Marine bioinvasion prevention: understanding ballast water transportation conditions and the development of effective treatment systems.

Katharine Jennifer Carney

A thesis submitted for the award of Doctor of Philosophy (PhD) at Newcastle University

School of Marine Science and Technology

Armstrong Building

Newcastle upon Tyne

NE1 7RU

July 2011

Abstract

Man's impact on the Earth is constantly increasing due to ever progressing technological developments. One of our major impacts is the transportation of organisms to new habitats, leading to alterations of existing ecosystems. Mechanisms responsible for the transportation of marine organisms are mainly associated with the shipping industry e.g. hull fouling, sea chests and ballast water. Ballast water has long been recognised as one of the major mechanisms by which aquatic organisms are transported to new environments. In 2004 the International Convention for the Control and Management of Ships' Ballast Water and Sediments was adopted and measures were implemented to reduce and control the number of future invasions.

This thesis has addressed aspects relevant to the future prevention of organism transport via ballast water. Firstly, during ballast water uptake organisms are exposed to potential damage whilst passing through a centrifugal pump. Upon reaching the ballast tanks they are stored in dark, confined conditions. These processes are not intended to damage individuals, but both could potentially kill organisms and reduce the discharge of live individuals. Both processes were examined in isolation to determine their effect on plankton survival. To manage ballast water introductions water treatment technologies have been investigated to determine their ability to kill plankton. This study assessed three technologies: a stainless steel 40μm screen filter, a UV light and a chlorine based chemical, for their potential in ballast water treatments. A further challenge facing researchers involved in developing ballast water treatment systems is accurately assessing the resulting mortality in plankton from treatments. Five common viability assessment methods were investigated and their application on test organisms and natural populations examined.

This thesis concludes that no significant mortality was caused to plankton by a centrifugal pump, and phytoplankton are able to survive long periods in dark confined conditions. Thus these processes will not prevent viable organisms reaching new destinations. The three treatments assessed were all effective on two

test species and could be utilised in large scale treatment systems on board vessels to minimise introductions. Finally, while viability is difficult to assess in plankton using viability stains it is possible to obtain accurate information if the methods used are properly optimised prior to use.

Acknowledgements

Well, this has been a long time coming….!

My first thank you is of course to my supervisors Ehsan and Jane. I am very grateful for the chance to work on BaWaPla and to be taken on to do a PhD - much more than I was expecting after signing up for a short masters project! Thanks as well to the extended BaWaPla group – Kayvan, Behrooz, and Shane and John from the early days. I'll not miss the Friday nights spent in the lab (in the dark!) with FlowCAM, but I definitely learnt a lot throughout the project and enjoyed the meetings in warmer climes!

In addition to BaWaPla the chance to work on the UKIERI project was a great experience. My three months in Goa were an interesting experience for me, and one I am very grateful to have had the opportunity to do. Thanks to the team at the NIO I worked with, including Dr. Anil and Dr. Sawant and to my office mates over there - Ravi, Priya, Shamina and Chetan. And of course Mani, don't worry - the end is near!!

Thanks to my family for seeing me through the last few years. Not much fun at times, but we got there in the end….!! The weekly chats, pep talks when needed them and visits were really appreciated. And Mum, I can now finally say – yes, I'm finished!!!! To my extended family – Pete, Em, Han, Vic & Gregg - thank you for the many visits, trips, phone calls, msn chats, Mario nights, dinners, etc etc etc. I think I owe you all a drink…!

Finally, thanks to everyone else! The guys at home, the Nottingham lot (aka the deserters!!), others from Uni and everyone who's been at the Dove over the last few years. Annie and Stu - I now have no excuse not to go running, so bully me all you want (within reason!)…..here's to the Great North in sub 2 hours!!!

Table of Contents

List of Figures

List of Tables

Chapter 1

General Introduction

Since the beginning of life on Earth, organisms have adapted to co-exist in carefully balanced ecosystems (Carson 1962). In the 1970s Lovelock described the Earth as a single living system comprising of all living organisms and material parts which make up its surface, he named this system 'Gaia' (Lovelock 1972). Lovelock hypothesised Gaia to be a self regulating system, which since its origin has maintained the conditions on Earth at the 'best balance' for life to survive. As such he determined that while man may pollute Gaia she is capable of reversing any detrimental impacts arising from our activities and restoring this 'best balance' (Lovelock 1979).

The evolution of mankind has brought about changes to the planets natural balance through our evolution and technological advances. With this however comes increasing detrimental impact on the ecosystem goods and services our planet provides. In a revised edition of 'Gaia: a new look at life on Earth' Lovelock retracted his earlier idea that mans activity could not permanently damage the Earth. In light of more recent scientific developments, e.g. ozone depletion and climate change, he recognised our ability to push beyond the limits at which Gaia alone can reverse our impact (Lovelock 2000).

You do not have to look far to see the effects of mankind on our planet, and we are modifying habitats at a faster rate than the environment and organisms are able to adapt or recover (Carson 1962). The atmospheric composition has been altered through the release of chemicals, e.g. CO_2, NO_x, SO_x and CFC's. The landscape both above and below the seas has changed due to drilling and quarrying. Seas and rivers have been drained due to extensive irrigation practices and pristine environments inhabited and used for farm land as population pressure increases. In past years these actions have been undertaken with little regard for the resulting effects on the

environment. However, due to scientific studies the impacts have been recognised as potential threats to mankind itself, and the consequences need to be addressed (Carson 1962).

In 1962 the publication of the book Silent Spring by Carson brought about an 'awakening' of the modern environmentalist movement. At its release Silent Spring was a highly controversial book in which Carson brought environmental issues regarding the use of pesticides to the attention of the general public. The book received heavy criticism, much of which came from the pesticide manufacturers who claimed that Silent Spring "was dangerous to the world because pesticides were cutting edge progress and to ban them would return the world to the dark ages of pestilence and famine" (Haynes 1989). This view shows a common vision at that time that the advancement and survival of the human race required our "mastery of the environment" (McCormick 1989). In the aftermath of Silent Spring and in light of other scientific advancements, including the earlier publication of Darwin's Theory of Evolution in 1859, a change in the view of our place in nature and the concept that survival of mankind is dependent on the maintenance of the Earth became widely realised (McCormick 1989; Lovelock 2000). Mankind has a duty to minimise our detrimental effects on the Earth not only for the benefit of mankind, but for the benefit of the Earth itself. The survival of each relies on the health of both (Carson 1962).

With this new environmentalist vision came the establishment of groups which aimed to protect the environment from the unsustainable actions of mankind. These include the Environmental Protection Agency, Greenpeace and Friends of the Earth. Management practices were implemented and one of the most successful has been Ecosystem-based management (EBM). The EBM approach takes into account all interactions, including human activity, existing within an ecosystem rather than focusing on just one aspect (Slocombe 1993; Christensen et al. 1996). This approach recognises the complexity, fluidity and unpredictability of biological systems and that by improving our understanding of ecosystems we can better predict their response to anthropogenic activity (Slocombe 1993; McLeod and Leslie 2009). This promotes an

adaptive management strategy and a precautionary approach to the management of natural systems in light of our incomplete understanding as to how ecosystem components interact. When implementing management practices it is important to recognise that due to their complexity we cannot manage the ecosystems themselves, merely the human influences acting on them (McLeod and Leslie 2009).

The philosophy behind this thesis is simply this: 'mankind impacts the Earth'. During its lifetime every living creature has an effect, an impact on its surroundings and the overall well being of the planet. Every action we take has a consequence, be it beneficial or detrimental. It is necessary for us to not only recognise the problems we cause but to act to repair the damage through effective management practices. This thesis addresses one anthropogenic impact on the environment: the transportation of aquatic organisms across the globe via shipping.

Organisms are naturally adapted to their environment and remain in specific regions due to natural or geographical barriers which prevent them from spreading. However, the human aided introduction of organisms to new environments has been occurring since ancient times (Elton 1958). These early introductions occurred through the transportation of plants and animals to new areas for agriculture, as well as the unintentional transfer of seeds and spores by nomad tribes (Leppakoski et al. 2002). This past movement of terrestrial, freshwater and marine organisms is undocumented and the extent of such introductions will be forever left unknown (Leppakoski et al. 2002).

Due to increased observations and surveys of global habitats recent introductions have been recorded and new invasions traced. While organisms can enter new ecosystems and fill a vacant niche (Elliott 2003), other introduced organisms have been observed to detrimentally impact the environment, the economy, human health, tourism, fishing and agriculture in the receiving area (E.g. Hallegraeff and Bolch 1991; Ruiz et al. 1997; Hall and Mills 2000; Herborg et al. 2003; Lewis et al. 2003; Bolch and de Salas 2007).

In 1908 the first marine invasive species, the diatom *Odontella sinensis*, was recorded by Ostenfeld in the North Sea where it is thought to have been introduced through shipping. Shipping, via hull fouling, ballast water and sea chests, is currently responsible for most introductions in the marine environment (Eno et al. 1997; Bax et al. 2003). In addition, deliberate introductions, e.g. for aquaculture, and the building of shipping canals, such as the Suez and Panama Canals, have resulted in many species colonising new areas (Galil 2008). The main factor in terms of increased introductions in the marine environment has been the growth of the shipping industry and the development of bigger, faster ships which carry larger volumes of ballast water. Since the observation of *O.sinenses* hundreds more introduced marine organisms have been recorded (see Chapter 2) with an estimated 10,000 organisms being transported in ballast tanks at any one time (Bax et al. 2003).

This thesis identifies one of the major mechanisms by which aquatic organisms are transported to new environments: ballast water. The studies completed have looked at the factors which are potentially detrimental to plankton survival during uptake and transportation in ballast tanks and the management techniques in practice and in development to treat the ballast water before discharge. This information will increase our understanding of these processes to aid future management strategies. The spread of organisms via ballast water is a clear example of anthropogenic activity detrimentally impacting the environment and effective management is vital.

Chapter 2

Literature Review

2.1 Bioinvasions: the problem, vectors and potential solutions

Across the globe marine and freshwater ecosystems are being invaded by non-native organisms. These invasions are referred to as 'bioinvasions'. Bioinvasions consist of the transport of plants, animals, bacteria, viruses and fungi to new environments where these newly introduced organisms have the potential to detrimentally affect ecosystems (Elton 1958). The reported scale of introductions into different oceans and seas varies greatly, and this is shown in Table 2.1.

Table 2.1. The number of reported non-native organisms in seas and oceans worldwide.

Area	Number of non-native organisms reported	Source
Atlantic and Channel coast	104	Goulletquer et al (2002)
Australia	129	Sliwa et al (2008)
Baltic Sea	125	Online database (Eds: Olenin, Leppakoski and Daunys): (http://www.corpi.ku.lt/nemo/alien_species_directory.html) Updated on 19.01.2010.
Black Sea	43	Gomoiu et al. (2002)
Brazil	53	Ferreira et al (2008)
British and Irish waters	79	Minchin and Eno (2002)
China and Korea	136	Seo and Lee (2008)
Marmara Sea	11	Ozturk (2002)
Mediterranean Sea	558 903	Galil (2008) Zenetos et al. (2008)
New Zealand	149	Hayden et al (2008)
Norwegian waters	211	Hopkins (2002)
North America: Chesapeake Bay Great Lakes San Francisco Bay	170 137 113	Fofonoff et al (2008) Ruiz et al. (1997) Cohen et al (2005)
North Sea: British coast Belgium Netherlands Denmark Germany Norway Sweden	52 44 94 30 59 38 28	Gollasch et al (2008)
South Africa	22	Griffiths et al (2008)
SE Pacific – Chile and Peru	51	Castilla and Neill (2008)

The variation in the number of reported non-natives in different areas can be due to differing scales of organism transportation throughout the globe, but in many cases it is due to a lack of scientific research and understanding of the problem on a local or regional level. In the absence of a comprehensive audit of species and regular surveys thereafter, the introduction of new organisms to an area can go unnoticed. A further hurdle to the reporting of invasive species is taxonomic expertise. It is common for invasive species to be misidentified as local species or simply named as 'new and undescribed' when identification of organisms from different regions is beyond the expertise of the taxonomist (Campbell et al. 2007). The Mediterranean Sea is heavily invaded (Table 2.1) and so there is a high search effort in this area to identify new species. Recent studies have recorded one new invader every week between 2003 and 2008 (Rilov and Crooks 2009). Prior to this, the Smithsonian Institute, Hebrew University of Jerusalem and the Sea Fisheries Research Station, Haifa, took part in the 'Cambridge Expedition to the Suez Canal'. This lead to high recorded numbers of invasive species in the Mediterranean Sea during the 1920s and the 1970s, the periods during which the surveys were carried out (Galil 2008). Programmes such as this enable us to monitor organism spread. While not performing these studies will lead to fewer reported introductions, the costs involved and time required to complete the studies do not allow them to be undertaken on a regular basis.

There are a number of ways in which organisms have successfully spread to new environments and this is possible due to easy transport routes, both natural and human-aided, throughout the oceans. Natural processes can aid the movement of organisms, for example, larvae can be naturally dispersed by currents (Cohen and Carlton 1997). Organisms can actively travel along canals or drift with the water movement, and floating debris can carry encrusting and small organisms to new environments (Cohen and Carlton 1997; Bax et al. 2003).

Most transport vectors are associated with human activity and those which are currently responsible for the most introductions are related to the shipping industry i.e. through hull fouling communities and the water in ballast tanks and sea chests (Bax et al. 2003; Sylvester and MacIsaac 2010). Recreational boating can spread organisms

through hull fouling, although on a much smaller scale to the shipping industry, and scuba diving can transport spores or bacteria which attach to the gear (Bax et al. 2003). Adults and juveniles can be transported on semi-submersible platforms or other long distance, slow moving platforms as well as in fisheries products (Cohen and Carlton 1997). The larvae of marine organisms can be transported in water with shipments of live fish (Cohen and Carlton 1997). Live organisms can escape or be released from research, private or public aquaria (Cohen and Carlton 1997). For example, the alga *Caulerpa taxifolia* was accidentally released from the Monaco aquarium into the Mediterranean Sea (Eno et al. 1997). Early observations deemed oyster culture the vector responsible for the transportation of most marine organisms across the globe (Elton 1958). Aquaculture, including oyster culture, is still considered to be the main route of macroalgal transport (Eno et al. 1997; Minuer et al. 2007) and it has been responsible for the movement of some organisms through intentional release for the purpose of replenishing stocks, discarded equipment i.e. nets, floats, traps, trawls and through the movement of gear, stock and food (Bax et al. 2003).

Although it is difficult to determine exact routes of introduction, as previously mentioned the shipping industry, through hull fouling, ballast water and sea chests, is now deemed to be the main vector for organism movement to new environments. In terms of relative importance, shipping exceeds other vectors in the number of organisms it has transported. Studies have tried to quantify the responsible transport mechanisms, and the status of introduction routes of foreign organisms present in British waters in the 1990s is displayed in Figure 2.1. This identifies shipping mechanisms as the main culprit, hull fouling as the main contributing vector, and mariculture also responsible for many introductions (Eno et al. 1997).

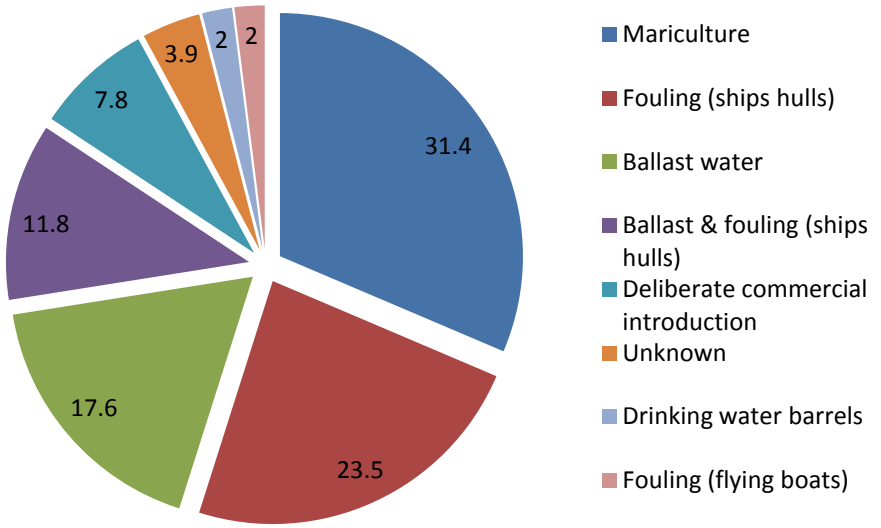

Figure 2.1. The estimated percentage of introductions via each vector to British waters. Adapted from Eno et al (1997).

Gollasch (2002) identified that 47 introduced species in the North Sea had been transported via ships, while only 36 had arrived associated with mariculture. Of the arrivals brought through shipping approximately 30 species were estimated to have been introduced via hull fouling (Gollasch 2002). In a recent study Gollasch (2007) also showed the importance of hull fouling for introducing marine organisms, see Figure 2.2. This study showed a reduction in the importance of aquaculture and non-shipping routes, while ballast water and shipping remained important vectors.

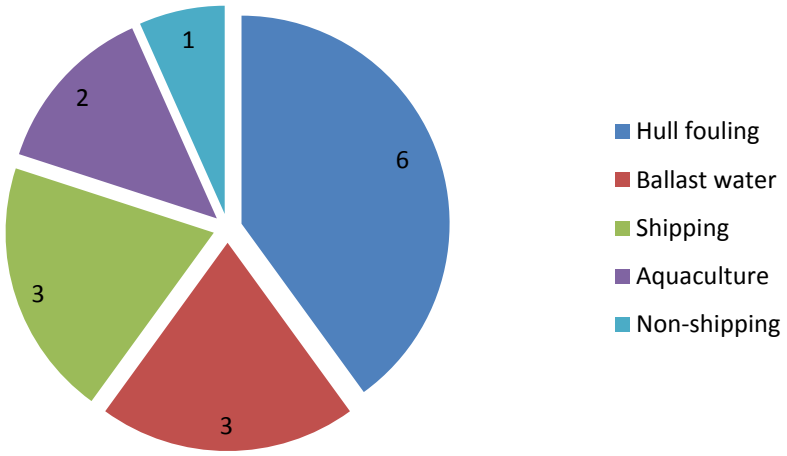

Figure 2.2. The number of geographical regions in which each vector was responsible for the most introductions, determined by Gollasch (2007). Three further locations showed equal introductions via two or more vectors.

Sea chests are required by ships to increase the efficiency of the ships pumps during water uptake by reducing air cavitation. They are present as recesses below the water level, sealed by grids with holes or slots of up to 35mm width. Small organisms are able to enter and have been found to grow to adult stages capable of releasing larvae into the water within these sea chests (Coutts et al. 2003; Coutts and Dodgshun 2007). Coutts and Dodgshun (2007) found 150 different types of organisms in sea chests of vessels from a number of different ports docked in New Zealand. The organisms observed included both mobile and sessile species. The authors suggested that sea chests are underestimated in their contribution to the movement of organisms to new locations and they are insufficiently researched.

Hull fouling has long been recognised as a method of organism transport and is a problem not only because of this, but also due to the increased drag imposed on the ship through the presence of hull fouling communities. These communities are composed of sessile organisms, for example the barnacle *Balanus improvisus*, and mobile organisms such as the crab *Hemigrapsus penicillatus* (Gollasch 2002). The presence of these communities is a problem for the shipping industry as the increased drag forces on the ship reduce the speed of the vessel and increase fuel costs. This has

lead to much research into effective antifouling paints which can be applied to ships to prevent fouling communities establishing themselves on the hulls. The current widespread use of successful antifoulants, in addition to the shift from wooden hulls to metal hulls have led to a decrease in the number of organisms able to foul ships hulls. While this has reduced the number of organisms transported via this vector hull fouling is still the most important vector for transporting organisms (see Figures 2.1 and 2.2) (Carlton 1985; Eno et al. 1997; Ruiz et al. 1997; Gollasch 2007).

Ballast water is ambient water which is loaded into ballast tanks and is required by vessels for stability and trim when the ship is empty of cargo (Gollasch et al. 2000b; Wonham et al. 2001; Drake et al. 2002; Lewis et al. 2003) to keep the propellers submerged when the ship is not fully loaded (Gollasch et al. 2000b), and to compensate for the altering weight of the vessel as cargo is loaded and unloaded at different ports (Lewis et al. 2003). The uptake of ballast water generally occurs as cargo is being unloaded from the vessel, water is pumped from the immediate water surrounding the vessel into the ballast tanks through filters which remove larger, adult organisms but do not prevent the uptake of plankton. As these filters do not affect the diversity of plankton which enters the tanks ballast water transport is non-selective. Phytoplankton such as diatoms and dinoflagellates, and zooplankton such as copepods, barnacle cyprids, isopods, radiolarians, mysids, euphausiids and bivalve, gastropod and crab larvae have all been found in ballast tanks (Carlton 1985).

The shipping industry is growing, in July 2010 the global shipping fleet stood at 53,005 vessels (Lloyds Register Fairplay 2009). Between 1998 and 2008 the UK shipping fleet alone increased from 416 to 842 vessels, vastly increasing the amount of ballast water required (Transport Statistics Great Britain 2009). In 2002 the IMO estimated that between 3 and 10 billion tonnes of ballast water was transported annually (IMO 2004). The scale of the ballast water problem grows as the shipping fleet increases. There is a vital need for adequate treatments to be developed to prevent this constant movement of organisms to new areas where they are establishing populations to the detriment of the local flora and fauna.

Not only are ships themselves transporting organisms, but in our efforts to reduce transit times between countries through the building of shipping canals, e.g. the Suez Canal, we have provided new routes for natural organism movement and invasions to occur (Carlton 1985; Bax et al. 2003). The Suez Canal was opened in 1869 to link the Red Sea with the Mediterranean Sea and is the world's densest shipping lane. In March 2010 a total of 1467 vessels travelled through the Suez canal, an average of 49 vessels per day (Suez Canal Traffic Statistics 2010). Since its opening an influx of organisms have travelled through the Suez Canal and 10% of established invasive species in the Mediterranean Sea are native to the Red Sea (Galil 2008).

The prevention of the spread of organisms via ballast water is a major concern due to the economic impacts of these organisms and impacts on the environment and human health. The different technologies which have been proposed as potential treatments include filtration, heat treatment, UV light, ozone, biocides and deoxygenation. However, none of these methods has proven effective against all organisms in all conditions. The likelihood of one treatment being able to work in every circumstance is very small due to the vast range of organisms present in the world's seas, oceans and rivers, in combination with the wide range of conditions they are able to tolerate and the number of ways they have adapted to survive unfavourable conditions.

2.2 The historical use of ballast water

Initially solid materials such as sand, shingle and beach detritus were all commonly used materials loaded onboard vessels by hand for ballast (Carlton 1985; Gollasch et al. 2000b; Minchin et al. 2008). This solid ballast was often unloaded directly to the dockside and reused by subsequent ships. In the 1880s water began to be used as ballast water and by the 1930s the majority of vessels had switched to use water as ballast (Minchin et al. 2008). This switch was aided by the development of steel ships and more efficient engines for the pumping of water onboard (Minchin et al. 2008). Originally ballast water was stored in empty cargo tanks for the duration of the voyage. The cargo often consisted of iron or crude oil and traces of chemicals from these

materials remained within the tanks and were thus present in the ballast water. These chemicals could be toxic and so when released with the ballast water they killed organisms in the destination port. However, these chemicals were useful in that they killed many of the organisms found within the ballast water and helped to prevent species introductions (Carlton 1985). In the 1980s tanks were designed specifically for the purpose of holding ballast, these are known as 'segregated' or 'dedicated' tanks (Carlton 1996). Their implementation was determined by the MARPOL convention 'The International Convention for the Prevention of Marine Pollution from Ships' (73/78) Annex I which entered into force in 1983. This change resulted in ballast water becoming cleaner, preventing the release of toxic chemicals during the discharge process but also enabling more organisms to survive within ballast tanks (Carlton 1985). Another factor which has led to cleaner ballast water is the recent global ban on the use of the antifouling paint tri-butyl tin (TBT) as a preventative for fouling on the hulls of vessels. When it was in use TBT contaminated port and coastal areas as chemicals leached from the hulls of ships into the water column. As it is no longer in use, TBT levels have decreased (Minchin and Gollasch 2003).

2.3 Organism survival within ballast tanks

To reach a new environment via ship transport organisms must survive three stages in the journey. These are the initial uptake into the tanks, the voyage itself and the discharge process (Wonham et al. 2001). During uptake into tanks organisms pass through the ballast pumps and could become damaged as they travel through by colliding with the impellers (Carlton 1985; Gollasch et al. 2000a). Suggestions have been made that this process is responsible for initial high levels of mortality in organisms entering ballast tanks (Gollasch et al. 2000a). By identifying the impact ballast water uptake has on plankton viability we could determine whether it actually aids treatment processes, or whether the observations previously made by Gollasch et al (2000a) attributed initial levels of mortality to an incorrect source. This has been addressed in Chapter 3.

Conditions within the tanks are extremely harsh and vary throughout the journey. The temperature of the water changes, mapping within a few degrees that of the outside seawater during transit (Gollasch et al. 2000a), pH, oxygen and salinity levels also vary depending on the conditions of the water at the location of uptake. There is no light within the tanks (Carlton 1985), and it has been suggested that many phytoplankton die within days of uptake due to the lack of light which prevents photosynthesis. This would then leave only mixo- or heterotrophic feeding options (Gollasch et al. 2000a).

