Research Worker ☐ (Tick if Yes)			
Equipment and Apparatus			
Lasers		N/A	
X Ray		N/A	
UV		N/A	
Other please detail		N/A	
The Research Worker has been trained on the use of all equipment he/she will use during his/her Research ☐ (Tick if Yes)			
Lone Working			
Working in Lab on own		M	Let someone know where I am
Collecting field samples on own		M	Let someone know where I am and what time will be back, bring a phone
Working outside normal college hours		M	Let someone know where I am and what time will be home, bring a phone
Human Testing			
Does any of the work involve testing of human subjects		N/A	
Are there any Other Health and Safety Hazards associated with the Research Work			
Slips, trips and falls		M	Where possible do not work alone, have emergency numbers to hand
Water		M	Where life vest and do not enter deep water

Signatures and Date	
Supervisor	16/2/11
Research Worker	Sara Meehan
Departmental Head	21 May 2014 Updated.

COMPLETED FORMS SHOULD BE SENT TO THE SCHOOL OR DEPARTMENTAL SECTARY FOR FILLING. A COPY NEEDS TO BE HELD BY THE SUPERVISOR AND RESEARCH WORK FOR THE DURATION OF THE WORK BEING UNDERTAKEN