Contrary to this observation, many planktonic organisms are able to form resting stages in unfavourable conditions, so while they are not present in an active form they remain viable. There are many names given to these resting stages, including 'spores' when referring to diatoms, 'cysts' for dinoflagellates (Furusato et al. 2004) and zooplankton can enter a 'resting state/diapause' (Conover and Siferd 1993). The formation of resting stages was originally thought to be a cell's response to nutrient limitation, but species of polar phytoplankton have been observed to produce these resting stages in conditions with sufficient nutrients. It is now thought that a reduction in light availability can induce organisms to enter these stages in order to increase their chances of survival (Peters and Thomas 1996). For phytoplankton this process involves the cell changing its structure to a more resistant 'resting' form, which will become active again once the cell enters non-limiting conditions. Diatom spores often have a thick outer shell made from silica which enables them to survive in cold, dark conditions. The cells survive by sealing their membranes and reducing metabolism to a minimal level (Peters and Thomas 1996). This conserves storage products required for maintenance (Peters 1996). Dinoflagellates can produce cysts through both sexual and asexual processes and these cysts show reduced cell metabolism of up to 98% (Rinalta et al. 2007). These resting stages are negatively buoyant and sink into the sediment where they are able to remain inactive for years, germinating when environmental conditions become favourable (Drake et al. 2007). The sediment that accumulates at the bottom of ballast tanks builds up over time, containing sediment deposits from a range of different ports of ballast water uptake (Hamer et al. 2000). Dinoflagellate cysts, diatom resting spores and copepod diapause eggs have been collected from ballast water tank sediment (Hallegraeff and Bolch 1991; Hallegraeff and Bolch 1992;

Hamer et al. 2000; Bailey et al. 2003; Pertola et al. 2006) and successfully germinated (Hallegraeff and Bolch 1991; Hallegraeff and Bolch 1992; Bailey et al. 2003; Pertola et al. 2006). The discovery of approximately 300 million viable cysts of the potentially toxic dinoflagellate *Alexandrium tamarense* in just one ballast tank highlights the importance of this vector as a transport mechanism for increasing the range of toxic species (Hallegraeff 2007).

Diapause is a common process in marine and terrestrial animals defined as 'a stage in the development of certain animals during which morphological growth and development is suspended and greatly retarded' (Andrewartha 1952). Zooplankton can enter diapause as a natural part of their life cycle. For example, many copepod species spend the winter months in a resting state and become active again as the water temperature increases: this has been observed in zooplankton living at high and mid latitudes (Conover and Siferd 1993). When in unfavourable conditions, the organisms are able to enter diapause or produce diapause eggs. When in diapause individuals seek deep water, regulate their buoyancy, decrease their activity and metabolism, change the metabolic substrate they oxidise during respiration and reduce levels of digestive enzymes and gut peristalsis (Conover and Siferd 1993). Diapause eggs are inactive but contain fertilised embryos which are maintained in a protective casing and can hatch if stimulated by favourable conditions (Bailey et al. 2003). An assessment of diapause eggs present in ballast water sediment observed that light was not required for eggs to hatch, and turbidity within tanks which can re-suspend eggs could stimulate hatching (Bailey et al. 2003). Diapause gives the organisms themselves and not just their eggs the chance to survive the voyage (Marcus 1980; Carlton 1985).

The response of organisms to ballast tank conditions is little understood. Organisms which enter these tanks are exposed to a set of environmental conditions which could enable the populations to thrive, or could result in mass mortality. A number of studies have looked at the composition of surviving organisms at the end of journeys (Medcof 1975; Williams et al. 1988; Hallegraeff and Bolch 1991; Carlton and Geller 1993; Dickman and Zhang 1999; Zhang and Dickman 1999; Hamer et al. 2000; Lewis et al. 2003; Pertola et al. 2006; David et al. 2007), with a view to determining the threat

posed by the ballast water discharged to the destination port. However, there are only five published studies to date which have monitored plankton populations within ballast tanks for the duration of journeys (Lavoie et al. 1999; Gollasch et al. 2000a; Gollasch et al. 2000b; Olenin et al. 2000; Klein et al. 2010). Even these few available studies highlight the wide variability of responses of different taxa to the conditions in ballast water. Gaining an understanding of the effects of ballast water transportation within the harsh conditions of the tanks could aid risk management processes to determine the threat posed by discharge from a specific journey. Assessing the effect of dark, confined conditions, the importance of geographical location and seasonality on the uptake of a plankton population, and the effect of nutrient availability in receiving waters on recovery potential will provide a more complete understanding of the transportation process. Recent studies have used temperature, salinity and shipping traffic data to assess invasion risks in specific regions (Keller et al. 2011; Liu and Tsai 2011), and by including the effects of ballast tank conditions on organisms risk management could be made more accurate. The effect of dark, confined conditions on phytoplankton has been investigated in Chapter 4.

Different conditions are experienced at the bottom of the tank where sediment builds up. As well as containing cysts and resting stages of phytoplankton and zooplankton (Hallegraeff and Bolch 1991; Hallegraeff and Bolch 1992; Hamer et al. 2000; Bailey et al. 2003; Pertola et al. 2006), it is well documented to be species rich with organisms forming stable communities (Gollasch et al. 2000a). The amphipod *Corophium acherusicum* has been found living in the sediment of ballast tanks 116 days after uptake into the tank (Gollasch et al. 2000a). The sediment in tanks is therefore very important for the transportation of these resting stages by providing a refuge from exchange and discharge practices, and it can also support the growth of planktonic larvae to adult stages. The only way to reduce the risk of transporting species in this sediment is to completely remove it as ballast water is exchanged. However, the ballast water exchange methods employed do not remove this sediment which causes it to build up within tanks (Hallegraeff and Bolch 1992). In some cases sediments have been observed to be thrown overboard in the destination port, thus releasing alien individuals into the new environment (Williams et al. 1988). The treatment of

sediments has been addressed by the IMO and measures put in place to minimise their discharge. In 1997 the MEPC requested that Port States have adequate reception facilities for sediments from ballast tanks, or that these sediments should be emptied during mid ocean ballast exchange and not into the destination port (MEPC 1997). The 2004 IMO Convention called for the Port States to ensure sediment reception facilities were available at ports in which cleaning or repair of ballast tanks takes place (IMO 2004). Any removal or disposal of sediments must be performed in accordance with protocols stated in the ships Ballast Water Management Plan, (a mandatory plan to be implemented by all vessels) (MEPC 2006a). In addition, the MEPC (2006b) G2 Guidelines advise on ways in which to design ships to minimise the uptake of suspended material.

2.4 Risks from invasive species

Organisms which successfully reach a new environment and establish populations can be referred to as 'alien', 'non-native', 'non-indigenous', 'introduced', 'exogenous' or 'invasive' species. All of these terms mean that the organism was introduced to a new area via human activity, but only the term 'invasive' species means that the organism is harmful to the receiving environment (Sandlund et al. 1999). Invasive species have been defined as *'playing a conspicuous role in the recipient ecosystems, taking the place of keystone species and/or being economically harmful'* (Elliott 2003). They impact native diversity by competing with and decreasing the abundance and diversity of local and endemic species, and are contributing to the global 'homogenisation' of the earth's biota (Ruiz et al. 1997; Hall and Mills 2000; Lewis et al. 2003). A major problem in determining the extent of species introductions is proving whether or not a species is foreign. Establishing whether an organism is in its native habitat is extremely difficult, and if the area has not previously been studied it may be assumed that an organism is native when it has been unknowingly introduced (Ruiz et al. 1997). It is not possible to conclusively determine which organisms will become problematic in a new area as they will often show no negative impacts in their native environment (Williams et al. 1988; Carlton and Geller 1993; Eno et al. 1997; Ruiz et al. 1997) and when in

different conditions in a novel community they are unlikely to behave as they do in their natural habitat (Ruiz et al. 1997). This unpredictability has been named 'ecological roulette' and it is therefore better to prevent organisms spreading rather than having to deal with the consequences afterwards (Carlton and Geller 1993). Some organisms can be present in a new environment for years before becoming problematic, and some introductions only occur years after the exchange of water from two locations has begun (Carlton 1996; Ruiz et al. 1997). It is even possible that foreign species can benefit their new environment, for example by creating commercial and recreational fisheries (Ruiz et al. 1997). This occurs if the organism is able to fill a vacant niche within the receiving ecosystem (Elliott 2003). The threats imposed on new environments due to invasive species can fit into three categories: health threats, economic threats and environmental threats. Each of these will now be discussed in more detail.

2.4.1 Impacts to health

Health threats can be brought to new areas by invasive species. The human pathogens *Vibrio cholera* and *Escherichia coli* have both been found to survive transport in ballast conditions (Mimura et al. 2005). Concentrations of bacteria and viruses in water are normally between 10^6 to 10^{11} per litre (Drake et al. 2002), which is six to eight times higher than that of other organisms (Ruiz et al. 2000). Viruses and bacteria are able to survive as they reproduce asexually, reproduce rapidly when conditions become suitable and form resting spores in adverse environments. They are also able to tolerate a wide range of salinity and temperatures and therefore can survive in more foreign ports than other organisms (Ruiz et al. 2000). Ruiz et al (2000) assessed ballast water from 15 ships docked in Chesapeake Bay for the presence of *V.cholera*, and all samples contained the virus, demonstrating the potential of ballast water to spread this life threatening disease (Ruiz et al. 2000).

Alexandrium tamarense is a toxic dinoflagellate which produces paralytic shellfish poison (PSP) and has been found in Scottish waters after an introduction via ballast water from North America. This species' ability to form cysts along with the short

voyage times associated with shipping around Europe leave it as a prime species for spreading further into Europe (McCollin et al. 2007b). The potentially toxic dinoflagellate *Pseudo-nitzschia* spp., has also been observed in ballast tanks (Hallegraeff 2007). PSP producing dinoflagellates are naturally found along coasts and during blooms they can affect fisheries and if ingested can prove fatal for humans. The occurrence of these blooms can affect bird and mammal populations occasionally, but the effects are usually seen in humans after consumption of infected shellfish (Hallegraeff and Bolch 1991). The range of places in which these dinoflagellate blooms are being found has increased, which is likely due to their transport to new areas via shipping, and this is leading to concerns for human health (Hallegraeff and Bolch 1991; Bolch and de Salas 2007).

2.4.2 Economic impacts

Economic losses result from the impacts of introduced species and the major industry affected is fisheries. Losses can be due to damaged gear or declines in fish abundance due to the effects of these invasive species. The diatom *Coscinodiscus wailesii* was introduced into the North Sea via ballast water and affects the fishing industry by clogging nets with excess mucus (Hallegraeff 2007). The ctenophore *Mnemiopsis leidyi* was introduced to the Black Sea in the 1980s. In 1989 a large bloom was observed which coincided with a decline in the abundance of the anchovy *Engraulis encrasicolus*. *E.encrasicolus* was the major fishery in the Black Sea with a high economic reliance (Kideys et al. 2005). Those *E.encrasicolus* which were caught in 1989 were small in size and showed signs of malnutrition, this was due to competition between *M.leidyi* and the anchovies for prey. The decline of the fishery in the Black Sea affected the bordering countries and Turkey alone estimated a US$1 billion loss between 1989 and 1993 (Kideys et al. 2005). The spread of *M.leidyi* did not stop in the Black Sea and by the 1990's it had reached the Caspian Sea where it affected the local Kilka (three species of *Clupeonella*) fishery. Landings of Kilka declined from 85,000 tonnes in 1999 to <24,000 tonnes in 2002. Iran estimated a US$125 million loss due to the fishery decline (Kideys et al. 2005).

The introduction of toxic phytoplankton can be a major economic problem for shellfish fisheries. Regular monitoring is in place to check the levels of toxic phytoplankton in waters surrounding shellfisheries to ensure the shellfish cannot ingest levels which would be harmful to humans. If the numbers of toxic species in the water increase above a set amount the fisheries are shutdown until the phytoplankton numbers decline.

The Zebra mussel *Dreissena polymorpha* is the main global invasive species of freshwater habitats. *D. polymorpha* originates from the Caspian and Black Seas and travelled to North America via ballast water where it causes many problems in rivers and lakes. *D. polymorpha* are able to colonise areas rapidly and in America it is commonly found blocking industrial pipelines. Earlier this decade their removal was estimated to cost US$5 billion each year (Aldridge *et al*., 2004).

The Chinese Mitten crab, *Eriocheir sinensis*, was first observed in Germany in 1912 and its spread to Europe is assumed to be within ballast water of ships trading between China and Germany. It has since spread throughout Europe through migration and shipping. It interferes with commercial and recreational fisheries by damaging nets and causing a loss of bait. In San Francisco the mitten crab has caused shrimp trawlers to abandon some fishing areas as they become tangled in nets and can damage shrimp during hauling (Herborg et al. 2003; Dittel and Epifanio 2009). In the Netherlands serious net damage was caused by a mass occurrence of *E.sinensis* (Herborg et al. 2003).

2.4.3 Environmental impacts

Introduced organisms can compete with native species for resources such as space and food and in many cases can lead to extirpations of native species. The loss of organisms from a system through competition with invasive species can lead to simplification of the food web (Hall and Mills 2000). Once an environment has been successfully invaded it may be left vulnerable to further invasions due to modification of the habitat by the newly introduced species (Ruiz et al. 1997). In the Caspian Sea *M.leidyi* not only

led to the decline of the Kilka fishery but also caused two predators of the Kilka - the white sturgeon, *Huso huso*, and the Caspian seal, *Phoca caspica*, to become highly endangered (Kideys et al. 2005). Endemic cladoceran species, which would be prey for the *M.leidyi*, have not been found in samples taken from the Caspian Sea since the invasion (Kideys et al. 2005). In San Francisco Bay the Chinese mitten crab *E.sinensis* has been observed to prey on freshwater shrimp, leading to concerns for the local endangered shrimp *Syncaris pacifica* (Dittel and Epifanio 2009). *E.sinensis* also causes river bank erosion due to its burrowing behaviour (Herborg *et al*., 2003). In some places, including Germany and the UK, this burrowing has damaged flood defences (Elliott 2003; Dittel and Epifanio 2009).

In addition to causing substantial economic costs associated with its eradication from industrial pipelines, the zebra mussel, *Dreissena polymorpha* also has environmental impacts. Individuals can filter 1-2L of water per day causing major shifts in plankton abundances in lakes and rivers, for example a reduction of 30-90% in phytoplankton biomass was observed in Lake Erie and Lake Huron (Ojaveer et al. 2002). Their filtering behaviour alters the clarity of the water which can result in decreases in fish abundance (Aldridge *et al*., 2004) and it increases competition for resources with other suspension feeders. They can also encrust the shells of native mussels, preventing them from feeding and resulting in mortality (Aldridge *et al*., 2004). Diving ducks *Fulica atra* are at risk as they have recently begun to exploit the mussels as a novel food source. The mussels have established in lakes polluted with heavy metals and accumulate toxins in their tissues and this has caused a decrease in the reproductive success of the ducks (Aldridge *et al*., 2004).

The risks of invasive species to human health, the economy and the environment have been described above and preventing the spread of organisms is the best solution. Successful introductions are more probable if the environment into which the organisms are released is similar to their native habitat. It is possible that global warming may decrease differences, for example temperature, between ports. This would consequently increase the number of successful establishments of foreign species and increases the threat of invasive species (Gollasch 2002; Lewis et al. 2003).

An example of this is the Finnish Lake District which has been protected from invasive species due to the natural low temperatures. However, there is expected to be an increase in global temperature estimated at 2°C - 5°C by the end of this century (UK Met Office 2009). This rise in temperature will have substantial consequences in areas such as the Finnish Lake District as it will allow species intolerant of the previous low temperatures to invade, settle and establish successful populations. Increased introductions into the northern hemisphere have been predicted as temperatures continue to rise (Pienimaki and Leppakoski 2004). The Laurentian Great Lakes in North America have received numerous introduced species, many of which have been deliberately introduced. Species of European origin which have successfully invaded are able to do so due to the similarity in the climates between Europe and the North America Lakes region (Hall and Mills 2000).

2.5 Minimising the spread of organisms via ballast water

It is clear that due to the ability of organisms to survive journeys within ballast water, and from the risks they pose after discharge that ballast water must be managed to minimise the transfer of species. However, there are many problems associated with this due to the volume of water that requires treatment in a short period of time. Most of the currently used or recommended management processes have various negative factors associated with them (Bax et al. 2003). The current recommended management technique for ballast water is to exchange it in the open ocean. This does not result in sufficient removal of organisms to prevent new introductions and is recognised as merely an interim measure to be used until effective treatment systems are available. Different treatment methods are being investigated and to put them into practice they must first meet important criteria i.e. they must be safe, economical, environmentally acceptable, non-toxic to non-target organisms when discharged, suitable for use by the current crew on ships, and they must be effective (Champ 2002). This study has assessed three treatment technologies to determine their potential application in ballast water treatment. Each technology is widely used for water treatment purposes

and the factors which could affect application in ballast water treatment were identified and assessed during Chapters 5, 6 and 7.

2.5.1 'Open Ocean Exchange' methods

Open ocean exchange methods involve the exchange of ballast water taken on at the origin port with water from the open ocean. This releases organisms native to coastal environments into the mid ocean and thus into conditions which they are not adapted to and theoretically should not survive in. The 2004 convention, which will be further discussed in Section 2.6, defined the conditions under which ballast water exchange should occur (Regulation B-4). Exchange should ideally take place in water that is at least 200 nautical miles from land and in water at least 200 meters deep. If this is not possible then exchange must occur at least 50 nautical miles from land and in water which is 200 meters deep (IMO 2004). If exchange cannot be completed in either of these conditions an area for exchange must be designated for use. The vessels then upload water from the mid ocean and the organisms present in this water should not be adequately adapted to survive if released alive into the destination port (Drake et al. 2002).

There are three types of open ocean exchange methods: 'empty-refill', 'continuous flow through', and 'dilution'. In the 'empty-refill' exchange method, tanks are emptied in the mid ocean by pumps until suction is lost and no more water can be pumped out. The pumps are located some centimetres above the bottom of the tanks and thus the tanks are never fully emptied. This will enable some organisms within the water column and those in the sediment at the bottom of the tanks to remain inside for the whole of the journey (Olenin et al. 2000), this will be further discussed. This method poses safety risks due to the vulnerability of vessels to excessive bending moments and stresses which may cause damage while the ballast tanks are emptied (Endresen et al. 2004). 'Empty-refill' is deemed the most effective of the mid ocean exchange methods, however due to the safety risks involved in the process in rough weather conditions it may not be possible to perform any ballast exchange (Perrins et al. 2006).

In the 'continuous flow through' system the IMO guidelines require water of at least three times the volume of the ballast tanks to be passed through the tanks as this can result in a 95% exchange of water (Champ 2002) if the water is mixed thoroughly (Anon 1997b). The requirement of such large water volumes increases the time required to complete the exchange process and consequently the cost involved. Less water can be passed through the system if it can be proved that there has still been a 95% exchange of water (MEPC 2005b). This process does not pose the same risks as the 'empty-refill' method as the tanks are never empty during the journey, but it does have a risk of over pressurisation of the tanks or the piping system which would damage the ship (Endresen et al. 2004). The 'dilution' method is similar and involves the replacement of ballast water by filling the top of the tanks at the same rate as the water at the bottom of the tank is being discharged (MEPC 2005b).

These ballast water exchange methods do not achieve 100% exchange of the water and organisms present in tanks. The amount of water exchanged has been recorded at 96-100% (Rigby and Hallegraeff 1994; Olenin et al. 2000; Wonham et al. 2001). The abundance of organisms after exchange can increase (McCollin et al. 2007b), although in most studies decreases in plankton abundance of between 42-99% have been observed (Dickman and Zhang 1999; Zhang and Dickman 1999; Gollasch et al. 2000b; McCollin et al. 2007b; McCollin et al. 2008; Klein et al. 2010). The efficiency of exchange in regional, shallow seas is lower than that in oceanic waters due to higher abundances of planktonic organisms in these shallower waters (McCollin et al. 2007b; McCollin et al. 2008).

In 1991 the International Maritime Organisation made it mandatory that these exchange processes were performed when possible to minimise species introductions, and so they are now the primary methods of managing ballast water (Murphy et al. 2004). However, it has been suggested that these exchange methods may actually be helping to disperse species and that island states 'downstream' of these mid ocean exchange areas may be at risk of species introductions (Anon 2006a). Their effectiveness has been questioned and a study by Wohnam et al (2001) observed that ocean water was not lethal to coastal organisms and that the survivorship of

zooplankton taken from the ballast tanks at the time of exchange was not significantly different after 48 hours when kept in ballast water or ocean water.

2.5.2 Filtration

Filtration is used to physically removal organisms from water in many applications, such as the purification of drinking water, swimming pool water and in the recycling of grey water, as a primary treatment. It is then commonly followed by a disinfection process, e.g. UV radiation or a chemical treatment. The earliest practised methods of water purification used filtration as early as 2000 BCE. In the 16^{th} – 18^{th} centuries France determined that every household should be supplied with clean, fresh water and employed sand filters to treat water prior to supply (Baker and Taras 1981). Filtration is now commonly used in a range of water treatment applications and shows good potential for use in ballast water (CEPA 2002).

Two types of filtration have been used in ballast water treatment: surface and depth filtration. These filters are employed as primary treatments, and by removing larger plankton and organic matter they improve the quality of the water for secondary disinfection. After early trials filtration was recommended as the most promising ballast water treatment technology by the National Research Council (1996) and it is utilised in seven of the ten treatment system which have gained Type Approval (MEPC 2010). Ballast water treatment using filtration will be further discussed in Chapter 7.

2.5.3 Ozone

Ozone has been used as a disinfectant since the late 1800s and is commonly employed in Europe to treat drinking water (Herwig et al. 2006). Ozone (O_3) is a strong oxidising agent which when added to seawater undergoes a series of redox reactions resulting in the production of a biocide. This biocide is produced in seawater due to the presence of bromine. Initially bromine ions (Br^-) are oxidised forming hypobromite ions (OBr^-). The hypobromite ions (OBr^-) can be hydrolysed to hypobromous acid (HOBr) and it is

the combination of these that form the biocide and result in mortality of organisms (Hess-Erga et al. 2008). Ozone is highly unstable and reacts rapidly with organisms, causing the destruction of cell walls before breaking down (White 1999). Organisms are killed quickly, but there is no potential to control regrowth without applying a subsequent treatment (National Drinking Water Clearinghouse 1999).

Ozone is created by bubbling compressed oxygen enriched air through a series of electrodes. It is produced within these electrodes and can then be piped into ballast water tanks. This method of production is beneficial as there is no requirement for chemical storage onboard vessels and the ozone can be dosed during uptake of seawater or during transit (Oemcke and van Leeuwen 2005). However, ozone has low water solubility and Herwig et al (2006) observed that ozone injected into ballast tanks was unable to mix evenly throughout the tanks due to the number of internal structures and platforms within the ballast tanks which limit water movement. This would be a major factor in ballast water applications as even distribution of the ozone would be vital for any ozone system to be capable of meeting the IMO regulations.

Herwig et al (2006) observed ozone treatment to be effective on dinoflagellates and microflagellates but during tests on shore crabs, mysid shrimps and Sheepshead minnows lower mortality levels were observed. Oemcke and van Leeuwen (2005) observed a 4-log inactivation of the dinoflagellate *Amphidinium* sp. at a dose of 4-11mgL^{-1} ozone with 6 hours contact time. A high dosage and long contact time was also required for inactivation of *Bacillus subtilis* spores (Oemcke and Van Leeuwen 2004) and so the authors concluded that the dosages required in both studies would be inappropriate for general use of an ozone system within ballast tanks. Further modifications to ozone treatment systems and testing would be required to accurately determine its future potential application.

2.5.4 Ultraviolet light

Ultraviolet (UV) light is the term given to light between 100-400nm. This range is split into four types of UV light: UV-A (315 - 400nm), UV-B (280 - 315nm), UV-C (200 -

280nm) and vacuum UV (100 - 200nm). UV-B and UV-C light have known germicidal effects identified in the late 1800s (Downes and Blunt 1877), but UV-A and vacuum UV are not germicidal (Kowalski 2009). The germicidal peak wavelength of UV irradiation was identified as 253.7nm in 1932 (Kowalski 2009) and this lies within the UV-C range.

The main way in which UV-C light affects organisms is through damaging nucleic acids i.e. DNA and RNA (Björn 1996; Herbert 2002; Liu 2005; Sassi et al. 2005; United States Environment Protection Agency 2006; Hess-Erga et al. 2008; Tsolaki and Diamadopoulos 2009). UV-C light is absorbed by bonds between base pairs in DNA and RNA molecules and causes the bond to become 'open' i.e. able to form new bonds. This can stimulate the formation of covalent bonds, named 'pyrimidine dimers', between these bases and neighbouring bases. These bonds alter the structure of the DNA, thus hindering or preventing DNA replication and results in the inactivation of organisms (Wright and Cairns ; Pini 1999; Goodsell 2001; Oguma et al. 2001; Martin et al. 2004; United States Environment Protection Agency 2006; Hess-Erga et al. 2008).

UV light is a widely used disinfectant in other applications, and has been utilised for water disinfection since the early 1900s (Clemence 1911; Carlson et al. 1985). UV disinfection has proved an effective ballast water treatment and combined with primary filtration it is used in three of the ten ballast water management systems with Type Approval certification (Gregg et al. 2009; MEPC 2010). The application of UV disinfection as a ballast water treatment technology will be further discussed in Chapter 5.

2.5.5 Laser irradiation

The use of pulsed laser irradiation has been investigated for its application in preventing biofouling and the formation of marine biofilms (Nandakumar et al. 2002; Nandakumar et al. 2003b; Nandakumar et al. 2004). Laser irradiation at 532nm causes immediate mortality to barnacle larvae (Nandakumar et al. 2002; Nandakumar et al. 2003b) and phytoplankton (Nandakumar et al. 2003c; Nandakumar et al. 2003a; Nandakumar et al. 2009). In addition to immediate mortality sub lethal effects have

been observed in *Balanus Amphitrite* as a reduction in swimming speed, the prevention of larval settlement and reduced moulting frequency (Nandakumar et al. 2002; Nandakumar et al. 2003b). The diatoms *Skeletonema costatum* and *Chaetoceros gracilis* showed destruction of frustules and draining of intracellular components after irradiation, although the exact mechanism of damage is undetermined (Nandakumar et al. 2003c). More recent research has assessed the applicability of laser irradiation as a ballast water treatment (Nandakumar et al. 2009) by assessing mortality in flowing conditions. Preliminary tests concluded that the technology could be effective although higher intensity lasers would be required for this purpose than is necessary in static applications (Nandakumar et al. 2009).

2.5.6 Heat treatment

Heating ballast water has been assessed as a ballast water treatment option (Anon 1997a; Hallegraeff et al. 1997; Rigby et al. 1999; Mountfort et al. 2001; Thornton and Chapman 2004; Quilez-Badia et al. 2008). High temperatures cause mortality to organisms through denaturing key proteins and causing membrane breakdown by increasing the mobility of molecules. These two processes inactivate vital metabolic processes in organisms, resulting in death (Rigby et al. 2004). Temperatures of 35-45°C have proved effective against zooplankton (Rigby et al. 1999; Mountfort et al. 2001), cysts of the dinoflagellates *Gymnodinium catenatum* and *Alexandrium tamarense* (Bolch and Hallegraeff 1993; Hallegraeff et al. 1997), and phytoplankton (Rigby et al. 1999). Higher temperatures are required to inactivate bacteria and viruses and 65°C exposure for 2 minutes was required to inactivate *Vibrio cholera* (Bolch and Hallegraeff 1993). There are associated problems with heat treatment, e.g. the energy required to maintain high temperatures, corrosion to tanks, safety issues associated with storing high temperature water for long durations, ensuring even heating throughout the tank, the feasibility of use on short voyages and added energy requirements if travelling through cold waters (Rigby et al. 1999; Mountfort et al. 2001; Quilez-Badia et al. 2008).

2.5.7 Deoxygenation

The removal of oxygen from seawater within ballast tanks has been investigated as a potential treatment for ballast water. In addition to killing potential invasive species, this method has the additional benefit of reducing corrosion levels within tanks, an added incentive for ship owners to employ such a treatment system. The removal of oxygen from tanks causes aerobic organisms within to suffocate (Browning et al. 2003). However, the effectiveness of these systems on organisms which are capable of surviving anaerobically may be minimal. After treatment the ballast water can be discharged and it is not thought that detrimental effects will be observed due to rapid reoxygenation of the water. If required the water could be reoxygenated prior to discharge (Tamburri et al. 2003).

There are various ways of creating anoxic conditions: producing a vacuum using a vacuum pump (Browning et al. 2003), adding a nutrient solution (McCollin et al. 2007a) or by bubbling nitrogen gas through tanks (Tamburri et al. 2002; Tamburri et al. 2003; Tamburri et al. 2006). These have been utilised by different companies for development and various deoxygenation treatment systems have been trialled for ballast water research.

One such system which gained type approval from the IMO in 2007 is the Venturi Oxygen Stripping™ system. This treatment removes oxygen from seawater by mixing 'oxygen stripping' gas into the seawater during uptake into the ballast tanks (Tamburri et al. 2006). The 'oxygen stripping' gas is composed of nitrogen, elevated levels of carbon dioxide and a trace amount of oxygen. This gas is injected into the ballast water during uptake using a Venturi injector, creating microfine bubbles. The oxygen diffuses into the bubbles; smaller bubbles have a higher surface area to volume ratio and so a greater surface for gas diffusion (Tamburri et al. 2003; Tamburri et al. 2006). The efficacy of this system has been investigated and significant reductions in live zooplankton have been observed, with >97% mortality in the invasive shore crab *Carcinus maenas* (Tamburri et al. 2003).

McCollin et al (2007a) added a nutrient mixture to seawater in order to create anoxic conditions. The mixture added contained glucose, sucrose, ammonium, nitrate and phosphate. This stimulated bacterial growth in the water to utilise the oxygen present, but it was designed to minimise the growth of sulphur reducing bacteria which can increase corrosion levels (McCollin et al. 2007a). This study observed a significant increase in zooplankton mortality in the treated tanks. The phytoplankton showed no effects due to the treatment and it was concluded to be unlikely that deoxygenation would be effective on phytoplankton (McCollin et al. 2007a). This is a significant limitation to the use of deoxygenation, and it would therefore need to be combined with an additional disinfectant which is effective on phytoplankton.

2.5.8 Biocides

Chemical treatments have been tested in previous ballast water studies and possess practical benefits, i.e. there are no scaling problems and they have simple dosing systems which are cheap to install (Wright et al. 2007a). Two of the major considerations when applying these treatments are the disposal of the ballast water after a chemical has been added and ensuring the chemical works in a concentration at which enough can feasibly be taken on board the vessel. The chemical must also be safe for handling by the crew, non-corrosive to the vessel and effective at a cost effective concentration (Gregg and Hallegraeff 2007).

Menadione is an organic oxidant toxic to a range of marine organisms. It is naturally present in plant cells but can also be produced commercially at low cost. Menadione is marketed commercially for ballast water treatment as Seakleen®. PERACLEAN® Ocean and Vibrex® are also commercially available ballast water biocides. PERACLEAN® Ocean contains peroxy-acetic acid and Vibrex® contains chlorine dioxide(Gregg and Hallegraeff 2007). Trials have proven the effectiveness of PERACLEAN® Ocean (Fuchs et al. 2001; Fuchs and de Wilde 2004; Gregg and Hallegraeff 2007; La Carbona et al. 2010) and SeaKleen (Wright and Dawson 2001; Cutler et al. 2004; Sano et al. 2004; Wright et al. 2007a; La Carbona et al. 2010) on plankton. In addition, PERACLEAN® Ocean has proven effective against dinoflagellate cysts while Seakleen® was ineffective (Gregg

and Hallegraeff 2007). Vibrex® caused 100% mortality of all organisms in preliminary tests, however this was due to the production of a noxious gas and so it is not safe for use on board a ship (Gregg and Hallegraeff 2007). The observations from these studies show that there is still scope for further chemical treatments as those currently available are not always effective or safe. Those that do work require high concentrations and long exposure times which may be longer than the voyage itself, further limiting their use.

Chlorine is the most commonly used biocide worldwide due to its ease of use and low cost, and is employed in many applications, e.g. in swimming pools and water for aquaculture and hospitals (Vianna da Silva and da Costa Fernandes 2003; Zhang et al. 2004). Preliminary studies (See Fuchs et al. 2001; Wright and Dawson 2001; Vianna da Silva and da Costa Fernandes 2003; Zhang et al. 2004; Faimali et al. 2006; Gregg and Hallegraeff 2007; Wright et al. 2007a; de Lafontaine et al. 2008) have shown that chlorine has potential to be used as a ballast water treatment.

An option available when using chlorine is the potential for electrolytic production. Electrolytic cells use the salts and ions naturally present in water to produce sodium hypochlorite which has been proven to kill heterotrophic bacteria such as *E. coli*. The sodium hypochlorite then returns to its original salts and ions and so should not cause any environmental damage (Anon 2006a; Anon 2006b). Testing using electrolysis has been carried out and some promising results have been observed (See Sousa et al. 2001; Sano et al. 2004; Gray et al. 2006; Matousek et al. 2006). The use of biocides for ballast water treatment will be further discussed in Chapter 6.

2.6 Regulations regarding ballast water

In the 1970's the International Maritime Organisation (IMO) recognised the problem of movement of organisms via ballast water. Through the Marine Environmental Protection Committee (MEPC) it began to look at ways to resolve the issue. In 1991 Resolution 50(31) – 'Guidelines for preventing the introduction of unwanted aquatic

organisms and pathogens from ships' ballast water and sediment discharges' was adopted by the MEPC. This was further updated in 1993 as Resolution A.774(18) and was adopted by the IMO Assembly. These guidelines outlined procedures to be followed by ships' when dealing with safe discharge of ballast water. Firstly they recommended that until effective treatment methods have been developed ballast water should be exchanged whenever possible. If this process cannot be completed then the Port State of the destination port should be informed and left to determine how the ballast water should be dealt with. The exchange of ballast water was discussed in Section 2.5.1, and while it can reduce the number of organisms present in ballast water it does not eliminate live organisms. Nor does it guarantee that the organisms discharged will be unable to survive in the receiving waters. In addition there are instances in which exchange is not performed, e.g. if it is unsafe due to bad weather or unsuitable sea conditions, or if it is operationally impractical e.g. on short voyages (MEPC 1997). The guidelines state that exchange of ballast water should be completed in waters 200nm from land and 200m deep, and where this is not possible in water 50nm from land and 200m deep. However, not all voyages will pass through an area as defined in the regulation and so exchange is an inadequate management practice for eliminating ballast water introductions.

The guidelines separately addressed the treatment of sediments and the importance of ensuring these are not discharged at the destination port due to the potential for cysts of harmful organisms to be present. Instead the sediment should either be flushed out during open ocean exchange, or removed to treatment facilities at the destination port (MEPC 1993). As previous observations have noted sediments being thrown overboard from vessels when in the destination port (Williams et al. 1988) it is necessary to ensure that where these sediment reception facilities are available they are strictly used by vessels.

In 1997 the IMO published Resolution A.868(20) – 'Guidelines for the control and management of ships' ballast water to minimise the transfer of harmful aquatic organisms and pathogens' which replaced the earlier Resolution A.774(18). These guidelines contained a number of instructions which if applied by vessels aimed to

reduce the risk of transport of organisms. Importantly the guidelines highlight the need for the vessels crew to be trained in the treatment and management of ballast water and sediments and for a log book which records ballast activity to be kept up to date. The records should include details of when and where the ballast water was taken onboard, the volume, water temperature and salinity. This information should be made available to the Port State if requested. All vessels should be provided with a management plan which details procedures specific to each ship which will minimise their risk of transferring species. In addition to these instructions the Port States themselves should provide reception and treatment facilities for vessels which should be utilised by vessels where available. The IMO recognised that adequate treatments for ballast water were not available at that time, and so called for developments in this area to be reported to them while in the meantime ships should still perform exchange where possible. The Port State has a further duty which is to inform ships of areas in which they should avoid taking up ballast i.e. areas which have known outbreaks of harmful organisms, areas which have current blooms of harmful phytoplankton, near sewage outfalls and dredging operations as well as in areas with high turbidity and poor tidal flushing. While these guidelines were useful, compliance was not mandatory and this led to countries adopting their own set of regulations. Fortunately these were generally based on the IMO guidelines and countries such as Australia, Canada, the UK, and a number of states within the USA determined their own regulations. However, because the shipping industry is a global industry and a voyage can involve passing through a number of different countries' jurisdictions these different regulations made it extremely difficult for vessels to comply (IMO Ballast Water Update, 2002).

In 2002 an update of the progress in ballast water research and regulation was published. It recognised the need for a uniform set of regulations to be implemented in all countries as at that time different rules were in place depending on the needs of the individual area (Raaymakers 2002). Regulation had mainly become focused on implementing the recording of ballast water activity and the IMO wished to increase the level of implementation of treatment methods. In addition to the need for more ships to exchange their ballast water the IMO recognised the necessity for a biological standard to be developed which treatment systems should endeavour to meet. To do

this more investment into ballast water treatment research was sought and symposiums were organised in London for researchers developing treatment systems to come together and share knowledge. The first of these symposiums was held in 2001 and the second in 2003. In addition the international project GloBallast was launched in 2000 which aimed to help developing countries design and implement ballast water strategies. This project was piloted in six countries: China, Iran, Ukraine, India, South Africa and Brazil and by 2010 had moved into the next phase and was working with more developing countries to improve their ballast water management.

In 2004 the 'International Convention for the Control and Management of Ships' Ballast Water and Sediments' was formed. The convention will not enter into force until 12 months after ratification by 30 states which represent 35% of the world's merchant shipping tonnage (IMO 2004), and at the time of writing (March 2011) 27 countries representing 25.32% of the worlds tonnage had ratified the convention. This convention consists of management and control requirements for ships and 15 sets of guidelines to support the aims of the convention. Two major standards were determined as part of this convention: Regulations D-1 and D-2. Regulation D-1 is the Ballast Water Exchange Standard which states that ships must exchange a minimum of 95% ballast water volume during exchange. Regulation D-2 is the Ballast Water Performance Standard and states the limit of the allowable number of viable organisms which can be discharged from vessels (IMO 2004), these levels are:

- Less than 10 viable organisms ≥50µm in minimum dimension per m^3
- Less than 10 viable organisms ≤50µm and ≥10µm in minimum dimension per ml
- These levels also included monitoring the levels of some indicator microbes:
    - Less than 1 colony forming unit (cfu) per 100ml or less than 1 cfu per 1 gram (wet weight) zooplankton samples Toxicogenic *Vibrio cholerae* (O1 and O139),
    - Less than 250 cfu *Escherichia coli* per 100ml,
    - Less than 100 cfu intestinal *Enterococci* per 100ml.

D-1 was the minimum standard to be imposed while systems capable of meeting D-2 were developed. D-2 is now being phased in and by 2016 all vessels will be required to meet these levels in their ballast water discharge. The determination and publication of D-2 was extremely important in terms of developing new ballast water treatments as the industry was finally given a standard which had to be met before the IMO will approve and certify systems for sale. The process of developing, testing and gaining certification for a treatment system requires a substantial amount of investment of both money and time from manufacturers, in addition to expertise in developing the elements of the treatment system and both the biological and technical aspects of testing a system. In 2005 MEPC 53 adopted G8 – 'Guidelines for the approval of ballast water management systems'. While these guidelines give guidance on completing tests they do not give a detailed, rigid testing protocol to be applied during all system testing in facilities across the world. This has led to variation in the testing protocols used and one of the major elements lacking from this is a biological protocol of how to determine whether or not the organisms present after treatment are viable. Various techniques have been used by biologists in many different applications to assess viability but in all methods limitations have been observed (E.g. Dressel et al. 1972; Crippen and Perrier 1974; Jochem 1999; Onji et al. 2000; Veldhuis et al. 2001; Buttino et al. 2004; Agusti et al. 2006; Reavie et al. 2010). These limitations include conditions under which the viability assessment technique is ineffective e.g. at specific temperatures or salinities, due to the method of killing, the organisms used, the requirement for expensive, specialist equipment or the time required for analysis. The determination of effective and reliable viability tools is vital for the accurate assessment of treatment systems under development. If testing centres use an ineffective stain this will affect the accuracy of the data obtained. Chapter 8 further discusses the problems faced by biologists when assessing organism viability and has used a systematic testing approach to identify effective stains and their optimum conditions for use.

By October 2010 ten treatment systems have received IMO Type Approval certification (MEPC 2010). A further eleven systems are expected to gain Type Approval by 2012 (Lloyds Register 2010; MEPC 2010). The systems which are approved use a

combination of more than one treatment per system and a range of different treatments are utilised in the nine systems. Filtration and ultraviolet light are used in three of the systems making it the most commonly used successful combination. In addition chemical, deoxygenation, electrochlorination, rapid oxidation and cavitation are used in the other six systems. As many more systems are in the approval process and others still in the earlier, development stage there will be a range of effective systems available when the D-2 standard comes into force.

2.7 Aims of this thesis

This chapter has described the mechanism by which organisms travel within ballast water tanks, the problems encountered in the receiving areas and the management options in place. It is clear that ballast water research has many areas to address and some of the current knowledge gaps have been highlighted. The aims of this thesis were:

- to investigate the effect on plankton viability of so called 'unintentional treatment' processes which occur during ballast water transportation i.e. travelling through the centrifugal pump and storage in ballast tanks,
- to investigate the biological effectiveness of three 'intentional treatment' technologies for potential use in ballast water treatment systems, i.e. filtration, UV irradiation and a chlorine based biocide, and to identify accurate and reliable methods of assessing the mortality caused to plankton.

Chapter 3

The effects of a centrifugal pump on plankton

3.1 Introduction

3.1.1 Damage to plankton by pumps

The first stage in the process of ballast water transportation is the uptake of water surrounding the vessel, which is used to fill the ballast tanks, using a centrifugal pump. This process could lead to organisms being damaged as they travel through the pump by colliding with the impellers (Carlton 1985; Gollasch et al. 2000a). Suggestions have been made that this is responsible for initial high levels of mortality in organisms entering ballast tanks (Gollasch et al. 2000a) but the effects of the pumps themselves have not been directly examined.

Previous studies have looked at the possibility of collecting plankton samples using pumps, generally with regard to effective methods of obtaining samples in good physical condition. In general, few problems have been reported, although some types of gelatinous and/or delicate zooplankton have been observed to sustain damage after passing through pumps. Those reported for centrifugal pumps are fish larvae (Harris et al. 1986), *Appendicularia* sp. tunicates (Mohlenberg 1987), copepod egg sacs (Rahkola et al. 1994) and the cladoceran *Holopedium gibberum* (Rahkola et al. 1994) and for a bilge pump ctenophores and fish eggs (Nayar et al. 2002). No damage has been reported for phytoplankton, although colonies of diatoms have been observed to break up after passing through a pump (Veldhuis et al. 2006). It is apparent from these studies that the extent and processes of damage are as yet undetermined. Some assumptions and suggestions have been put forward by authors, but empirical evidence of this damage is still lacking.

3.1.2 Centrifugal pump mechanism

Centrifugal pumps contain an impeller which rotates rapidly, pulling water in through the centre of the pump and forcing it out at the other side. They must be filled with water before use as they are unable to form a vacuum to pull water in initially, but the process of speeding up water and pushing it out of the other side will draw more water into the pump once in use. Figure 3.1 shows the basic mechanism of the centrifugal pump.

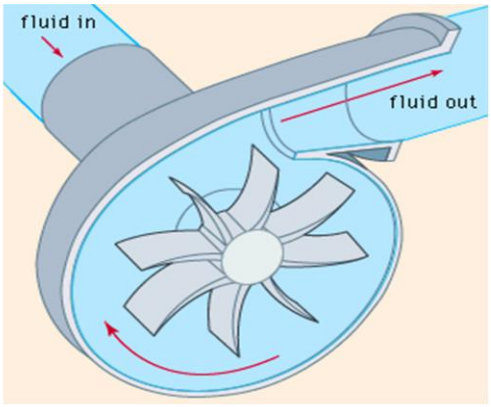

Figure 3.1. Mechanism of the centrifugal pump. (Adapted from www.britannica.com)

3.1.3 Aims of this study

Qualitative evidence has been reported in previous studies to describe organism damage after passing through centrifugal pumps (Section 3.3.1). Rahkola et al (1994) published data of the percentage of damaged individuals for four zooplankton test species after travelling through a centrifugal pump, but other qualitative data is lacking. The aim of this study is to quantify phytoplankton and zooplankton damage by a centrifugal pump by assessing the mortality of a natural plankton community before and after passing through a centrifugal pump. To the best of the author's knowledge this is the first study to collect this quantitative data.

In order to prevent bioinvasions through ballast water transportation it is vital to understand the impact each part of the process has on the organisms involved. The first stage which could have an impact on organisms' health is the process of being pumped into ballast tanks. This study aims to determine whether this affects the viability of organisms present in the water. The data collected is not only important for knowing the effect of the pump itself, but in ballast water research isolating the effects of the pump from those of a treatment system being assessed will prevent over estimations of a system's effectiveness. This study will show whether the process of pumping water onboard could itself be considered part of the treatment of ballast water, or whether the observations previously made by Gollasch et al (2000a) attributed initial levels of mortality to an incorrect source.

3.2 Methodology

3.2.1 The pump

A Grundfos LPE 80 160/149 centrifugal pump was used during tests (Figure 3.2). The pump contained stainless steel impellers. The pipe outlet diameter of the pump was 3" but during testing this was reduced to 2" to accommodate the pipes used. The technical specification and dimensions of the pump are displayed in Table 3.1, Table 3.2 and Figure 3.3.

Figure 3.2. Centrifugal pump used during tests.

Table 3.1. Technical specification of pump

Grundfos: LPE 80 160/149 centrifugal pump	
Flow rate (maximum)	$41m^3hr^{-1}$
Rotation frequency	2900rpm
Motor power	5.5kW
Power supply	3 x 380-415V
Full load current	12.00A
Pipe diameter	3"

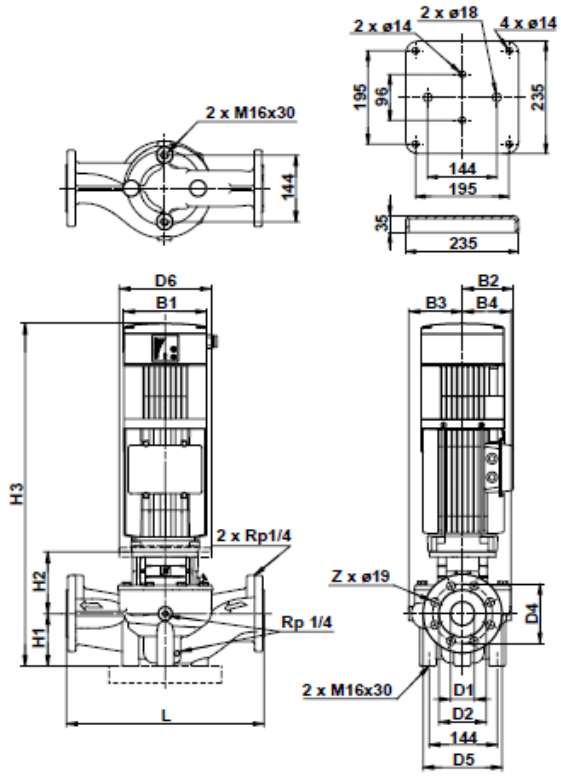

Figure 3.3. Dimensions of the pump.

Table 3.2. Dimensions of the pump.

Dimensions (mm)													Z	Weight (kg)		Ship.
D1	D2	D4	D5	D6	B1	B2	B3	B4	H1	H2	H3	L	(poc.)	Net	Gross	Vol (m^3)
80	140	160	200	298	220	134	156	150	127	200	882	525	8	95	128	0.340

3.2.2 Experimental set up

Seawater for the control samples was collected using 10 litre containers from Cullercoats Bay. The centrifugal pump was used to pump seawater straight from the sea for each test. A pipe was laid down the beach from the Dove Marine Laboratory and secured at low tide ready for sampling at high tide.

In order to obtain sufficient organisms for testing, 100 litres of water was sampled for zoo- and phytoplankton tests. The samples were put into storage containers and

filtered with 50µm and 10µm sieves to separate the zoo- and phytoplankton. Three replicate samples were taken for both 'Control' and 'Pump' samples. The pump was tested at approximately 2300 rpm.

For zooplankton viability assessment samples were stained using neutral red vital stain. 3ml 0.1% neutral red stain was added per 100ml sample and left for 1 hour. After this 4ml 1N sodium acetate was added and then fixed using 50ml 4% formaldehyde. Samples were stored over night at 2-3°C. Before assessment glacial acetic acid was added drop wise until the solution turned magenta. The sample was filtered and washed with 10µm filtered seawater before being placed in a Petri dish for examination. All organisms were examined using a Meiji microscope at 20-45x magnification. The organisms were identified to taxon and the number of live and dead organisms was recorded. Live organisms at the time of neutral red addition were observed to stain red and dead organisms remained unstained.

Phytoplankton viability assessment used the mortal stain Evans blue. 1% solution (w/v) Evans blue was added at 2:1 (sample:stain) and left for 1 hour. Samples were then filtered using a 10µm sieve and rinsed with 0.45µm filtered fresh seawater before counting. The sample was concentrated to a known volume and a 1ml aliquot was examined in a Sedgewick Rafter counting cell. Cells which were dead prior to staining were stained blue, whereas individuals alive before staining did not exhibit any colour change. For analysis samples were made up to 100ml with fresh 0.45µm filtered seawater and a 1ml aliquot was removed and examined using an Olympus CK X31 microscope at 200-400x magnification. The number of live and dead organisms was recorded and the percentage mortality of each sample was calculated.

3.2.3 Statistical analysis

The percentage mortality of each individual taxon in each of phytoplankton and zooplankton were calculated separately for each replicate. Only those taxa which had representatives recorded in all replicates of a given treatment were included in the formal analysis. This data was Arcsine square root transformed. The data did not show

normal distribution in an Anderson Darling normality test. The ANOVA was proceeded with as it is deemed robust enough to cope with such aberrations from ANOVA assumptions (Underwood, 1997). Levene's test was used test for homogeneity of variance (phytoplankton: p = 0.476).

3.3 Results

3.3.1 Effects of the centrifugal pump on phytoplankton

In total 23 taxa were present in the samples (Table 3.3). Twelve of these taxa were statistically analysed and no significant two-way interaction was present between the factors Pump and Taxon (p = 0.803, F = 0.6, df = 10). There was also no significant effect on phytoplankton mortality by the factor Pump (p = 0.671, F = 0.18, df = 1). The percentage mortality of each taxa in the Control and Pump Tested samples are shown in Figure 3.4.

Table 3.3. The average number of live and dead phytoplankton before and after the pump. Values are means ± SE of three 100 litre replicate samples.

Species	Average number of live phytoplankton before pump (±SE)	Average number of dead phytoplankton before pump (±SE)	Average number of live phytoplankton after pump (±SE)	Average number of dead phytoplankton after pump (±SE)
Actinophycus sp.	5.7±4.3	12.7±9.4	8.3±8.3	3.0±3.0
Asterionella sp.	0.0±0.0	0.0±0.0	0.7±0.7	2.0±2.0
Ceratium sp.	0.0±0.0	1.0±0.6	0.0±0.0	0.7±0.3
Chaetoceros sp.	0.0±0.0	4.0±4.0	0.0±0.0	1.7±1.7
Corethron sp.	6.3±3.2	80.0±26.1	1.3±1.3	12.0±6.0
Coscinodiscus sp.	2.3±1.9	0.3±0.3	0.3±0.3	0.0±0.0
Cylindrotheca sp.	9.0±4.7	86.7±33.9	10.0±7.0	130.3±36.4
Dinophysis sp.	0.0±0.0	0.0±0.0	0.3±0.3	0.0±0.0
Fragilariopsis sp.	31.0±13.5	7.0±6.0	35.3±16.6	7.0±5.1
Manguinea sp.	7.0±1.5	0.3±0.3	10.3±1.9	1.3±1.3
Navicula sp.	8.7±3.8	2.7±1.8	10.7±2.7	4.0±3.1
Odontella sp.	0.7±0.3	12.3±1.2	0.0±0.0	1.3±0.9
Paralia sp.	296.7±115.4	24.3±17.0	50.3±21.9	6.0±2.6
Pleurosigma sp.	15.3±3.3	2.0±0.6	8.0±7.0	3.3±2.0
Prorocentrum sp.	1.0±0.6	34.0±18.0	1.7±0.9	4.0±1.2
Protoperidinium sp.	1.7±0.9	2.0±0.6	0.0±0.0	1.0±1.0
Pseudo-nitzschia sp.	54.3±19.4	18.7±3.8	288.3±81.5	37.3±13.7
Rhizoselenia sp.	5.7±3.5	11.7±5.7	1.0±0.6	1.3±0.9
Silicoflagellate	110.0±27.6	2.3±1.3	19.0±9.8	0.3±0.3
Thalassionema sp.	14.0±6.0	186.3±35.5	0.7±0.7	22.7±10.7
Thalassiosira sp.	2.3±0.9	4.3±4.3	2.0±1.2	2.3±2.3
Triceratium sp.	0.7±0.3	39.3±23.7	0.0±0.0	4.0±4.0
Unidentified diatom	11.7±5.9	1.0±0.6	2.0±2.0	0.3±0.3

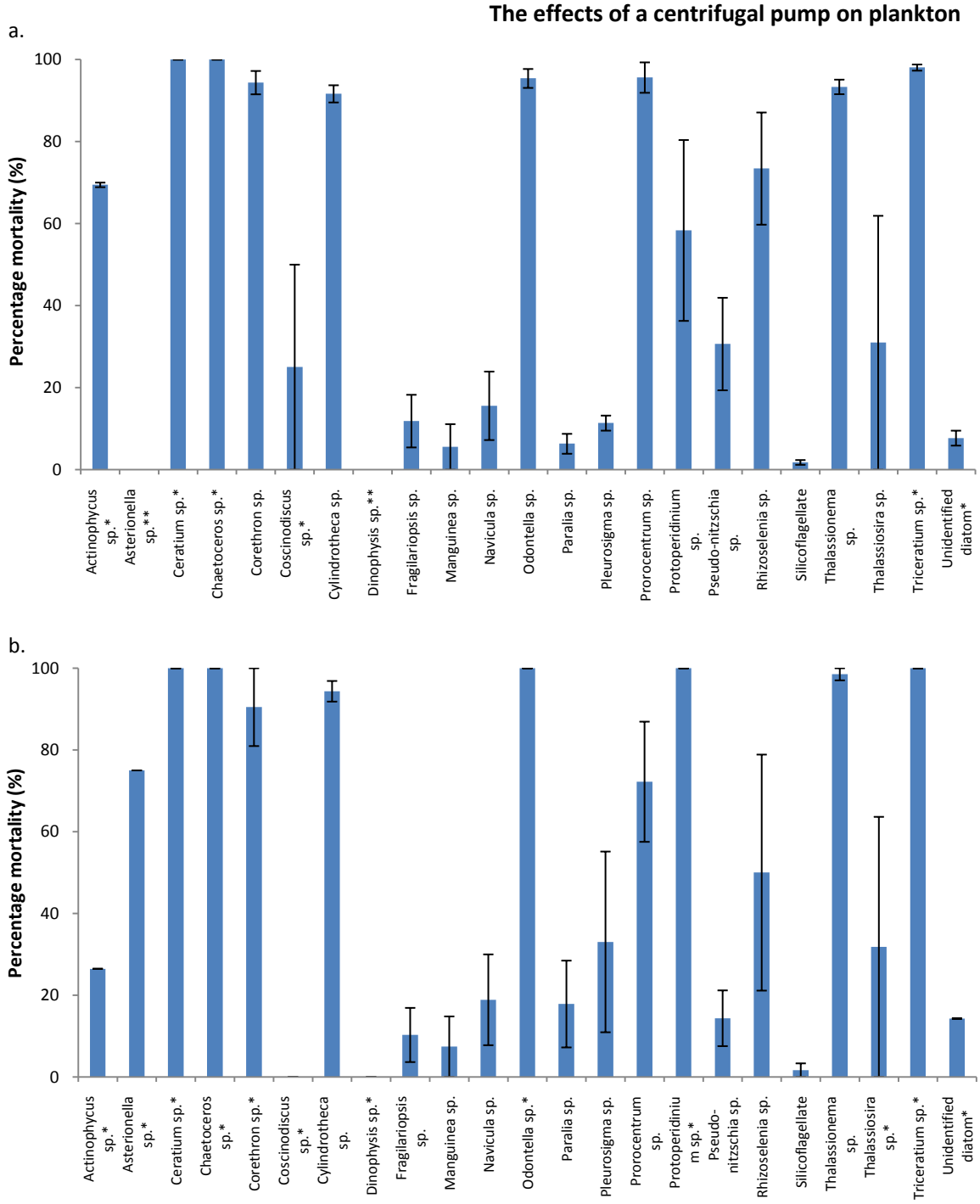

Figure 3.4. Mean percentage mortality for each phytoplankton taxon in a. Control samples and b. Pump treated samples. Values are means ± SE of three 100 litre replicate samples. * = samples with <3 replicates, ** = absent from all replicates.

3.3.2 Effects of the centrifugal pump on zooplankton

There were insufficient numbers of organisms present in the zooplankton samples to carry out statistical investigations (Table 3.3). The low number of organisms obtained in the samples which were tested with the centrifugal pump prevents any significant effects from the pump being determined. As a result, this study is unable to statistically determine a change in the mortality of zooplankton due to the pump.

Table 3.4. Mean percentage mortality of zooplankton organisms (±standard deviation) and the mean number of individuals (Italics) in each replicate (±standard deviation). Values are averages of three 100 litre replicates. * = taxon was present in only one replicate, ** = taxon was present in only two replicates.

Organism	Control	After pump
Acartia clausi	0% (0)** *2 (0.52)*	Not present
Copepodite	20% (-)* *5 (1.60)*	0% (-)* *0.17 (0.41)*
Hyperiid	0% (-)* *1 (0.41)*	Not present
Littorina littorea larvae	0% (0) *7 (1.94)*	Not present
Microsetella sp.	55.6% (9.6) *15 (1.87)*	80.6% (17.3) *2 (1.41)*
Mysidacea sp.	Not present	0 (0)** *0.33 (0.52)*
Pseudocalanus elongates	50% (0) *2 (0.52)*	Not present
Semibalanus balanoides cyprids	66.7% (33.3) *12 (1.41)*	Not present
Unidentified copepod	50% (-)* *2 (0.52)*	Not present
Zaus sp.	4.8% (8.2) *15 (2.81)*	0 (-)* *0.17 (0.41)*

3.4 Discussion

Within the scope of this study tests show that the centrifugal pump tested did not have a significant effect on phytoplankton mortality. This lack of damage could be explained by their small size, <50µm, and structure. Of the 21 taxa found in this study 16 were diatoms that possess a hard silica cell wall which will protect them during collisions. In addition, no examples of damage to phytoplankton were recorded in the literature, and phytoplankton were assessed by Nayar et al (2002) and Veldhuis et al (2006). These observations suggest that during ballast water uptake phytoplankton are not damaged or killed when pumped into ballast tanks, and thus begin the journey in good health. There is the potential for mortality during transportation due to the harsh conditions imposed on organisms within the ballast tanks, and this will be addressed in Chapter 4. However, these results highlight that treatment systems must be effective in killing phytoplanktonic organisms.

The zooplankton data collected suggests there was no significant increase in mortality due to the pump, although statistical analysis was not performed and these results should be treated with caution. Zooplanktonic organisms can be delicate and unlikely to withstand colliding with the hard surface of the impellers at the speed and pressure of ballast water uptake, without some resulting damage (as described in Section 3.1.1.). However, smaller and more robust zooplankton, e.g. copepods, have been observed to show little damage due to centrifugal pumps: Mohlenberg (1987) recorded damage to <2% of the copepods in samples and Nayar et al (2002) observed that damage after pumps was negligible (except in delicate ctenophores and fish eggs). The data presented in this study, and that present in the literature, show that the impact on zooplankton is varied. It is possible that some species will enter ballast tanks in reduced health, but other organisms could survive the voyage and be released alive, meaning that effective treatments are required.

3.5 Conclusions

The aim of this study was to collect quantitative data to determine the effect of a centrifugal pump on plankton and this data was obtained. This study agrees with the previous literature that phytoplankton mortality does not increase after passing through the centrifugal pump tested. The data also suggests that zooplankton were not significantly damaged by the pump. Thus, when assessing potential treatment systems it permits the effects of the treatment technology to be isolated from the effects of the pump. However, it also shows that the uptake of ballast water is not a treatment step in itself, and cannot be relied upon to decrease the number of viable organisms entering ballast tanks.

Chapter 4

The effects of prolonged darkness on temperate and tropical marine phytoplankton, and their implications for ballast water risk management

K.J.Carney, J.E.Delany, S.Sawant, E.Mesbahi

Accepted for publication in Marine Pollution Bulletin, March 2011.

4.1 Introduction

The transport of ballast water in international traffic is deemed to play a key role in the unintentional movement of non-indigenous aquatic species across their geographical borderlines. Resolution of this problem has been fraught with difficulties given the implications for international trade, and the administrative complexity of assuring compliance by many governments (Raaymakers 2002). Attempts to perfect removal of organisms have adopted a range of technologies (e.g. filtration, biocides, UV light and deoxygenation). However, the engineering and biological challenges involved are significant given the size, scale and sheer diversity of the organisms concerned, and the fact that thousands of tonnes of water are required to be processed in a short period of time. Efforts are being channelled into finding ways of significantly reducing the risk of organisms being successfully transported. Management policy will thus be based on effective treatment systems capable of meeting the D-2 discharge standard (IMO 2004) and complemented by risk analysis. A tool to aid more effective risk analysis development is a greater understanding of the population dynamics of transported organisms within the ballast water environment, the resultant discharge density at the

journey's end and the potential ability for colonisation of the new environment by these introduced organisms.

Ballast tanks are hostile environments with no light to support growth of autotrophs over long periods, which consequently affects grazing populations. Food and nutrient availability, temperature, and chemical regime also differ from the natural state and will influence survivorship of transported organisms. A number of studies have examined the composition of surviving organisms at the end of journeys with a view to determining the threat posed by ballast water discharged to the destination port (Medcof 1975; Williams et al. 1988; Hallegraeff and Bolch 1991; Carlton and Geller 1993; Dickman and Zhang 1999; Zhang and Dickman 1999; Hamer et al. 2000; David et al. 2007). However, there are only five published studies to date which have monitored zooplankton and phytoplankton populations within ballast tanks for the duration of journeys, and thus investigated the role of the internal tank conditions on inoculate density (Lavoie et al. 1999; Gollasch et al. 2000a; Gollasch et al. 2000b; Olenin et al. 2000; Klein et al. 2010). Even these few available studies highlight the wide variability of responses of different taxa to ballast water conditions. It is possible that even for an individual species, different journeys could present either incubator or hostile space (Gollasch et al. 2000a).

Little research has been conducted on how the conditions within ballast tanks influence phytoplankton population dynamics. This is a significant oversight as these groups are most likely to be influenced by the lack of light within ballast tanks and are themselves key food sources for grazing fractions of the plankton community. Strategies employed to survive prolonged darkness include the formation of resting stages (Marcus 1980; Conover and Siferd 1993; Drake et al. 2007; Rinalta et al. 2007), reducing cell metabolism (Peters 1996; Jochem 1999; Rinalta et al. 2007; Shi et al. 2007; Wu et al. 2008) and switching from autotrophic to heterotrophic feeding (Furusato et al. 2004; Tuchman et al. 2006). Tolerance is not equal in all species and the ability of organisms to survive transport could depend on the length of darkness they are likely to experience in their native environment, as has been demonstrated in a number of studies on polar and temperate species (Anita 1976; Peters 1996; Peters

and Thomas, 1996). These studies did not however explore responses to the dark experimental conditions in assemblages of several taxa. Survival of organisms can be affected by the presence of other species, e.g. through grazing, competition for resources or through an increase in the availability of metabolites due to cell death and lysis of darkness intolerant species. Therefore, in this study an assemblage of natural phytoplankton was studied rather than a single species culture. Further investigations into the differential capability in this regard between taxa from different latitudes will provide valuable information to develop risk analysis of likely discharge scenarios from such voyages.

On release into destination port waters, the successful establishment of an invasive species depends not only on the numbers of viable organisms discharged, but also on the physico-chemical properties of the receiving waters and of the resident biota (Eno 1996; Gollasch and Leppakoski 1999). Seasonality too will have an influence by determining the abundance and diversity of organisms present on transport (Gollasch and Leppakoski 2007), and also on the growth rates and colonisation capability of any given species on discharge. A number of studies have examined the viability of discharged phytoplankton and their potential to colonise after transport (Hallegraeff and Bolch 1992; Kelly 1993). Monitoring the on-growth of organisms in different media subsequent to the dark period is vital to understanding the role of the quality of receiving water on the colonisation success of populations. A single study (Kang et al. 2010) has investigated the role of varied nutrient conditions on on-growth of phytoplankton populations isolated from ballast tanks. However, it could not be demonstrated that all organisms were completely eliminated from the tanks on discharge between journeys. This, coupled with open water de-ballasting and exchange and a lack of in-transit monitoring means that the exact history of dark exposure experienced by the test organisms used by Kang et al (2010) was unknown. Studying the population response of test assemblages in controlled conditions can help to build a more comprehensive picture of colonisation capability enabling more accurate prediction of discharge outcome.

This study has examined the effects of prolonged darkness on naturally sourced phytoplankton assemblages in a controlled environment, and their subsequent ability to recover when returned to a light regime. The objectives were to (i) monitor the dynamics of these assemblages over time and in relation to changes in inorganic nutrients with a view to understanding the role of lysed and degenerating 'peer' cells in sustaining the surviving cohort (ii) investigate the role of biogeographic origin of phytoplankton assemblages and their relative ability to withstand prolonged dark conditions (iii) test the responses of phytoplankton sourced in different seasons and (iv) to assess the colonization success of these observed assemblages, whose recent light-exposure history is known, in media of different nutrient concentrations.

4.2 Methodology

Tests were performed at the National Institute of Oceanography, Goa, India in February and March 2009. Tests at the Dove Marine Laboratory, Newcastle University, UK were performed in October - November 2009 (autumn) and April - May 2010 (spring).

4.2.1 Experimental set up

Locally sourced seawater was collected for all experiments on the day of experimental set-up, from a pier immediately adjacent to the respective laboratories and filtered by 100µm to remove larger organisms. Three replicate samples (280ml Goa, 180ml Newcastle) were taken on Day 0 to determine cell abundances, chlorophyll α and nutrient levels before the seawater was split between six experimental containers. The total volume of seawater in containers was 5L (Goa) and 3.5L (Newcastle). Three of these containers were covered for the initial four weeks to prevent light reaching the water ('Dark-regime' treatment), the other three containers served as 'Controls'. After the Dark experimental period the coverings were removed from the Dark-regime treatment containers to reintroduce the phytoplankton assemblages to light conditions, and maintained under a similar regime as the Control containers for the remainder of the experimental period. In Goa the light regime was 11.5:12.5 (L:D) and

in Newcastle 12:12 (L:D). Similar experimental containers were used in the two locations with the key features of being transparent, allowing gas exchange and enabling ease of sample collection. In Goa, transparent polyethylene bags were adapted as experimental containers and in Newcastle glass bottles (Fisherbrand cat: 02-911-918) were used. Tubes were inserted into each container to allow air exchange. In Goa the temperature of the experimental containers was maintained at a mean of 31±0.13°C with a mean daily variance of 3.8±0.13°C, by suspension in an outdoor pool. In Newcastle the experimental containers were placed in an incubator for the duration of the test, and maintained at temperature of 11°C.

Samples were taken for phytoplankton abundance, chlorophyll α and inorganic nutrient levels from each container on Days 0, 1, 4, 7, 14, 21 and 28 (experimental dark period), and at Day 36 and 43 (after reintroduction to light regime). In the Newcastle experiments, samples were also collected on Day 29, and for the autumn experiments, sampling was completed on Day 36.

Additional experiments were carried out at Newcastle University to determine population regrowth response under different nutrient regimes, following re-introduction to light conditions. Three different nutrient regimes were assessed: (i) the original incubation water that organisms had been maintained in for the initial 28 day period (ii) a fresh supply of seawater sourced locally and (iii) nutrient depleted water. On day 29 the contents of each of the six containers were split between the three media. From each vessel 650ml was filtered by 10µm and the filtered organisms were rinsed into a new experimental container with 650ml of either incubation water, fresh seawater or nutrient depleted water. Before use the new media were filtered using Whatman grade 42 filters. Once the experimental containers were established, samples for analysis of cell abundance, chlorophyll α and nutrient levels were taken.

In the autumn Newcastle experiment, 'nutrient depleted' water was prepared following the methodology of David and Sleep (1989) but using *Tetraselmis suecica* instead of *Skeletonema costatum* as the nutrient scavenger. However, analysis carried out subsequent to the end of the experiment showed that high levels of nutrients

were present on Day 29 and this technique was therefore deemed not to have worked. This particular treatment therefore represents a further nutrient regime and not one of nutrient-depleted. In the spring experimental run Low Nutrient Seawater was obtained from OSIL.

4.2.2 Parameters measured

4.2.2.1 Chlorophyll α

Samples (100 ml) were filtered through pre-ashed 0.7 µm pore GF/F Glass fiber micro filters using a vacuum pressure <400 mm Hg. The filters were then extracted in 90% acetone in the dark at 4ºC for 24 hours, centrifuged and then analysed with a Turner Designs Trilogy™ fluorometer (Parsons et al. 1984).

4.2.2.2 Inorganic nutrients

50ml of seawater was collected and gravity filtered using 10µm mesh to remove large particles. In Goa nutrient analysis was carried out immediately following the methods described by Parsons et al (1984) for nitrate, nitrite, ammonia, phosphate and silicate. At Newcastle samples were frozen at -20°C and analysis subsequently performed using a Bran and Luebbe Autoanalyser 3. Nitrate, nitrite, ammonia, phosphate and silicate were measured simultaneously.

4.2.2.3 Viability assessment

In spring and autumn Newcastle experimental runs only viability was assessed using the vital fluorescent stain Fluorescein diacetate and FlowCAM. The stain was prepared following the method outlined in Jochem (1999). A 5ml sample was assessed and 166µl of FDA working stock was added. The sample was incubated in the dark at room temperature for 5 minutes and then assessed using FlowCAM with a 100µm depth flow cell at 10X magnification.

FlowCAM assessment was performed in the dark using TriggerMode with only Channel 2 switched on for particle detection (emission frequency 525±15nm) at a threshold of 400. FlowCAM is equipped with a 488nm laser which excites the FDA stain (excitation frequency 490nm) and then detects cells emitting a fluorescent signal (FDA emission frequency 515±15nm) above the threshold level of 400. The files were manually assessed and cells per ml calculated by FlowCAM.

4.2.2.4 Cell number

A 10ml sample was taken and fixed using Lugols Iodine. All cell counts were performed using FlowCAM. Samples were run in AutoImage mode using a 100µm depth flow cell and 10X objective and a 5ml aliquot was assessed.

4.2.3 Statistics

Two and three way ANOVA tests were used to assess for significant differences between Control and Dark treated samples in all variables measured between light/dark treatment, days and water type where applicable. Where significant differences were found Tukey tests were applied. Dark period and regrowth period data were assessed separately.

4.3 Results

4.3.1 Tropical population

Total cell numbers of 1204±422 cells per ml were present in Day 0 samples. Chlorophyll α levels in both the Dark-regime and Control samples decreased during the initial 28 day dark experimental period, but the decrease was much more apparent in the Dark-regime, where levels decreased from 3.2µg/l on Day 0 to the minimum level of 0.15µg/l on Day 14 (Figure 4.1 a). A two way ANOVA of chlorophyll α levels, (Fixed

factors: Light/dark regime, 2 levels; Day, 7 levels) showed that this difference was significant between levels in Control and Dark treatments (2-way ANOVA; $p = <0.001$, $F = 39.14$, d.f. = 1) and also between Days (2-way ANOVA; $p = 0.010$, $F = 3.53$, d.f. = 6), with no significant interaction between factors. Changes in phytoplankton populations as evidenced by the chlorophyll α levels coincided with changes in nitrate, nitrite and silicate levels (Figure 4.1 b-d), while phosphate and ammonia levels did not change accordingly (Figure 4.1 e-f). In Dark-regime samples this was manifested as an overall increase in nitrate, nitrite and silicate levels over the 28 days. No such increase was observed for nitrate and nitrite levels in the Control samples. For silicate, levels in Control returned to Day 0 levels after an initial decline. Two way ANOVA showed significant interactions between factors Light/dark regime and Day for all three nutrients: nitrate (2-way ANOVA; $p = <0.0001$, $F = 8.75$, d.f. = 6), nitrite (2-way ANOVA; $p = <0.0001$, $F = 9.54$, d.f. = 6) and silicate (2-way ANOVA; $p = 0.036$, $F = 2.66$, d.f. = 6). Tukey multiple comparisons showed that this was due to the differences between the dark maintained and Control samples within certain sampling dates.

During the period of re-exposure to light chlorophyll α levels in Dark treated samples increased during the first week to 3.01µg/l, almost equal to Day 0 value. During the same period Control chlorophyll α levels continued to decrease (Figure 4.1 a). Nitrate, nitrite and silicate levels all decreased throughout the re-exposure period (Figure 4.1 b-e). A significant interaction between the factors Dark/Control treatment and Days for nitrate concentration was observed (2-way ANOVA; $p = 0.005$, $F = 8.86$, d.f. = 2). Tukey multiple comparisons showed that this was due to the differences between the Dark-regime and Control samples on Day 29. A significant interaction between Dark/Control treatments and Days was also found for silicate levels (2-way ANOVA; $p = <0.001$, $F = 64.18$, d.f. = 2). Tukey multiple comparisons showed that this was due to the differences between the Dark-regime and Control samples on Days 36 and 43. Nitrite levels showed a significant difference between Control and Dark-regime treatments (2-way ANOVA; $p = 0.046$, $F = 5.08$, d.f. = 1).

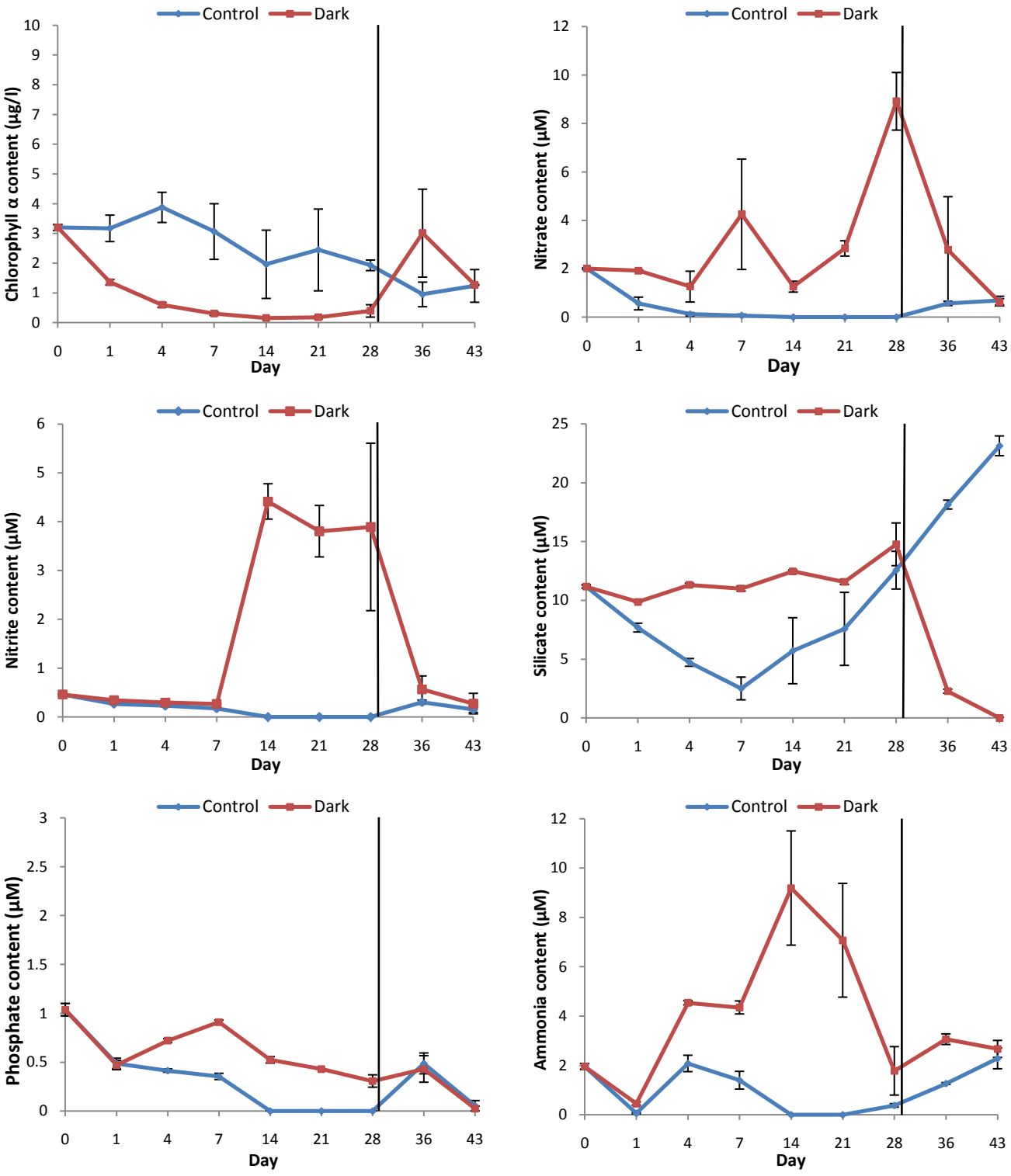

Figure 4.1 a-f. Chlorophyll α, nitrate, nitrite, silicate, phosphate and ammonia (a-f) concentrations on all sampling days of the tropical population experiment performed in Goa. All values are the mean of three replicates ± SE. The line indicates removal from Dark conditions.

4.3.2 Temperate population: Autumn

In this experiment chlorophyll α levels were lower than the tropical population, and few live cells were present: 1.67µg/l chlorophyll α and 2.3 live cells per ml were present in Day 0 samples. During the dark experimental period live cell numbers and chlorophyll α levels in the Dark-regime samples showed an overall decrease (Figure 4.2 and Figure 4.3a). Control treated samples showed an overall increase in both chlorophyll α levels and live cell numbers. A significant interaction was found between the factors Dark/Control treatment and Days for both chlorophyll α levels (2 way ANOVA; $p = <0.001$, $F = 21.23$, d.f. = 6) and live cell numbers (2-way ANOVA; $p = <0.001$, $F = 19.67$, d.f. = 6). Tukey multiple comparisons showed that for both chlorophyll α levels and live cell numbers the significant interaction was due to differences between the Dark-regime and Control samples within a number of sampling dates. Nitrate, nitrite and silicate levels showed little change and concentrations did not increase to high levels towards the end of the dark period (Figure 4.3b-c and Figure 4.4a-c), as was observed in the tropical population.

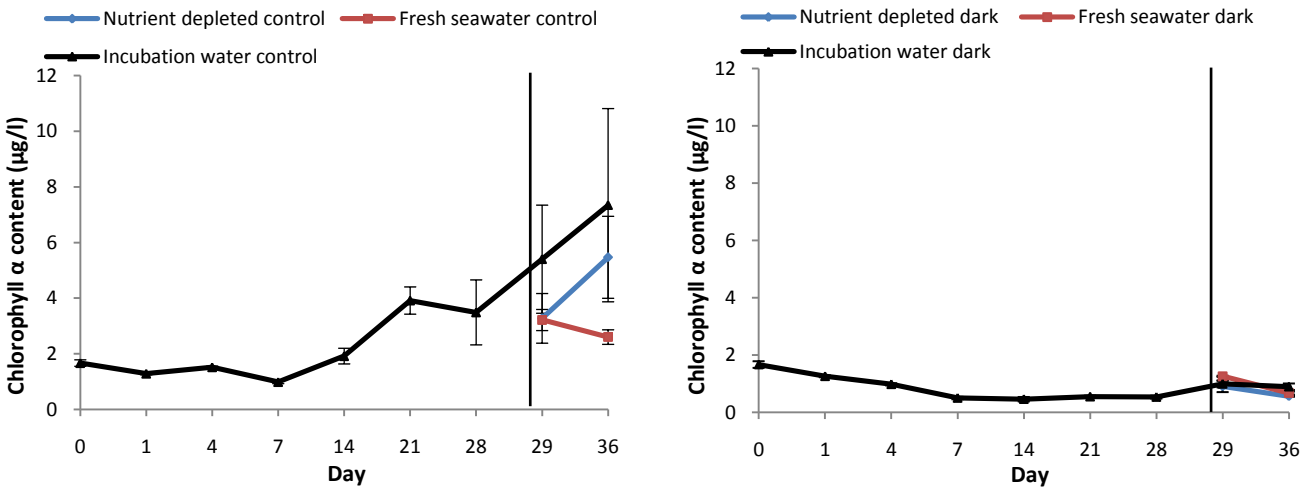

Figure 4.2. The temperate population experiment performed in autumn in Newcastle. Figures show chlorophyll α levels on all sampling days. Values from Control and Dark treated samples are separated and the line indicates removal from Dark conditions. All values are the mean of three replicates ± SE.

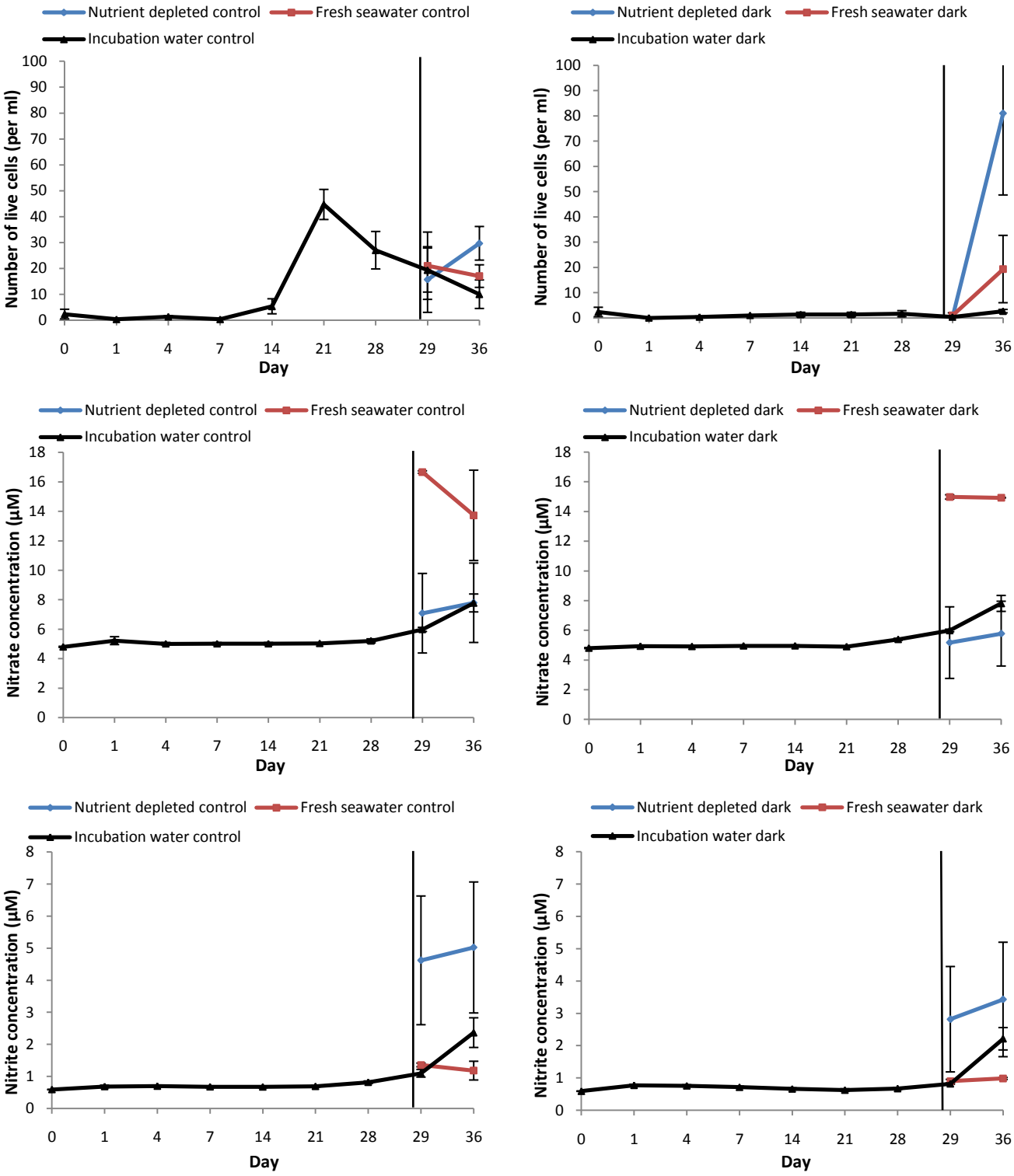

Figure 4.3a-c. The temperate population experiment performed in autumn in Newcastle. Figures show live cell numbers, nitrate and nitrite (a-c) concentrations on all sampling days. Values from Control and Dark treated samples are separated and the line indicates removal from Dark conditions. All values are the mean of three replicates ± SE.

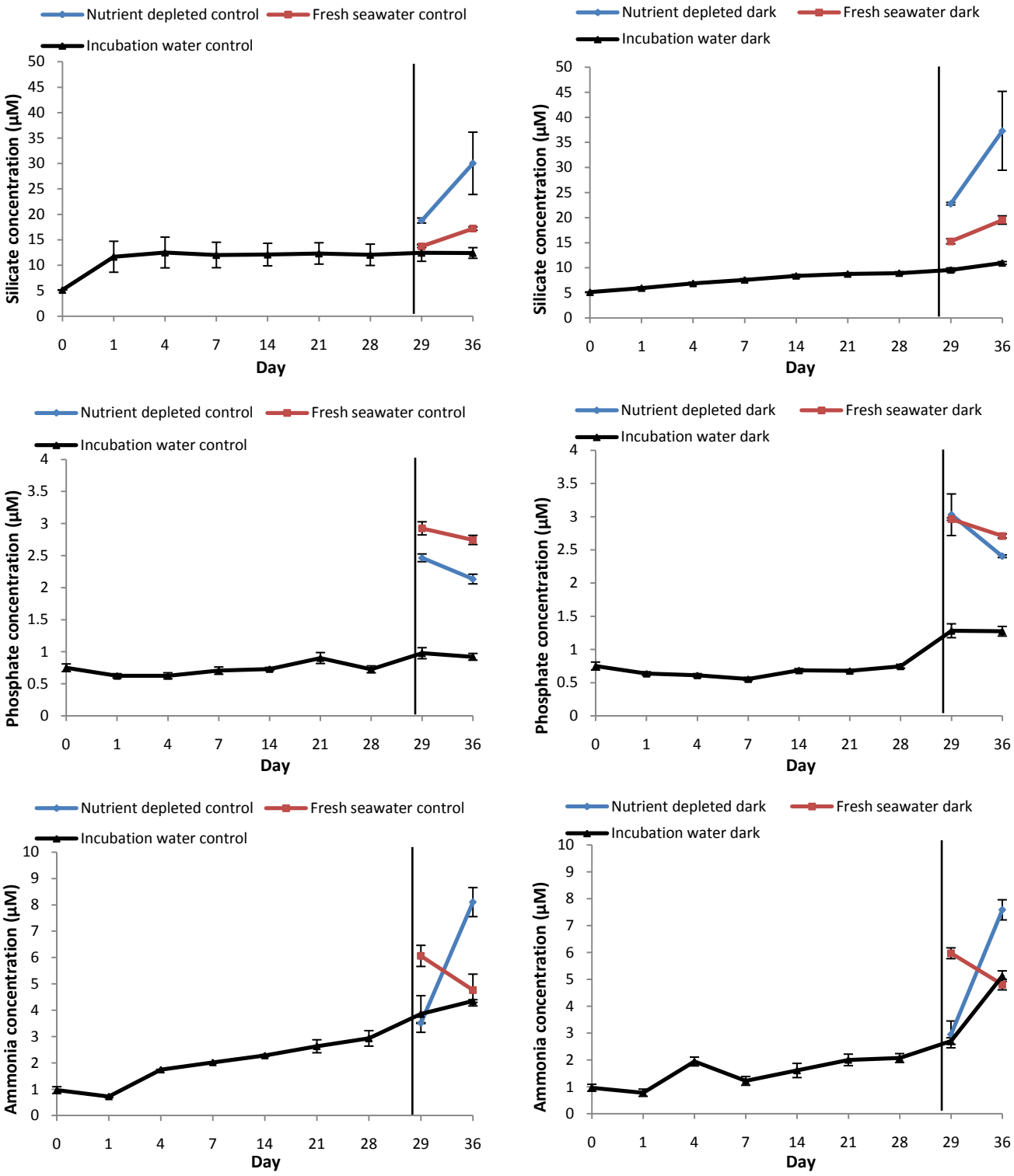

Figure 4.4a-c. The temperate population experiment performed in autumn in Newcastle. Figures show silicate, phosphate and ammonia (a-c) concentrations on all sampling days. Values from Control and Dark treated samples are separated and the line indicates removal from Dark conditions. All values are the mean of three replicates ± SE.

During the re-exposure to light, the effects of three different nutrient regimes were assessed: (i) the original incubation water (ii) locally sourced fresh seawater and (iii) a third prepared nutrient regime that proved to have high inorganic nutrient content. During this period (Days 29-36) chlorophyll α levels in Control samples continued to increase, but no increase was observed in Dark-regime samples. However, live cell numbers in the Dark-regime samples did rise, and this increase was highest in the (iii) high nutrient water type. Significant two way interactions were found in the numbers of live cells recorded between factors Day and Water Type (3-way ANOVA; $p = 0.003$, $F = 5.74$, d.f. = 2), Tukey multiple comparisons showed that this was due to differences between Days within the (iii) high nutrient level water type, and between all three Water types on Day 36. A significant interaction was also found between factors Day and Dark/Control treatments (3-way ANOVA; $p = <0.001$, $F = 7.34$, d.f. = 1). Tukey multiple comparisons showed that this was due to differences between the Dark-regime and Control samples on Day 29. The increase in live cell numbers which was not reflected in chlorophyll α levels was due to the increase in numbers of an indeterminate flagellate and dinoflagellate cysts. Definitive identification was not possible but the most probable genus for the flagellate is *Hemistasia* sp., which is a heterotrophic genus. Nutrient levels did not show a consistent change in any of the three water types (Figure 4.3b-c and Figure 4.4a-c).

4.3.3 Temperate population: Spring

In the spring experiment higher numbers of live cells and higher chlorophyll α levels were present than in the autumn experiment, with 14 live cells per ml and 3.8µg/l chlorophyll α present on Day 0. During the dark experimental period (Days 0-28) chlorophyll α levels and live cell numbers in Dark-regime samples showed an overall decrease, and during the same period chlorophyll α levels and live cell numbers in the Control samples increased (Figure 4.5 and Figure 4.6a). A significant interaction was found between the factors Dark/Control treatment and Days for both chlorophyll α levels (2 way ANOVA; $p = <0.001$, $F = 39.55$, d.f. = 5) and live cell numbers (2-way ANOVA; $p = 0.001$, $F = 5.28$, d.f. = 6). Tukey multiple comparisons showed that this was due to the differences between the Dark-regime and Control samples within certain

sampling dates in both cases. Nutrient levels in initial samples were high (Figure 4.6b-c and Figure 4.7a-c). Dark-regime treated samples showed a decrease in nitrate, nitrite and phosphate during Days 0-28, while in the same period an increase in silicate concentration was observed. In Control treated samples nitrate, nitrite, silicate and phosphate levels all decreased throughout the dark experimental period, and this decrease was more than that observed in the Dark-regime treated samples.

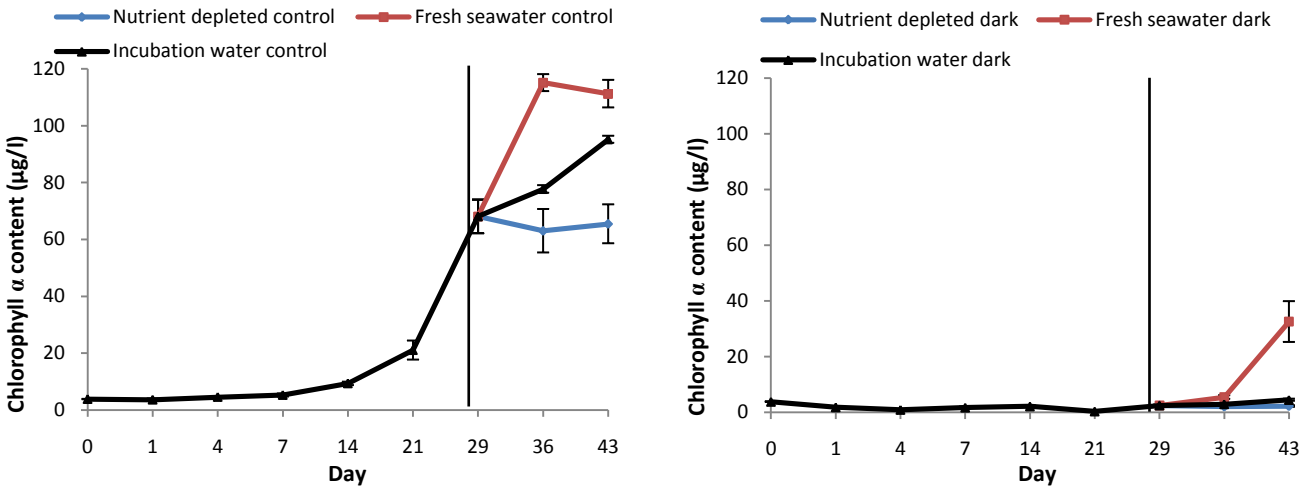

Figure 4.5. The temperate population experiment performed in spring in Newcastle. Figures show chlorophyll α levels obtained on all sampling days. Values from Control and Dark treated samples are separated and the line indicates removal from Dark conditions. All values are the mean of three replicates ± SE.

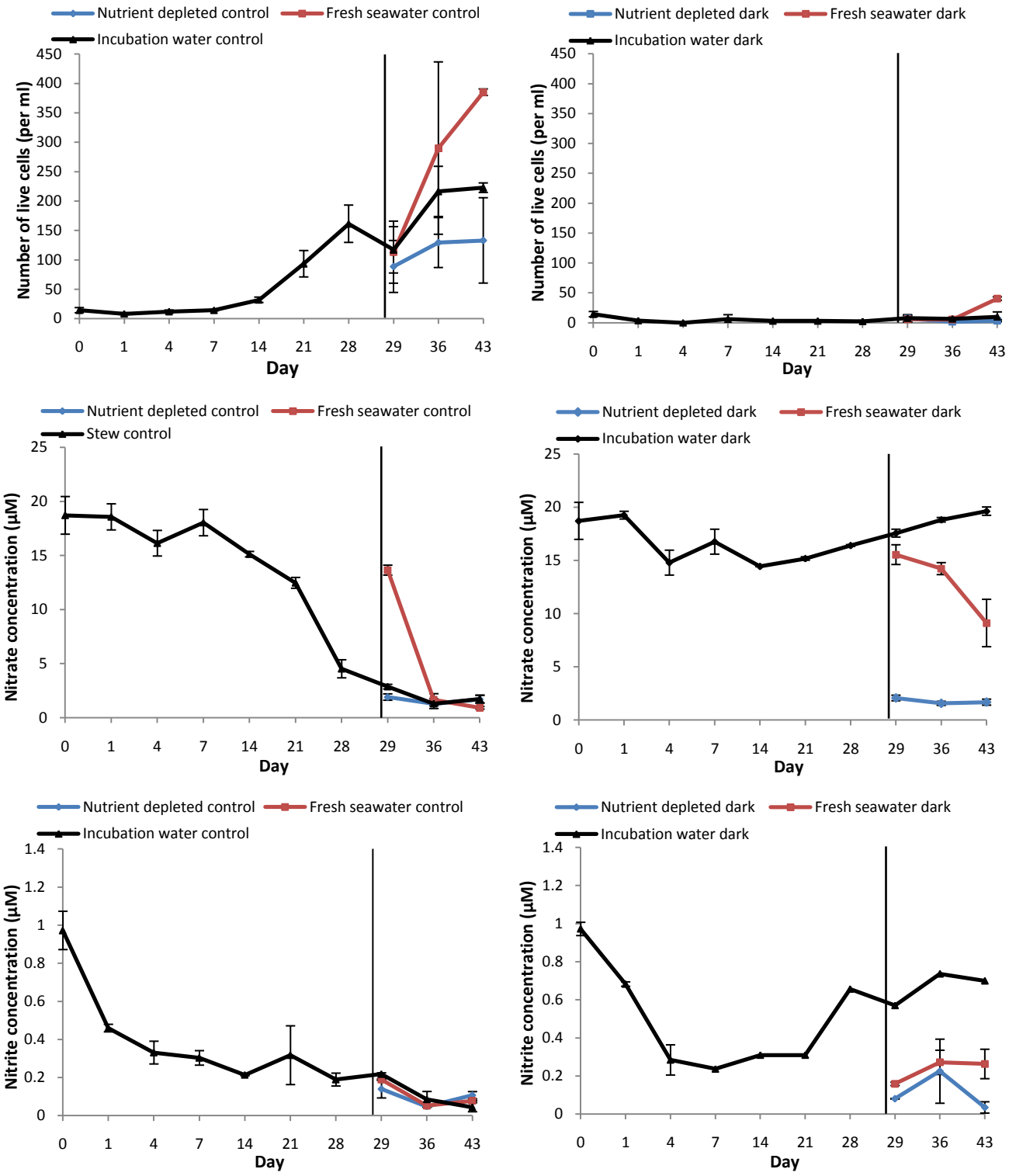

Figure 4.6a-c. The temperate population experiment performed in spring in Newcastle. Figures show live cell numbers, nitrate, nitrite (a-c) concentrations obtained on all sampling days. Values from Control and Dark treated samples are separated and the line indicates removal from Dark conditions. All values are the mean of three replicates ± SE.

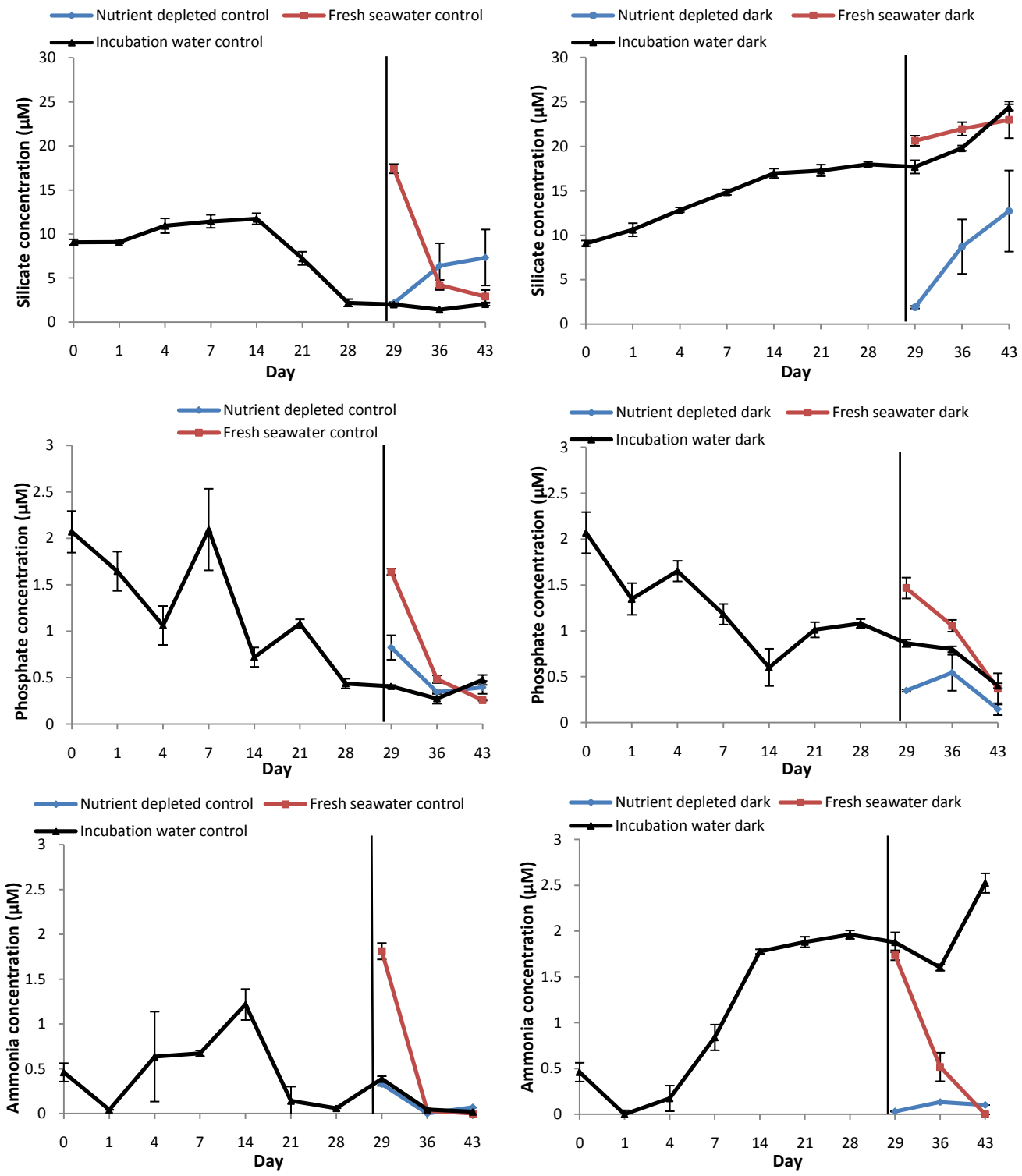

Figure 4.7a-c. The temperate population experiment performed in spring in Newcastle. Figures show silicate, phosphate and ammonia (a-c) concentrations obtained on all sampling days. Values from Control and Dark treated samples are separated and the line indicates removal from Dark conditions. All values are the mean of three replicates ± SE.

During the re-exposure to light phase (Days 29-43) chlorophyll α concentrations in the Control samples continued to increase, except in the nutrient depleted water. When returned to the light on Day 29 population regrowth was observed in those samples introduced to the locally sourced fresh seawater (water type ii). Two diatom taxa were observed to grow in this water type: nitzschioid pennate diatoms and *Thalassiosira* spp.. The nitzschioid pennate diatoms in Dark-regime treated samples increased from 0.3±0.3 live cells per ml on Day 29 to 5±2 live cell per ml on Day 43 and *Thalassiosira* sp. increased from 6±5 live cells per ml on Day 29 to 18±2 live cells per ml on Day 43. Nutrient levels in the Dark-regime treatment in locally sourced fresh seawater (water type ii) showed decreases in nitrate, phosphate and ammonia, while nitrite and silicate showed little change. Control treated samples in fresh seawater showed a decrease in all nutrients. The nutrient depleted water did not promote population growth (Figure 4.5 and Figure 4.6a), and little change in nutrient levels was observed. The incubation water maintained the Dark-regime exposed population for the two week period, (Figure 4.5 and Figure 4.6a) and decreases in nitrate, nitrite, silicate and ammonia levels were observed.

4.4 Discussion

The experiments completed in this study demonstrate that organisms from both tropical and temperate locations were able to survive the 28 day dark period and, when returned to a light regime, were in sufficient health to reproduce and photosynthesise. The ability of organisms to survive prolonged dark conditions is related to the likelihood that they would experience these conditions in their natural environment, as shown by various authors (Antia 1976; Peters 1996; Peters and Thomas 1996). This would suggest that assemblages of tropical origin, which experience sunlight in the range of 11 to 13 hours per day, might be less able to adapt and survive than a temperate assemblage. However, the observations made in this study show that even populations accustomed to experiencing a limited variance in sunlight were able to maintain their photosynthetic equipment during an extended dark period and begin photosynthesis quickly when returned to a light regime. These

findings expand our understanding of the effect controlled dark conditions have on plankton assemblages by testing organisms from a new biogeographic area. The level of growth observed in the tropical population was comparable to that of the temperate population in incubation water after the dark period.

Observations made from initial tests in Goa showed high levels of nitrate, nitrite and silicate were present in dark treated samples at the end of the dark experimental period, which was attributed to cell lysis. The cellular matter released by dead and lysing cells is further broken down into dissolved organic matter. This increases the levels of nutrients and organic matter available for utilisation by cells, either for cell repair and growth when returned to light or to support heterotrophic populations during the dark period (Bunt and Lee 1972; Wulff et al. 2008; Hess-Erga et al. 2010). Subsequent tests on the temperate population were therefore used to investigate the role of nutrient availability on population recovery following their return to light. The type of water organisms were exposed to did have a significant effect on population recovery. When the water was depleted of nutrients growth was limited, as seen in the Newcastle spring experiment. But when nutrients were available i.e. in the fresh seawater, incubation water and nutrient depleted water (Newcastle autumn experiment), recovery and growth of populations was observed through increased chlorophyll α levels and live cell numbers. Kang et al (2010) was the first study to investigate the potential growth of phytoplankton populations transported in ballast tanks in a range of seawater conditions which simulated those post discharge, in order to determine the effect different nutrient regimes have on population recovery. An understanding of how these varying nutrient regimes affect survival could be used to predict the response of phytoplankton populations to different water types upon discharge. This could be used to aid the development of future management strategies as discharge could occur into a variety of different nutrient conditions, from stagnant nutrient-poor dock waters to nutrient-rich coastal waters.

The incubation water in this study, and the ballast water used by Kang et al (2010) reflected the effect of nutrient release through peer-lysis on population recovery. Kang et al (2010) observed growth in the ballast water, and the incubation water used in this

study supported growth in all experiments during the recovery period, although the growth during this time did not exceed original chlorophyll α levels. On discharge at port, conditions of receiving waters may differ substantially from those of the native habitat from which transported organisms originated. Individuals can, however, be buffered to a degree by the accompanying large volume of ballast water and associated nutrients. This could prove important for colonisation success in poorly flushed waters in particular.

The species which showed most growth in experiments were diatoms: *Amphora* sp. in the tropical assemblage and *Thalassiosira* sp. and nitzschioid pennate diatoms in the temperate populations. Kang et al (2010) demonstrated that bloom forming diatoms and pennate diatoms appear to be the species most likely to become successfully established in the studied ports. The species observed in this study can be harmful, for example, some species of nitzschioid pennate diatoms and *Amphora* sp. are blooming diatoms and known producers of the neurotoxin domoic acid. *Pseudo-nitzschia* sp. and *Amphora coffaeiformis* have caused amnesic shellfish poisoning through human consumption of contaminated mussels (Shimizu et al. 1989; Wright et al. 1989) and have been responsible for bird kills in Monterey Bay, California (Fritz et al. 1992). The occurrence of blooms of *Thalassiosira* spp. has been linked to observations of damage to fisheries and aquaculture through physiological injuries to the gills of fish and bivalves (Takano 1956; Kent et al. 1995; Miyahara et al. 1996). These recordings show that the species which survived in these experiments could cause negative impacts if successfully transported by ballast water.

The effect of seasonal changes showed different organisms exhibited growth in the recovery period of the autumn and spring experiments. In both experiments the diatom *Thalassiosira* spp. was present in early samples, but it only showed population recovery in the spring experiment. Temperature, light intensity and photoperiod were identical in both experiments and therefore other factors were responsible for this difference in growth. Nutrient availability could be a potential factor as nitrate, nitrite and silicate concentrations were higher in autumn samples than spring samples at the end of the dark experimental period. Another factor which is significant in the success

of invasive species is propagule pressure i.e. the number of individuals released into an area to which they are not indigenous (Johnston et al. 2009). *Thalassiosira* spp. are dominant diatoms in the spring blooms of many temperate locations (Harris et al. 1995; Popovich and Gayoso 1999) and are thus present in high numbers in the spring months. The number of *Thalassiosira* spp. present in autumn samples was less than those present in spring, and so the input density of organisms in autumn may have been insufficient to establish a successful population. This is a relevant factor in risk management for ballast water discharge as the input of organisms from the same shipping route will vary considerably with season, and species present throughout the year will pose a greater threat during certain months. Season has a significant effect on propagule pressure and must be considered when identifying high risk scenarios.

Research into assessing the risk of successful invasions posed by ballast water discharge has been approached in a variety of ways by different authors (Hayes 1998; Hayes and Hewitt 1998; Gollasch and Leppakoski 1999; Hayes and Hewitt 2000; Hayes and Sliwa 2003; Behrens et al. 2005; Gollasch and Leppakoski 2007; Barry et al. 2008). This research has sought to identify transport routes with a high risk of successful introductions and species which pose a high risk of invasion to certain areas, in order to implement management techniques to minimise the possibility of successful transportation. Effective risk assessment relies on understanding a complex matrix of factors (e.g. the environmental similarity between the origin and destination port through temperature and salinity data, assessment of food availability, presence of predators, habitat structure, ballast water volume and frequency of input, and the length of voyage) which all play an important role in the potential success of any invasive species. In addition to identifying high risk scenarios through reported invasions and predictive models, controlled laboratory experiments such as this and Kang et al (2010) are able to increase our understanding of the factors involved in successful transportation via ballast water, and aid the development of effective management techniques.

4.5. Conclusions

This study monitored phytoplankton assemblages from two geographic locations during a prolonged dark period and assessed their ability to recover when returned to a light regime. Recovery was observed in both temperate and tropical assemblages. Nutrient availability during recovery had a significant effect on growth: nutrient depleted conditions prevented growth while the availability of nutrients facilitated growth in phytoplankton assemblages from both locations. The role of seasonality was addressed and was observed to affect the dominant species present. This could alter the risk posed by ballast discharge throughout the year. The data obtained increases our understanding of ballast transportation and discharge conditions on phytoplankton. This study achieved its aims, and the inclusion of this information in ballast water risk assessment techniques could be used to aid effective management of ballast water transportation.

Chapter 5

The potential application of UV irradiation for ballast water treatment

5.1 Introduction

5.1.1 Discovery of UV light

Early observations of the effects of what we now know to be UV light involved the darkening of silver chloride soaked paper when exposed to sunlight. In 1801 Ritter observed that this darkening was due to light rays just beyond the violet end of the spectrum. He went on to determine that these light rays were not causing the darkening through heat, and so he referred to the rays as 'deoxidizing' to highlight their chemical reactivity (Hockberger 2002). Research investigating the effects of light irradiation continued, and in 1842 Becquel and Draper first identified the wavelength spectrum of UV light to be between 340 and 400nm (Hockberger 2002). Further studies continued and in 1862 Stokes was able to extend the known region to 182nm (Masschelein 2002), i.e. UVB, UVC and the high end of vacuum-UV. We now know that UV light consists of light of wavelengths 100 - 400nm and in the light spectrum it is situated between X-ray light and visible light (Figure 5.1). UV light itself is then split into four different types: vacuum UV (100-200nm), UV-C (200-280nm), UV-B (280-315nm) and UV-A (315-400nm).

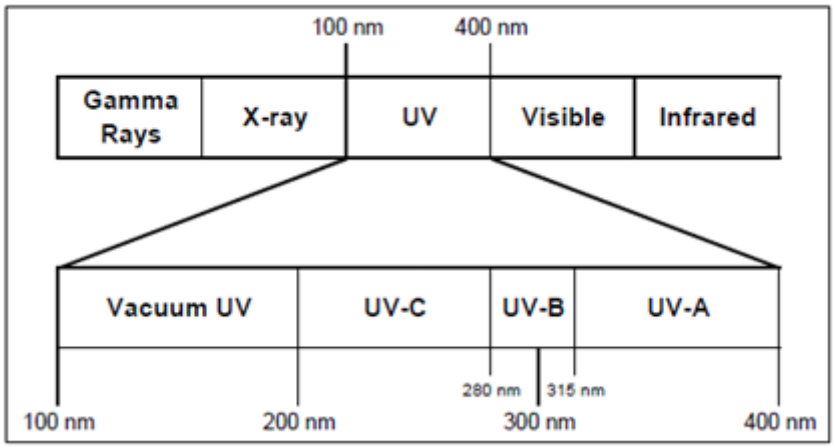

Figure 5.1. The UV light spectrum. Figure adapted from EPA (2006).

5.1.2 The germicidal effect of UV light

The initial suggestion that UV light may have a bactericidal effect was by Downes and Blunt (1877). Ward (1892) proved through experimentation that light was responsible for mortality in *Bacillus anthracis* spores, and he attributed this to UV wavelengths. However, it was Barnard and Morgan (1903) who were able to finally identify UV rays of 226-328nm as bactericidal. Newcomer (1917) further assessed the bactericidal effect of UV light and determined that >280nm wavelengths produced less bactericidal effect and a slight peak in strength was observed around 260nm. In 1932 the germicidal peak was isolated to be 253.7nm by Ehrismann and Noethling (Kowalski 2009). While UV-B and UV-C have been shown to have a germicidal effect, UV-A light (315-400nm) and vacuum UV (100-200nm) are not germicidal (Kowalski 2009).

An early study by Arnold (1933) observed a reduction in phytoplankton photosynthesis due to increased UV-C (253.7nm) irradiation. This was confirmed by Holt et al (1951), who in addition observed a delayed reduction in respiration in *Scenedesmus* sp. after irradiation at 253.7nm, and further studies observed an inhibitory effect of UV light on photosynthesis (Steeman Nielson 1964; Jitts et al. 1976). While early studies concentrated on the effects of UV-C irradiation attention turned to the effects of UV-B exposure after it was discovered that the ozone layer, the main protection against high UV-B exposure on Earth, was being depleted. Lorenzen (1979) showed experimentally

that in the absence of UV-B irradiation there was an increase in ^{14}C incorporation by phytoplankton. Further studies have since shown that UV-B irradiation inhibits photosynthesis in phytoplankton (e.g. Steeman Nielson 1964; Jitts et al. 1976; Smith et al. 1980; Worrest et al. 1981; Worrest 1983; Paerl et al. 1985; Gala and Giesy 1991). Phytoplankton are more susceptible to UV-B damage than higher plants and animals as they do not possess protective epidermal layers which absorb UV light (Hader and Worrest 1991). Some phytoplankton do possess protective pigments e.g. zeaxanthin, which limit damage caused by UV irradiation (Demers et al. 1991), but sunlight has been observed to kill diatoms at high irradiation levels (Calkins and Thordardottir 1980). Photosynthetic activity is reduced because the photosynthetic pigments (phycobilins, carotenoids and chlorophylls) are bleached and the reactive centre of Photosystem II is damaged, membrane integrity changed and lipid content reduced (Hader and Worrest 1991). Mortality, decreased growth and reduced reproductive capability are all resulting factors of UV-B exposure; for a detailed review of the effects of increased UV irradiation on aquatic organisms see Hader and Worrest (1991).

The main way in which UV-C light affects organisms is through damaging nucleic acids i.e. DNA and RNA (Björn 1996; Herbert 2002; Liu 2005; Sassi et al. 2005; United States Environment Protection Agency 2006; Hess-Erga et al. 2008; Tsolaki and Diamadopoulos 2009). UV-C light is absorbed by bonds between base pairs in DNA and RNA molecules and causes the bond to become 'open' i.e. able to form new bonds. This can stimulate the formation of covalent bonds, named 'pyrimidine dimers' (Figure 5.2), between these bases and neighbouring bases. These bonds alter the structure of the DNA, thus hindering or preventing DNA replication and result in organism inactivation (Wright and Cairns ; Pini 1999; Goodsell 2001; Oguma et al. 2001; Martin et al. 2004; United States Environment Protection Agency 2006; Hess-Erga et al. 2008).

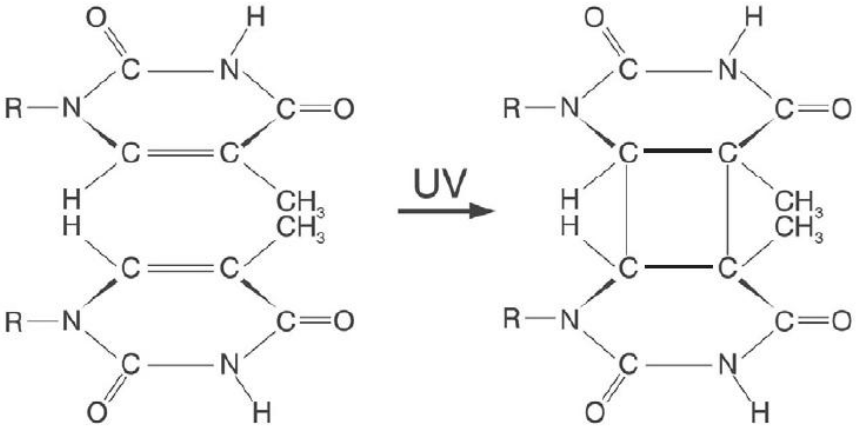

Figure 5.2. The formation of pyrimidine dimers due to UV light absorption. Taken from Martin et al (2004).

The formation of pyrimidine dimers can be reversed by many affected cells, although viruses are unable to do so (Wright and Cairns). Human cells have groups of proteins which work together to locate these bonds and remove a section of approximately 30 base pairs around the bond (Goodsell 2001). Some microorganisms are also able to remove these pyrimidine dimers and there are two main methods used for this: photorepair and darkrepair (United States Environment Protection Agency 2006). Photorepair requires light between 300 and 500nm to stimulate enzymes which break the covalent bonds formed by UV exposure (Wright and Cairns). Dark repair does not require light but can be performed in the presence of light. Dark repair processes include excision repair – this is completed by enzymes which remove the section of DNA or RNA containing the pyrimidine dimers and then the gap is filled by a newly formed section (United States Environment Protection Agency 2006). When UV irradiation is applied for disinfection purposes the ability of organisms to repair damage should be addressed and the disinfection protocol should be used to minimise the possibility of repair occurring.

5.1.3 Development of UV lamps

As research identified the germicidal properties of UV light its application in artificial lamps was being investigated. In 1835 Wheatstone invented a mercury vapour lamp and in 1850 Stokes invented a quartz arc lamp which produced UV light at 185nm. Over the next few decades these discoveries were further modified into a commercially viable mercury vapour UV lamp by Hewitt in 1901 (Hockberger 2002). These early lamps were used for artificial lighting, but the development of tungsten lamps in 1906 gave a more efficient product, replacing UV light in this application (Hockberger 2002). As the germicidal effect of UV became known scientists began to develop other purposes for UV light and in 1906 the first mercury UV water sterilizer was invented by De Mare (von Recklinghausen 1914).

UV light is created by applying voltage to a gaseous mixture, resulting in the discharge of photons. The wavelength of UV light emitted is dependent on the gas used for production and the power level of the lamp (United States Environment Protection Agency 2006). Mercury vapour is commonly used as it creates UV light with good germicidal properties. There are two types of mercury vapour lamp used for disinfection: low and medium pressure. Low pressure lamps are regularly used in drinking water applications as they emit monochromatic light at the germicidal frequency, i.e. 253.7nm UV-C light. Medium pressure lamps emit polychromatic light of ≈210-320nm, but emit a higher intensity light and so result in more energy output than low pressure lamps (Wolfe 1990).

Once UV light is emitted from the lamp it interacts with surrounding materials either by absorption, reflection, refraction or scattering (Liu 2005). Absorption of UV light transforms it into different forms of energy leaving it unavailable for disinfection purposes. The other processes: refraction, reflection and scattering, change the direction the light is travelling in but do not reduce the available energy (United States Environment Protection Agency 2006). In addition to UV absorption the transmittance of UV (UVT) is an important factor when assessing water quality and UV behaviour.

UVT is defined as 'the percentage of light passing through a material, e.g. water, over a specified distance' (United States Environment Protection Agency 2006).

5.1.4 Applications of UV light for disinfection of liquids

Initial investigation of UV irradiation for water disinfection was related to its application in drinking water treatment. The opening of the first UV disinfection treatment works for drinking water was in 1910 in France (Clemence 1911; Carlson et al. 1985). However, at this time the technology was not sufficiently advanced, attempts failed due to high maintenance and operating costs and UV became replaced by chlorine treatment for many years. After widespread use it became apparent that chlorination produced potentially carcinogenic and toxic by-products and that some of the common waterborne pathogens, including *Cryptosporidium* sp. and *Giardia* sp., were resistant to chlorine. In the US this became apparent after an outbreak of Giardiasis, caused by the protozoan parasite *Giardia lamblia* (Carlson et al. 1985; Harris et al. 1987). Subsequently, UV irradiation was again investigated for application in drinking water disinfection to replace chlorination, with many studies assessing UV induced inactivation of *Cryptosporidium* sp. and *Giardia* sp. (Rice and Hoff 1981; Carlson et al. 1985; Bukhari et al. 1999; Craik et al. 2001; Shin et al. 2001; Linden et al. 2002; Mofidi et al. 2002; Morita et al. 2002). After success in this application UV treatment was investigated for use in wastewater, sewage and swimming pool water disinfection.

The first research into the use of UV in sewage treatment was in 1975, and when combined with a primary treatment 99% bacterial inactivation was obtained. The authors recommended UV for further development and use in this application (Oliver and Cosgrove 1975). Investigations into utilising UV treatment for secondary effluent in wastewater disinfection began due to disease outbreaks from contaminated water supplies. Its use was found to be successful and is now widely implemented (Harris et al. 1987; Chrtek and Popp 1991; Carnimeo et al. 1994; Gehr et al. 2003; Kruithof et al. 2007).

In the 1990s UV disinfection was initially used for swimming pool water disinfection (Leigh 2010). Its application was again to replace the use of chlorine, due to the production and presence of harmful by-products produced by the chlorine. For swimming pool water treatment it is vital that *Cryptosporidium* sp. and *Giardia* sp. are eliminated, these species are both harmful human pathogens which can be transmitted to the water by infected swimmers (Leigh 2010). The resistance of these two species to chlorination was a major factor for the change to UV disinfection (Leigh 2010).

UV irradiation is used in hospitals mainly to prevent the contamination of water supplies with *Legionella* sp., which previous disinfection practices had been unable to achieve (Farr et al. 1988; Liu et al. 1995). The utilisation of UV systems in the disinfection of hospital water supply has been widely successful (E.g. Knudson 1985; Farr et al. 1988; Liu et al. 1995; Ferrato et al. 2009). A controlled experiment performed by Liu et al (1995) observed that UV irradiation was able to kill and prevent regrowth of *Legionella* sp. for a three month period. This was only possible when combined with primary filtration which prevented the build up of scale on the UV lamp quartz sleeve and so maintained 100% UV irradiation (Liu et al. 1995). Its application for other sterilisation, including bone marrow transplant units, has also been recommended (Matulonis et al. 1993). A recent application of UV-C light in medical practices has been the successful disinfection of liquid nitrogen for the vitrification of human oocytes and embryos (Parmegiani et al. 2010).

In the beverage industry UV irradiation is used as an alternative to thermal pasteurisation as it kills pathogens while minimising flavour and nutritional content changes in fruit juices and cider (Harrington and Hills 1968). Low pressure UV-C disinfection systems have been found successful in reducing pathogenic populations, including *C.parvum* oocysts, to below allowable levels (e.g. Wright et al. 2000; Hanes et al. 2002; Koutchma et al. 2004; Tran and Farid 2004; Keyser et al. 2008; Caminiti et al. 2009). The application of UV disinfection for juices is more challenging due to higher levels of soluble solids and suspended matter which cause reduced penetration of UV light into the liquid. It is therefore necessary to adapt the UV systems in this application, and turbulent flow systems have been developed to ensure that the UV

will irradiate all of the liquid (Guerrero-Beltran and Barbosa-Canovas 2004; Keyser et al. 2008).

The application of UV irradiation in seawater began later than its application in freshwater and initial studies addressed the use of UV irradiation for seawater used in oyster cultivation. This use was to replace chlorination techniques which had been observed to have adverse effects on oysters (Kelly 1965). Initial systems involved the recirculation of seawater through the system over a period of 24 hours and treatment resulted in negligible levels of coliform bacteria present (Kelly 1971). This reduction in coliform bacteria confirmed the successful application of UV disinfection in oyster sterilisation plants (Kelly 1971). More recently research has begun to look at the application of UV disinfection to prevent disease outbreaks in re-circulating aquaculture systems (Mamane et al. 2010).

In the 1990s investigations began into the application of UV irradiation to suppress algal blooms both in freshwater and marine environments. This research included the development of a UV boat containing 20 UV-C lamps in two tubes to pump in and disinfect water in situ (Iseri et al. 1993). Further research had determined this method to be effective on the bloom forming phytoplankton *Microcystis aeruginosa*, *Chlorella ellipsoidea*, *Chlorella vulgaris*, *Scenedesmus quadricanda*, *Heterosigma akashiwo*, *Chattonella marina*, *Gymnodinium mikimotoi* and *Peridinium bipes*, with more research on natural populations recommended before widespread implementation (Iseri et al. 2004; Tao et al. 2010).

UV light has been demonstrated as a feasible ballast water treatment in preliminary studies (Jelmert 1999; Sutherland et al. 2001; Sutherland et al. 2003; Waite et al. 2003; Wright et al. 2004; Sassi et al. 2005; Wright et al. 2007b; Gregg et al. 2009). The use of UV has mainly been trialled in combination with a preliminary treatment e.g. filtration or hydrocyclonic separation (See: Jelmert 1999; Sutherland et al. 2001; Sutherland et al. 2003; Waite et al. 2003; Wright et al. 2007b). These combinations improved the efficiency of the UV treatment by removing larger particles from the water, including zooplankton and larger phytoplankton.

Table 5.1. UV dose ($mJcm^{-2}$) applied and the resulting inactivation of bacteria, phytoplankton and zooplankton observed.

Organism	**UV Dose ($mJcm^{-2}$)**	
Bacteria:		
Bacillus subitilis	60	3.0 log inactivation (Kruithof et al. 2007)
	120	4.0 log inactivation (Kruithof et al. 2007)
Cryptosporidium parvum oocysts	2.2	3.0 log inactivation (Oguma et al. 2001)
	3 (LP)	3.4 log inactivation (Clancy et al. 2000)
	3 (MP)	3.0 log inactivation (Clancy et al. 2000)
	10	2.0 log inactivation(Craik et al. 2001)
	19	3.9 log inactivation (Bukhari et al. 1999)
	20	3.2 log inactivation (Kruithof et al. 2007)
	25	3.0 log inactivation(Craik et al. 2001)
	120	4.5 log inactivation (Kruithof et al. 2007)
	230	2.0 log inactivation (Morita et al. 2002)
Escherichia coli	6	3.0 log inactivation (Oguma et al. 2001)
Free living bacteria	60	2.0 log inactivation (Waite et al. 2003)
	350	3.0 log inactivation (Hess-Erga et al. 2008)
	2120	4.0 log inactivation (Hess-Erga et al. 2008)
Giardia lamblia	3	2.0 log inactivation (Mofidi et al. 2002)
	63	0.6 log inactivation (Rice and Hoff 1981)
Giardia muris	3	2.0 log inactivation (Mofidi et al. 2002)
	20	2.3 log inactivation (Kruithof et al. 2007)
	120	3.2 log inactivation (Kruithof et al. 2007)
	165	2.0 log inactivation (Carlson et al. 1985)
Legionella pneumophila	3	3 log inactivation (Antopol and Ellner 1979)
Pasteurella piscicida	10.8	6.0 log inactivation (Sugita et al. 1992)
Streptococcus sp.	47.3	5.0 log inactivation (Sugita et al. 1992)
Vibrio anguillarum	21.97	5.0 log inactivation (Sugita et al. 1992)
	122	3.0 log inactivation (Liltved et al. 1995)

Vibrio salmonicidia	122	3.0 log inactivation (Liltved et al. 1995)
Yersinia ruckeri	2.7	5.0 log inactivation (Liltved et al. 1995)
Phytoplankton:		
Isochrysis	92	100% mortality (Jelmert 1999)
Microcystis aeruginosa	75	Growth stopped (Alam et al. 2000)
Pavlova	92	100% mortality (Jelmert 1999)
Biomass: chlorophyll α	375	56% reduction (Sassi et al. 2005)
	180	Growth stopped (Wright et al. 2004)
Zooplankton:		
Artemia sp.	563	78% mortality (Sassi et al. 2005)
Artemia franciscana	258	90% mortality (Sutherland et al. 2003)
Artemia nauplii	92	100% mortality (Jelmert 1999)
Natural zooplankton population	563	56% mortality (Sassi et al. 2005)
	180	95% mortality (Wright et al. 2004)
	200	88.7% mortality (Wright et al. 2007b)

5.1.5 Factors to address for the application of UV irradiation on ballast water

It is clear that ultraviolet irradiation is an effective disinfectant technology in a wide range of applications. In order to develop an ultraviolet light disinfection system for ballast water purposes it is vital to assess the practical issues which have arisen in other applications, and determine how they could be applied/dealt with in ballast water treatment. The factors which have been addressed are the lamp type, the removal of suspended particles, the dose required for disinfection and the potential for organism recovery.

Two types of lamp are available for germicidal purposes: low and medium pressure. Studies which have compared the inactivation caused by the two lamp types have observed very similar mortality levels (Carlson et al. 1985; Clancy et al. 2000; Craik et

al. 2001), and so neither lamp offers a germicidal advantage. Therefore the decision should be made with respect to economic factors, i.e. low pressure lamps are more expensive to run and maintain as they require more bulbs, due to their lower intensity irradiation, and therefore incur higher maintenance costs and require more space (Carlson et al. 1985; Clancy et al. 2000; Craik et al. 2001). For application in ballast water treatment, where cost is a major selling point of any potential system, every effort should be made to reduce capital, operational and maintenance costs where possible. Therefore, medium pressure UV lamps are generally preferred and utilised.

The presence of suspended particles in water leads to a reduction in UVT and thus reduces UV disinfection. In some cases this has been attributed to protection of target organisms through their attachment to shielding particles (Qualls et al. 1983; Harris et al. 1987; Lindenauer and Darby 1994; Emerick et al. 1999; Loge et al. 1999; Templeton et al. 2005; Li et al. 2009). It is also possible for the particles themselves to absorb the UV irradiation, thus leaving less available UV light for disinfection (Bitton et al. 1972). The size of particles can directly affect the resulting UV disinfection efficiency, and larger particles (>20µm) reduce disinfection more than smaller particles (>5µm) (Madge and Jensen 2006). For coliform bacteria studies have shown that particles ≤2µm are able to shield the target organisms and block UV light transmission (Emerick et al. 1999; Loge et al. 1999; Templeton et al. 2005). Therefore, the use of UV disinfection is often combined with a primary treatment, and this has been recommended to obtain higher disinfection efficiency (Oliver and Cosgrove 1975; Emerick et al. 1999; Loge et al. 2001). For ballast water treatment this would mean the development of combined treatment systems utilising a primary technology to pre-treat the seawater before UV irradiation. The most effective primary technologies for this application are those which physically remove particles from the seawater, i.e. filtration and hydrocyclones.

The required UV dose varies with each application and the resistance of the target species. For ballast water there is no minimal dose which must be applied, and the available data on species inactivation shows a vast range in the required dose for different species (Table 5.1). In order to establish the required dose it is vital that

systems are thoroughly tested prior to installation onboard a vessel to ensure a high level of disinfection.

The ability of organisms to repair UV damage is widely reported, and has been observed to vary due to experimental conditions and the type of organisms assessed (E.g. Oguma et al. 2002; Zimmer and Slawson 2002; Guo et al. 2009). The UV dose applied affects repair capability, and when exposed to higher UV doses (i.e. >40mJcm^{-2}) photoreactivation is negligible or prevented in coliform bacteria (Harris et al. 1987; Lindenauer and Darby 1994; Hoyer 1998; Bohrerova and Linden 2006; Hu and Quek 2008; Guo et al. 2009). This is thought to be due to the formation of such high numbers of pyrimidine dimers that repair is not possible (Guo et al. 2009). In addition, when compared to low pressure lamps, irradiation of *Escherichia coli* by medium pressure UV lamps results in less repair (Oguma et al. 2002; Zimmer and Slawson 2002). When applied to ballast water research and the doses applied by previously tested UV systems, i.e. ≥60mJcm^{-2}, repair should be minimal or prevented. This will be even more likely if the organisms are exposed to double UV irradiation (upon uptake and discharge).

5.1.6 Aims of this study

This study assessed the biological efficiency of UV irradiation on plankton for potential use in ballast water treatment. Factors identified in the previous literature (described in Section 5.1.5) which could affect use of UV irradiation in this application have been assessed during four series of tests.

5.2 Methodology

In total four series' of tests were completed over a three year period. The tests were designed to identify the required dose for plankton mortality, the effect of primary filtration and the potential for application of a UV treatment system in ballast water disinfection.

5.2.1 UV system tested

The UV lamps tested were supplied by ATG-Willand. In all tests medium pressure mercury lamps were used. In Test Series' 1-3 a parallel lamp with one 7300W bulb was used. In Test Series 4 a perpendicular lamp with eight 3500W bulbs was used. The lamp designs are displayed in Figures 5.3 and 5.4.

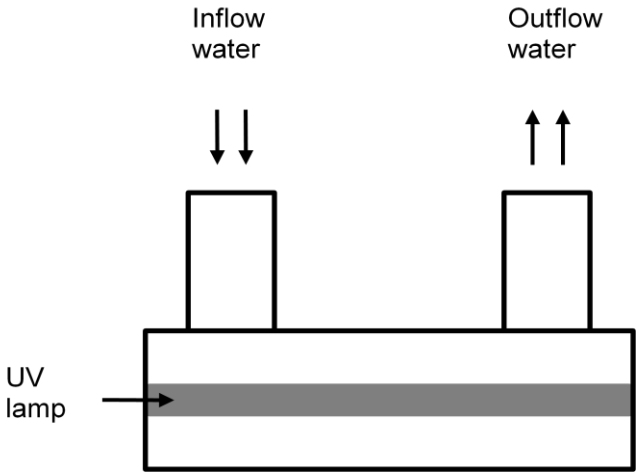

Figure 5.3. The parallel flow UV lamp configuration showing the UV bulb parallel to the flow of water though the UV chamber.

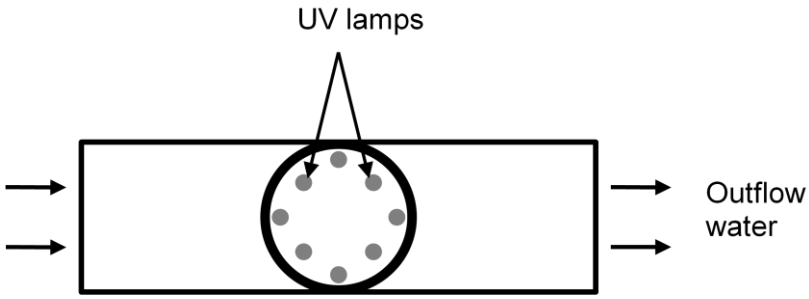

Figure 5.4. The cross flow UV lamp configuration showing the eight bulbs. Water flows through the UV chamber from left to right and across the eight UV bulbs.

5.2.2 Test series 1

Series 1 tests were performed at the Dove Marine Laboratory in December 2007. All tests assessed the combination of a self cleaning 40µm stainless steel screen filter and subsequent UV exposure on the mortality of zoo- and phytoplankton. Four flow rates were tested; 1.6, 2.2, 4.8 and 5.5m^3hr^{-1}. Samples were collected immediately after treatment. In all tests seawater was pumped from Cullercoats Bay using a centrifugal pump and passed through the filter/UV system. Three replicate 20L 'Control' samples and three replicate 100L 'Treated' samples were each collected for zooplankton at two points in the system. Three replicate 1L 'Control' samples and three replicate 10L 'Treated' samples were each collected for the phytoplankton at two points in the system. Figure 5.5 shows the sampling points in Series 1 tests.

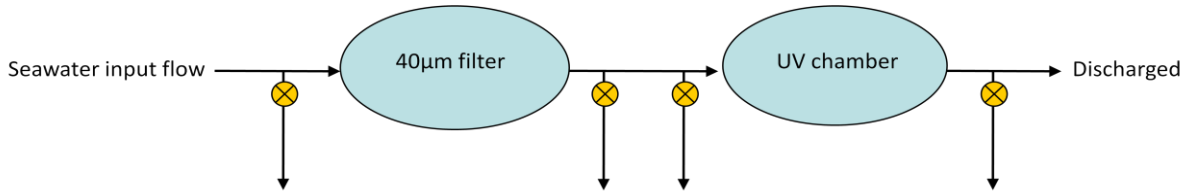

Figure 5.5. Shows the four sampling points used. 'Control' samples were obtained before the filter and before the UV system, and 'Treated' samples were obtained after the filter and after the UV system. ⊗ - sampling points.

5.2.3 Test series 2

Tests were performed at the Dove Marine Laboratory in March and April 2008. All tests assessed the UV system in isolation; the filter was not applied in these tests. The flow rate of water through the UV system was altered and four flow rates were considered: 3, 4, 10 and 12.5m^3hr^{-1}. A 1000L storage tank was filled with sand-filtered seawater pumped from Cullercoats Bay. The test organism *Tetraselmis suecica*, a single celled green alga, was added to the tank and the water was then passed through the UV system. Only phytoplankton were assessed in these tests as it was assumed that

the inclusion of the filter would remove a high number of the zooplankton, and therefore the UV light would be required for phytoplankton disinfection. Three replicate 1L 'Control' samples and three replicate 10L 'Treated' samples were collected. The test set up and sampling points are shown in Figure 5.6.

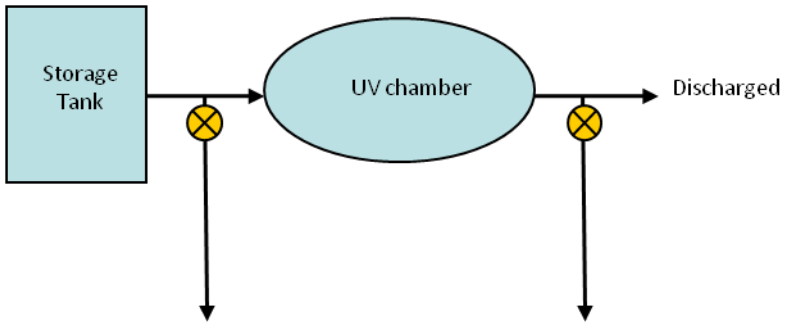

Figure 5.6. Test procedure for series 2 UV tests. Arrows indicate the direction of water flow and the sampling points are shown. ⊗ - sampling points.

5.2.4 Test series 3

Testing consisted of three experimental runs performed at the Dove Marine Laboratory in April and June 2008. The flow rate of water through the UV system was altered and three flow rates of 2, 5.5 and 10 m^3hr^{-1} were considered. To increase the turbidity of the water kaolin was added to the tank of seawater prior to testing. Kaolin clay (particle size <40µm) was used as it remains in suspension in seawater and it is UV absorbent. Tests were performed as described in Section 5.2.3.

5.2.5 Test series 4

Four filter/UV tests were performed in series 4. Two tests were performed at the Dove Marine Laboratory in May/June 2009. Two further tests were performed at a test site in Blyth, UK, in August/September 2009.

For tests performed at the Dove Marine laboratory a 65,000L storage tank was filled with natural sand-filtered seawater. The test organisms *T.suecica* and *A.salina* were

added to the tank. At the Blyth test site seawater was pumped directly into a large storage tank to which the test organisms were added. In all four tests on Day 0 samples were collected 'Before filter', 'After filter' and 'After UV'. Three replicates were collected at each sampling point. For *A.salina* analysis 20L was collected 'Before filter', 100L 'After filter' and 1000L 'After UV'. For *T.suecica* analysis 1L was collected 'Before filter', 1L 'After filter' and 10L 'After UV'. After treatment organisms were pumped back into a 'Treated' tank and left for five days. Untreated organisms were kept in a 'Control' tank in identical conditions for the five day period. In the tests performed at the Dove Marine Laboratory it should be noted that the storage tanks were open and exposed to both light and air. In the final two tests performed at the test site in Blyth the storage tanks were sealed and organisms were not exposed to outside conditions for the five day holding time. On the fifth day samples were collected from the control tank. Water from the treated tank was passed back through the UV system and samples were collected 'Before UV' and 'After UV'. For *A.salina* analysis 100L was collected from the control tank, 20L 'Before UV' and 1000L 'After UV'. For *T.suecica* analysis 1L was collected from the control tank, 1L 'Before UV' and 10L 'After UV'. The test procedure is described in Figure 5.7.

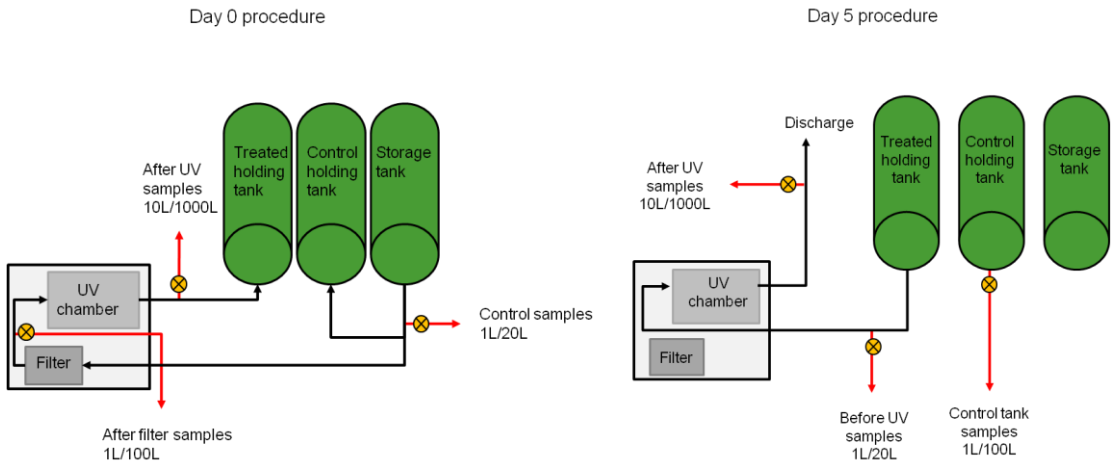

Figure 5.7. Test procedure for the UV/filter test. Arrows indicate the direction of water flow and the sampling points are shown.

5.2.6 Viability assessment

In all tests zooplankton/*A.salina* samples were concentrated by 50µm mesh and rinsed using 0.45µm seawater before being analysed using a Meiji microscope at 10-40X magnification. The entire sample was examined manually and all *A.salina* within were classed as live or dead by visually looking for internal or external movement. If no movement was immediately apparent the organisms were 'prodded' to determine whether movement could be induced. If nothing was observed the organisms were classed as dead. The total number of organisms per 1000L was calculated.

In Test series 1-3 all phytoplankton samples were stained using Evans blue using the following method:

> 1% solution (w/v) Evans blue was added at 2:1 (sample:stain) and left for 1 hour. Samples were then filtered using a 10µm sieve and rinsed with 0.45µm filtered fresh seawater before counting. The sample was concentrated to a known volume and a 1ml aliquot was examined in a Sedgewick Rafter counting cell. Cells which were dead prior to staining were stained blue, whereas samples alive before staining did not exhibit any colour change. All samples were examined using an Olympus CK X31 microscope at 200-400x magnification. A viability assessment (live/dead) was made for each sample.

In Test series 4 *T.suecica* samples were assessed by FlowCAM using a 100µm depth flow cell and 10X objective. Live/dead assessment used the fluorescent vital stain Fluorescein diacetate (FDA) and was performed as described in Section 4.2.2.3.

5.2.7 Statistics

No statistical analysis of Test series 1 and 2 was performed due to low organism numbers. For Series 3 and 4 results all data was checked for normality using the Kolmogorov smirnov Normality test and equal variance using Levene's Test. If data was found to be non normal they were transformed using common transformation

methods. A 1-way ANOVA or Kruskal Wallis test was used in Test series 4 analyses to determine the effect of Factor Treatment (Before filter, After filter, After UV, Control tank, Before UV, After UV) on the number of live organisms. Post hoc tests were applied where required.

5.3 Results

5.3.1 Test series 1

Organism numbers in Series 1 were very low as natural seawater and natural plankton populations were relied upon to supply test organisms. It was apparent that natural populations could not be used reliably and so in subsequent experiments test species were added to the input water. The experimental conditions and results of each test in Series 1 are displayed in Table 5.2.

Table 5.2. Experimental conditions of the UV/filter tests performed in Series 1 in which both zooplankton and phytoplankton were analysed. All samples are the mean of three replicates ± standard error. (BF = Before Filter, AF = After Filter, BUV = Before UV, AUV = After UV).

Treatment	**Flow rate (m^3hr^{-1})**	**UV Transmission UVT (%)**	**UV dose ($mJcm^{-2}$)**	**Number of live Zooplankton (*n*±StDev) per m^3**	**Number of live Phytoplankton (*n*±StDev) per ml**
UV/Filter	1.6	88.8	3350	**BF** - 350±50 **AF** - 60±40 **BUV** - 50±50 **AUV** – 3±6	**BF** – 10.7±5.4 **AF** – 5.5±3.1 **BUV** – 4.5±2 **AUV** – 3.8±2.3
UV/Filter	1.6	78.3	2310	**BF** - 300±100 **AF** - 180±1 **BUV** - 50±50 **AUV** - 0±0	**BF** – 7.0±1.6 **AF** - 28800±18052 **BUV** – 8.2±3.9 **AUV** – 3.6±0.9
UV/Filter	2.2	94.2	2910	**BF** - 400±100 **AF** - 80±70 **BUV** - 100±50 **AUV** - 0±0	**BF** – 5.7±1.5 **AF** – 3.8±1.2 **BUV** – 6.7±11.8 **AUV** – 3.1±0.5
UV/Filter	4.8	84.9	980	**BF** - 450±100	**BF** – 9.6±1.9

				AF - 20±20 **BUV** - 50±50 **AUV** - 0	**AF** – 2.7±1.2 **BUV** – 24.3±27.3 **AUV** – 3.5±0.4
UV/Filter	5.5	92.9	1120	**BF** - 550±100 **AF** - 30±40 **BUV** - 0±0 **AUV** - 0±0	**BF** – 20.2±1.2 **AF** – 2±1.5 **BUV** – 3.4±1.4 **AUV** -1±0.3

These tests showed a reduction in live zooplankton and phytoplankton after treatment. Although low organism numbers were obtained which prevented statistical analysis it is clear that filtration physically removed most of the zooplankton. This then left the UV light to kill the remaining phytoplankton. Therefore, in further small scale tests it was decided to assess the UV itself on phytoplankton in isolation without filtration or the presence of zooplankton.

5.3.2 Test series 2

In Series 2 tests the test species *T.suecica* was added to the input water. The experimental conditions of each test in Series 2 are shown in Table 5.3. In these tests (and all subsequent tests) the exposure time, i.e. the length of time required for a particle to travel past the UV light (which is determined by the flow rate of the water) was calculated, along with the log inactivation in live *T.suecica* after treatment (Log inactivation = - log(live *T.suecica* discharge/live *T.suecica* intake).

Table 5.3. Experimental conditions of the UV tests performed in Series 2.

Treatment	**Flow rate (m^3hr^{-1})**	**UV Transmission UVT (%)**	**UV dose ($mJcm^{-2}$)**	**Exposure time (s)**	**Log inactivation of live *T.suecica***
UV	3	90.8-96	2200	5.35	0.96
	4	95	1700	4.01	1.06
	10	95	730	1.60	0.27
	12.5	95	560	1.28	0.21

As can be seen in Table 5.3, the highest reduction in *T.suecica* was observed at a flow rate of 4m^3hr^{-1} and a UV dose of 1700$mJcm^{-2}$. This could be attributed to the high exposure time (4.01 seconds) of cells to the UV light. The relationship between UV dose and the survival rate of *T.suecica* is displayed in Figure 5.8. Survival rate was calculated as:

$$Survial\ rate\ = \frac{\text{Number of live } T.\ suecica \text{ after UV exposure}}{\text{Number of live } T.\ suecica \text{ before UV exposure}}$$

Figure 5.8 clearly shows that as the flow rate increases, the UV dose decreases and the survival rate increases. Table 5.3 confirms this and shows that as the flow rate increases, the exposure time and UV dose reduce.

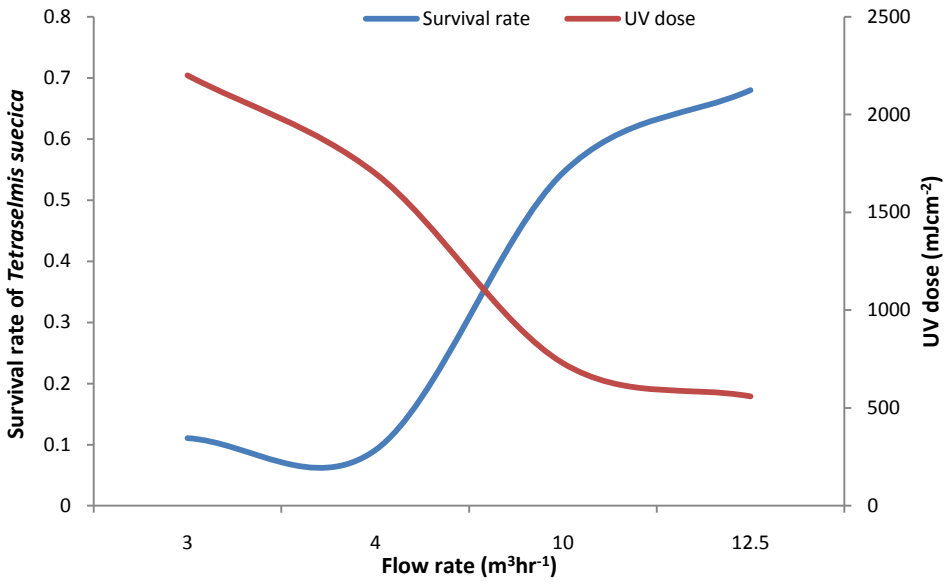

Figure 5.8. The survival rate of *Tetraselmis suecica* after UV treatment in relation to the UV dose applied and flow rate.

Series 2 tests show that UV irradiation is effective at reducing live *T.suecica* numbers, and increasing the exposure time increases the resulting mortality. The results presented in Table 5.3 also suggest that changes in UVT affect the mortality, this was observed as higher mortality at a dose of 1700$mJcm^{-2}$than 2200$mJcm^{-2}$. *T.suecica* cells exposed to the higher dose (2200$mJcm^{-2}$) also had a longer exposure time and yet

lower mortality resulted from treatment, which could be due to lower UVT in this test (Table 5.3). Lower UVT is due to the presence of suspended particles, and therefore *T.suecica* cells could have been shielded from irradiation or the UV could have been absorbed by non-target particles. Series 3 tests were performed to further assess the effect of turbidity.

5.3.3 Test series 3

Series 3 tests assessed the efficiency of UV irradiation under increased turbidity by adding kaolin to the test water, and therefore the UVT was reduced. This caused the UV dose to decrease significantly when compared to Test series 2: the UV doses applied at a flow rate of 10.0m^3hr^{-1} at UVT of 95% (Series 2) and 70.3% (Series 3) were 824$mJcm^{-2}$and 286$mJcm^{-2}$ respectively. Even under these more challenging conditions a 60% reduction in live *T.suecica* cells was observed at 70.3% UVT, UV dose of 286$mJcm^{-2}$and an exposure time of 1.6 seconds. The reduction observed in this test was higher than in the previous test series, and the reason for this cannot be confirmed. However, it could be due to differing behaviour of the suspended particles, i.e. varying levels of UV absorption and UV scattering due to the properties of the particles present.

Table 5.4. Test conditions for Test series 3 increased turbidity tests (kaolin added) with the UV system.

Treatment	**Flow rate (m^3hr^{-1})**	**UV Transmission UVT (%)**	**UV dose ($mJcm^{-2}$)**	**Exposure time (s)**	**Log inactivation of live *T.suecica* (%)**
UV	2	82.8	2215	8.0	2.3
UV	5.5	83.5	824	2.9	1.4
UV	10.0	70.3	286	1.6	0.2

Tests using the small scale UV system showed that UV irradiation was effective in reducing *T.suecica* viability. To ensure high efficiency when treating zoo- and phytoplankton a filter would be required for primary treatment and the removal of

larger particles and organisms. The system was still effective when challenged with high turbidity, and if the UV dose was increased further a higher reduction in live *T.suecica* cells could be obtained in low UV transmission conditions. All of the information obtained in Series' 1-3 was used to develop a large scale combined filter/UV treatment system for testing in Series 4.

5.3.4 Test series 4

As preliminary small scale tests had shown the UV treatment to be successful, a large scale system was manufactured and tested in four large scale tests at two different testing locations. These tests assessed a cross flow lamp configuration (See Section 5.2.1) using a larger volume of water and higher flow rates to show whether the combined filter/UV system had potential for further development in ballast water treatment. The tests also looked at adjusting the UV dose to ensure the power requirement was minimised whilst still obtaining high mortality levels.

5.3.4.1 Test 1

The experimental conditions for Test 1 are shown in Table 5.5. In this test two of the UV lamps were switched off to reduce the UV dose. However, it was determined that in future tests to obtain a higher mortality it would be more effective to switch on all lamps but reduce their output power. This method ensures that organisms cannot pass through the chamber at a high distance from a UV lamp, and thus receive a lower UV dose which would increase their chance of survival.

Table 5.5. The test conditions on the first and fifth days in Test 1.

Day	Lamp power (%)	Flow rate (m^3hr^{-1})	UV Transmission UVT (%)	UV dose ($mJcm^{-2}$)	Exposure time (s)	Log inactivation of live *A.salina*	Log inactivation of live *T.suecica*
0	75	38	82.9	745	1.95	3.2	1.6
5	75	69	86.7	488	1.07	-1.5	1.2

The number of live *A.salina* present was significantly different between samples (Kruskal wallis; p = 0.029, χ^2= 12.5, d.f. = 5). The filter showed 99.9% removal of *A.salina* individuals, and so it is not possible to make any conclusions about the effectiveness of the UV irradiation itself.

The number of live *T.suecica* present was significantly different between samples (One-way ANOVA; p = <0.001, F = 44.08, d.f. = 5). Further analysis showed that the Before Filter sample on Day 0 was not significantly different from the After Filter sample (Least significant difference; p = 0.690). *T.suecica* cells are between 10-20µm in size which is smaller than the mesh size of the filter, therefore the filter was not expected to remove a large number of these cells and the UV was required to kill these smaller organisms. On Day 0 there was a reduction from 3833 live *T.suecica* per ml (After Filter) to 124 live *T.suecica* per ml (After UV), this shows 96.8% mortality due to UV irradiation. Samples collected on Day 5 contained very low numbers of *T.suecica* and so no conclusions could be made about the effect of the UV treatment on the fifth day.

5.3.4.3 Test 2

The experimental conditions in Test 2 are shown in Table 5.6.

Table 5.6. The test conditions on the first and fifth days in Test 2.

Day	Lamp power (%)	Flow rate (m^3hr^{-1})	UV Transmission UVT (%)	UV dose ($mJcm^{-2}$)	Exposure time (s)	Log inactivation of live *A.salina*	Log inactivation of live *T.suecica*
0	100	66.2	92.5	931	1.12	3.9	1.5
5	100	59.2	88.8	844	1.23	1.4	1.4

The number of live *A.salina* present was significantly different between samples (Kruskal wallis; p = 0.014, χ^2= 14.3, d.f. = 5). The filter showed 99.8% removal of *A.salina* individuals.

The number of live *T.suecica* present was significantly different between samples (One-way ANOVA; p = <0.001, F = 53.8, d.f. = 5). Further analysis showed that the filter again did not show significant removal (Least significant difference; p = 0.394). The UV treatment on Day 0 showed 94.8% mortality in *T.suecica* cells, while samples collected on Day 5 again contained very low numbers of *T.suecica* and no conclusions could be made about the effect of the second UV treatment.

5.3.4.4 Test 3

The experimental conditions of Test 3 are shown in Table 5.7.

Table 5.7. The test conditions on the first and fifth days in Test 3.

Day	Lamp power (%)	Flow rate (m^3hr^{-1})	UV Transmission UVT (%)	UV dose ($mJcm^{-2}$)	Exposure time (s)	Log inactivation of live *A.salina*	Log inactivation of live *T.suecica*
0	50	43.5	83.6	477	1.68	4.5	0.6
5	100	64.6	70.5	371	1.14	1.2	2.3

The number of live *A.salina* present was significantly different between samples (One-way ANOVA; p = <0.001, F = 58.7, d.f. = 5). The filter showed 99.9% removal of *A.salina* individuals in this test and so it is difficult to make any conclusions about the effectiveness of the UV itself. However, in this test for the 5 day holding period the organisms were kept in sealed holding tanks i.e. no light or air exchange was permitted. The change in holding tanks led to high numbers of live organisms being present in samples from the control tank on the fifth day of the test. So although it is not possible to make conclusions about the UV on *A.salina* it is possible to conclude that the filter/UV combined system caused a significant reduction in organisms after

the five day test (Least significant difference; p = <0.001) and no live organisms remained after UV treatment on the fifth day.

The number of live *T.suecica* present was significantly different between samples (One-way ANOVA; p = <0.001, F = 15.1, d.f. = 5). UV treatment on Day 0 again showed a high mortality (73.5%), and a significant reduction in live cells (Least significant difference; p = 0.003). Samples collected on Day 5 contained high numbers of live *T.suecica* in the control tank and significantly lower live cells in the treated samples; Before UV Day 5 (Least significant difference; p = <0.001) and After UV Day 5 (Least significant difference; p = <0.001). Although there was no statistically significant reduction in live cells due to UV treatment on the fifth day (Least significant difference; p = 0.307) there was a reduction and no live cells were present in the After UV samples.

5.3.4.5 Test 4

The experimental conditions for the fourth test are shown in Table 5.8.

Table 5.8. The test conditions on the first and fifth days in Test 4.

Day	Lamp power (%)	Flow rate (m^3hr^{-1})	UV Transmission UVT (%)	UV dose ($mJcm^{-2}$)	Exposure time (s)	Log inactivation of live *A.salina*	Log inactivation of live *T.suecica*
0	100	91.8	78.5	350	0.8	4.7	1.6
5	100	127.0	73.3	213	0.58	1.3	2.3

The number of live *A.salina* present was significantly different between samples (Kruskal wallis; p = 0.016, χ^2= 13.9, d.f. = 5). The filter showed 99.9% removal of *A.salina* individuals in this test. Due to low numbers after filtration it is not possible to make conclusions about the UV on *A.salina* but it is again possible to conclude that the filter/UV combined system caused a significant reduction in organisms. No live organisms were present after UV treatment on the fifth day.

The number of live *T.suecica* present was significantly different between samples (One-way ANOVA; p = <0.001, F = 28.9, d.f. = 5). A significant reduction in live *T.suecica* after UV treatment on Day 0 was observed (Least significant difference; p = 0.001). Samples collected on Day 5 contained high numbers of live *T.suecica* in the control tank and significantly lower live cells in the treated samples; Before UV Day 5 (Least significant difference; p = <0.001) and After UV Day 5 (Least significant difference; p = <0.001). Although there was no statistically significant reduction in live cells due to UV treatment on the fifth day (Least significant difference; p = 1.000) there was a decrease and no live cells remained in After UV samples.

The four tests performed in Series 4 show that the combined filter and UV system was capable of treating water at high flow rates to mortality levels which would be suitable for ballast water treatment. The results are only applicable to the combined system as the primary filtration is vital for the high removal of *A.salina* which improves the conditions for the UV disinfection. High levels of mortality were observed in *T.suecica* immediately after UV treatment and also after a five day storage period. No obvious recovery was observed in all tests in the samples taken prior to UV treatment on Day 5, which suggests the *T.suecica* were not able to repair UV damage in the dark conditions.

5.4 Discussion

This study showed that UV irradiation was highly effective against *T.suecica*. In the final tests in Series 4 100% mortality was observed after double UV irradiation and a five day holding period. In the published studies a dose of >75mJcm^{-2} was sufficient to cause significant mortality to phytoplankton, but there is limited data available on few species (Table 5.1). In Test 4 (Series 4) a dose of 350mJcm^{-2} was applied on Day 0 which resulted in a 97.5% reduction in live *T.suecica*, and after treatment on Day 5 no live cells were present in samples. It may be possible to optimise the UV treatment further and reduce the required dose whilst obtaining 100% mortality, and this would require further testing.

Due to the high removal of the filter in this study it was not possible to determine the efficiency of the UV irradiation itself on zooplankton. Previous studies have observed microalgae and zooplankton to require higher doses of UV irradiation than bacteria and viruses due to their large size and pigmentation (Gregg et al. 2009). Some organisms have shown a tolerance to UV irradiation and one mechanism which acts to reduce the effects of UV is photoprotection e.g. by pigments. This is defined as 'the filtering out of harmful UV radiation by photoprotective compounds before they reach genetic material or vital structures' (Sutherland et al. 2003). Reflection, refraction and absorption of UV light by the exterior tissues of organisms can protect their vital structures, and the quantity and quality of this exterior tissue could be important to their survival rate (Sutherland et al. 2003). The UV doses applied in the final and most effective test in this study, i.e. 350mJcm^{-2} (Day 0) and 213mJcm^{-2} (Day 5) are higher than most used in previous literature (Table 5.1) and so would be expected to cause high mortality to zooplankton. When primary filtration is applied few zooplankton will remain in samples for UV irradiation and the combined treatment should result in full mortality.

As reported in Section 5.3.3 when the UVT was reduced the UV dose also decreased. The effect suspended particles had on the resulting mortality varied, and this may have been due to the behaviour of the particles present in the water. As previously stated, particles can shield organisms from UV irradiation and some can also absorb the UV themselves, leaving it unavailable for disinfection (Bitton et al. 1972). The small scale tests which assessed UVT did not use a primary filter, but simply by including this in the treatment system set up the number of organic particles in the water and the turbidity would be reduced, and both of these factors affect the UV dose.

A major benefit to UV treatment is that it can be applied both during uptake and discharge of ballast water. This enables it to remove any repair or regrowth of organisms which occurs in the tank after the first UV treatment (Herbert 2002; Sutherland et al. 2003; Wright et al. 2007b). These cells may be at reduced health due to the first UV exposure and subsequent five day dark storage, and thus further

treatment on discharge could cause mortality. Damage caused by UV exposure can be reversed by many affected cells, and there are two main methods used for this: photo-repair and dark-repair (United States Environment Protection Agency 2006). Photo-repair requires light between 300nm and 500nm to stimulate enzymes which break the covalent bonds formed by UV exposure (Wright and Cairns). Dark repair does not require light but can be performed in the presence of light. Dark repair processes include excision repair – this is completed by enzymes which remove the section of DNA or RNA containing the pyrimidine dimers and then the gap is filled by a newly formed section (United States Environment Protection Agency 2006). In Series 4 Tests 1 and 2 the storage tanks used were open to the environment and sunlight, providing the opportunity for UV damaged cells to perform both light and dark repair. The storage tanks used in Tests 3 and 4 were entirely sealed and dark. In this set up only dark repair would be possible and the chance of organisms repairing themselves was reduced. This would also be the case when onboard vessels and would result in higher mortality of organisms.

UV light damages the nucleic acids within cells but does not affect the metabolic processes, such as respiration, therefore cells do not die immediately (United States Environment Protection Agency 2006). Wright et al (2007b) suggested that if left for long enough after treatment mortality of organisms exposed to UV irradiation could reach 100%. Waite et al (2003) observed a significant decrease in chlorophyll α after an 18 hour holding period, but not immediately after UV irradiation. The subsequent decline could have been due to the time taken for the chlorophyll α to degrade as this can occur over a period of hours to days, and so a reduction would not be observed immediately after treatment (Waite et al. 2003; Sassi et al. 2005). This study observed samples within 6 hours of collection. Within this time scale it may not be possible to observe the resulting damage from UV treatment and readings taken 24 hours subsequent may be more accurate and representative. It is therefore possible that a lower UV dose could be sufficient to kill organisms as delayed mortality was not assessed by this study. If this is the case then it could reduce running costs of the UV system and it would therefore be beneficial to research further.

Resting stages of organisms are more resistant to disinfection treatments due to the hard structure of their outer shells. As described in Chapter 2, the resting stages of marine plankton are tougher and more resilient than active organisms. Therefore it is likely a higher UV dose will be required. Whilst this research has not been performed on marine resting stages, many studies have assessed the oocysts of *Cryptosporidium* sp. and *Giardia* sp. for UV treatment of drinking water. The results have shown varied inactivation rates (Table 5.1) and the tests were performed under static conditions using collimated beam apparatus. Whilst this information is vital for giving an idea of minimum dose requirements, when applied in a fluid system this dose will be higher. It is therefore necessary to include these organisms in future research.

Developing UV systems for onboard ballast water treatment would not be too difficult as the basic technology is available. When scaling systems up to high flow rates the size of the equipment and thus the space needed for a UV system would not be unfeasible for shipboard use (Herbert 2002). The systems are long lasting: medium pressure lamps are reported to have a lifetime of 2000-5000 hours (Sassi et al. 2005). UV systems require little maintenance and already have a long history of use within the marine environment (Herbert 2002). All of these factors make UV technology a good candidate for further research regarding its use in ballast water treatment.

5.5 Conclusions

The aim of this study was to determine the biological effectiveness of UV irradiation on plankton and this was achieved through the tests completed. A dose of 350mJcm^{-2} caused 97.5% mortality of *Tetraselmis suecica* and double exposure on Day 0 and Day 5 of system tests killed all organisms, the second exposure used to kill any surviving organisms and those which had repaired initial damage. For application in ballast water treatment the inclusion of a primary filter is required for removal of zooplankton. Further testing could determine whether a reduced dose was able to cause full mortality of organisms, this could reduce the power requirements of the system and thus the operational costs.

Chapter 6

Assessing a chlorine based biocide for potential application in ballast water treatment

6.1 Introduction

6.1.1 The use of chemicals as biocides:

Biocides are employed to kill microorganisms in many industries, for example in the treatment of drinking water, water to supply hospitals and swimming pools, and in sewage and wastewater treatment (National Research Council 1996; Vianna da Silva and da Costa Fernandes 2003; Zhang et al. 2004). There are two types of biocides: oxidising and non-oxidising. Both types work similarly to pesticides and interfere with normal cell functions. They can affect reproductory and neural functions, inhibit enzymes, destroy cell membranes and walls or work to disrupt critical cell processes, e.g. inhibit respiration (National Research Council 1996; Chelossi and Faimali 2006; Tsolaki and Diamadopoulos 2009). Chlorine is the most commonly employed oxidising biocide for water treatment as it is easy to use and cost-effective (Gregg et al. 2009). This chapter will discuss the potential application of a chlorine based biocide for application in ballast water treatment.

6.1.2 Chlorine

6.1.2.1 History of use and development

The first recorded use of chlorine for disinfection was in 1830, when it was used to treat water supplies in a hospital (Water Pollution Control Federation. Technical Practice Committee. 1976). The requirement for disinfection came after it was realised

that water supplies were responsible for diseases spreading throughout hospitals, as well as entire towns and cities. Once this was established it was vital that water sources were disinfected to kill diseases, and early practices used chlorinated lime for this purpose (Water Pollution Control Federation. Technical Practice Committee. 1976; White 1999). As disinfection practices progressed water treatment became used more regularly and the first large scale wastewater treatment plant using chlorinated lime opened in 1854 in London, England. In 1859 Hofman and Frankland showed that the addition of 47.9mg/L chlorinated lime delayed putrefaction of wastewater by four days, and in 1884 Dibdin used chlorinated lime to deodorize the Thames River (Dibdin 1903). With the success of these studies chlorinated lime disinfection continued, although other ways of applying chlorine were investigated as chlorine in chlorinated lime would degrade when in storage.

The production of chlorine using electrolysis – this is the process of passing an electric current through water containing sodium chloride to produce a disinfectant solution – (Matousek et al. 2006) was first developed in 1851, when Watt obtained an English patent for his electrolytic system design. However, at this time the technology did not exist to actually manufacture the system and it was 1890 when the Elektron Company in Germany first commercially produced chlorine by electrolysis. In 1893 the first electrolysis treatment plant opened in the US using the Woolf process – an electrolysis process which produced chlorine from brine. This treatment plant was successfully used until destroyed by fire in 1911 (Water Pollution Control Federation. Technical Practice Committee. 1976; White 1999). After this time interest in electrolytic production dropped as the methods used were inefficient and attention turned to liquid and gaseous chlorine for chlorination methods. Electrolysis will be further discussed in Section 6.1.2.3.

In 1774 Scheele discovered gaseous chlorine, but it wasn't until 1887 that it became utilised via the Powers Process for chlorination of wastewater at the first large scale plant in the US. The Powers Process produces chlorine through the reaction between manganese oxide, sodium chloride and sulphuric acid (Water Pollution Control

Federation. Technical Practice Committee. 1976). In 1903 chlorine gas was used to treat drinking water in Belgium, and in 1910 Darnall first used compressed gas from steel cylinders to chlorinate water. Compressed gas is still the most common method used to date for chlorination, but the process itself has been modified. In 1912 Keinle developed a way to disinfect drinking water by pumping compressed chlorine gas into a chamber in the opposite direction to the water flow, which enabled the gas to disinfect the water. This was further modified by Ornstein who began use with his system in Delaware, 1913. In 1915 Ornstein patented a device to measure and add chlorine gas to water at a determined amount (Water Pollution Control Federation. Technical Practice Committee. 1976).

The first recorded use of liquid chlorine for disinfection was at wastewater disinfection plants in Pennsylvania and Wisconsin in 1914. The delay in its use was due to availability, liquid chlorine only became available in 1890 in Europe, and 1909 in the US. This, combined with the development of effective chlorinators, e.g. the Ornstein chlorinator, enabled chlorination to be widely implemented for water disinfection (Water Pollution Control Federation. Technical Practice Committee. 1976).

6.1.2.2 Germicidal properties

Chlorine is one of the most reactive elements and in nature is only found combined with other elements. The most effective compound, in biocidal terms, formed by chlorine is hypochlorous acid, and this has been shown experimentally by Akin et al (1982). When added to water, chlorine forms hypochlorous (HOCl) and hydrochloric (HCl) acids:

$$Cl_2 + H_2O \leftrightarrow HOCl + H^+ + Cl^-$$

The pH of the water to be disinfected affects the type of compounds formed by chlorine after addition. This is because hypochlorous acid is a weak acid and between the pH range 6-8 it undergoes partial dissociation and forms both hypochlorous acid and hypochlorite ions:

$$HOCl \leftrightarrow H^+ + OCl^-$$

When exposed to pH6 the solution produced would contain 80% hypochlorous acid and 20% hypochlorite ions, at pH8 this is reversed.

Hypochlorous acid is similar in structure to water and is neutrally charged. It is therefore able to pass through negatively charged cell walls, as well as slime layers and the protective layers of microorganisms (www.lenntech.com/water-disinfection/disinfectants-chlorine.htm). Once inside the cell wall hypochlorous acid destroys enzyme groups by replacing hydrogen atoms in the enzymes with chlorine atoms. This results in a change in the cell's molecular structure causing it to breakdown and die (www.lenntech.com/water-disinfection/disinfectants-chlorine.htm ; White 1999). Surface damage observed after chlorine treatment is minimal and the permeability of cell walls does not alter significantly due to chlorine treatment. It is the reaction of chlorine within the cell with internal components that causes mortality of organisms (Cho et al. 2010).

6.1.2.3 Production by electrolysis

It is common in industry for chlorine to be produced by electrolysis (White 1999). As previously stated, this is the process of passing an electric current through water containing sodium chloride (NaCl), e.g. seawater, to produce a disinfectant solution (Matousek et al. 2006). Inside the electrolytic cell chlorine is produced at the anode:

$$Cl^- \longrightarrow Cl_2\,(aq) + 2e^-$$

The chlorine is then hydrolysed to form hypochlorous acid:

$$Cl_2 + 2H_2O \longrightarrow 2HOCl + 2H^+$$

The hypochlorous acid then dissociates to form hypochlorite ions in alkaline solutions:

$$HOCl \longrightarrow OCl^- + H^+$$

The solution produced by electrolysis contains seawater, sodium hypochlorite, hydrogen gas and hypochlorous acid (Matousek et al. 2006).

Early use of electrolysis declined due to the development of effective chlorinators and the higher efficiency of alternate chlorination methods, e.g. using liquid and gaseous chlorine. However, attention returned to electrolysis after an evaluation of the hazards associated with storing liquid and gaseous chlorine (White 1999). Electrolytic generation systems are low risk as there is no requirement for storage of chemicals which prevents large spills (White 1999).

6.1.2.4 Application of chlorine for water disinfection

As previously described, the first applications of chlorine for water treatment was in wastewater and drinking water disinfection. This began in the 1800s, with the first recorded use in 1832 to prevent disease spreading through a hospital via the water supply and in 1854 the first large scale wastewater treatment plant opened in London, England (Water Pollution Control Federation. Technical Practice Committee. 1976). The first application of in drinking water disinfection was in 1893 in Belgium, and in the US in 1908. These early applications used chlorinated lime, but in 1887 technology advanced and chlorine gas was used in a treatment plant in the US. The free chlorine doses currently applied in these applications are a maximum of $5mgL^{-1}$ free chlorine can be used for drinking water treatment, although on delivery chlorine levels must have dropped to $0.2mgL^{-1}$ (World Health Organisation 2010). For wastewater disinfection treatment plants generally apply a dose of $0.5\text{-}1.0mgL^{-1}$ residual chlorine to adequately disinfect wastewaters (Brungs 1973).

The disinfection of swimming pools was first addressed in the early 1900s, and while it was clear that chlorination would disinfect the water researchers were unsure of the effects of chlorine to bathers (Stovall et al. 1923). While chlorination has continued adverse health effects have been observed, including the presence of carcinogenic by-products and some respiratory effects, e.g. a link to increased asthma cases (See

Nemery et al. 2002 and references therein). Current standards for the treatment of swimming pools with chlorine state that <3mgL^{-1} must be used for disinfection (World Health Organisation 2009). While this is a high enough level to kill many bacteria (Table 6.1) the resistance of some harmful pathogens, including *Cryptosporidium parvum* and *Giardia* spp. to chlorination is a limitation to its use in swimming pool water disinfection. Industry is now looking at different technologies for swimming pool water disinfection (Leigh 2010).

The tolerance of some pathogens to chlorination is a major limitation for the use of chlorine. *Cryptosporidium parvum* and *Giardia lamblia* are harmful pathogens that can be carried in wastewater, drinking water and swimming pool water. While chlorination has been used to disinfect these waters it has been observed that some pathogens have a tolerance to this treatment and high chlorine doses are required for their inactivation, e.g. Korich et al (1990) (Table 6.1).

Mussels are particular pests in power stations and chlorination is aimed at preventing the settlement of larval stages as they pass through the power station. Power stations dose the entrance to the condensers continuously at 0.2 mgL^{-1} NaOCl to prevent settlement (Thompson et al. 1997). Continuous low levels of chlorine (0.02mgL^{-1}) have been observed to prevent feeding behaviour in the mussel *Mytilus edulis* and over long exposure periods can result in mortality. Over shorter periods it retards growth in adult mussels due to a reduction in feeding time; mussels shut their valves when chlorine is present in the water and so only open to feed during any periods in which chlorination is stopped (Thompson et al. 1997; Rajagopal et al. 2003). Concentrations of <1.5mgL^{-1} have been observed to reduce filtration rate, foot activity index and byssus thread production in the mussels *Brachidontes striatuluas*, *Dreissena polymorpha*, *M.edulis* and *Mytilus leucophaeta* (Rajagopal et al. 1997; Rajagopal et al. 2003).

The application of chlorination for ballast water treatment has been tested in preliminary studies (Sano et al. 2004; Gray et al. 2006; Matousek et al. 2006). Sano et al. (2004) and Gray et al (2006) examined the toxicity of sodium hypochlorite on

marine organisms. Gray et al (2006) determined that the high levels of sodium hypochlorite required for effective treatment (Table 6.1) show that it would not be suitable to carry this amount for shipboard use in full ballast tanks. However, it could be a potential treatment for ships carrying low amounts of ballast water, e.g. NOBOB vessels (Gray et al. 2006). Matousek et al. (2006) used electrolysis to produce hypochlorite and determined that 3.5mgL^{-1} resulted in bacterial reduction with minimal regrowth over 10 days, chlorophyll α levels dropped to below detection within 5 hours of hypochlorite addition and zooplankton levels were reduced to <10 live organisms per litre at only 1.0ppm (Matousek et al. 2006). In contrast, Zhang et al (2004) observed that 20mgL^{-1} was needed for bacterial inactivation, although some *E.coli* cells were still viable. 60mgL^{-1} killed all algae and protozoa in a natural population, while a lower dose was effective against zooplankton: 40mgL^{-1} killed all amphipods and 2mgL^{-1} resulted in no live *Artemia* sp. The variation in dose observed in the studies highlight the necessity of preliminary tests for each different chlorine system. It is vital to establish the dose required in the conditions of use for each specific application.

Table 6.1. Chlorine dose (mgL^{-1}) applied and experimental conditions of previous studies and the inactivation of bacteria, phytoplankton and zooplankton observed (- indicates information not available).

Organism	Temperature (°C)	pH	Exposure time	Chlorine dose (mgL^{-1})	
Bacteria:					
Coxsackie B 5	25-28	7	2 minutes	0.2	≥99.7 inactivation (Kelly and Sanderson 1958)
Cryptosporidium parvum oocysts	-	-	90 minutes	80	99% inactivation (Korich et al. 1990)
	20	-	48 hours	2	100% inactivation (Carpenter et al. 1999)
	30	-	24 hours	2	100% inactivation (Carpenter et al. 1999)
	20	-	12 hours	10	100% inactivation (Carpenter et al. 1999)
	30	-	6 hours	10	100% inactivation (Carpenter et al. 1999)
Enterococci sp.	-	-	20 minutes	5	100% inactivation (Bergendahl and Stevens 2005)
Escherichia coli	20	7.1	-	0.085	1 log reduction (Cho et al. 2010)
	-	-	1 minute	1.1	4 log reduction (Rice et al. 1999)
	-	-	20 minutes	5	97% inactivation (Bergendahl and Stevens 2005)
Giardia lamblia oocysts	5	6	30 minutes	2.5	99% inactivation (Rice et al. 1982)
	5	7	1 hour	2.5	99% inactivation (Rice et al. 1982)
	5	8	1hour	2.5	99% inactivation (Rice et al. 1982)
	25	-	10 minutes	1.5	100% inactivation (Jarroll et al. 1981)
	5	6	10 minutes	8.0	100% inactivation (Jarroll et al. 1981)
	5	7	10 minutes	8.0	100% inactivation (Jarroll et al. 1981)
	5	8	30 minutes	8.0	100% inactivation (Jarroll et al. 1981)
Giardia muris oocysts	25	7	10 minutes	3	99% inactivation (Leahy et al. 1987)
	25	5	10 minutes	4	99% inactivation (Leahy et al. 1987)
Legionella pneumophila	35	-	27 days	0.5	100% inactivation (Cooper and Hanlon 2010)
Polio virus	0	6	10 minutes	0.1	99% inactivation (Weidenkopf 1958)
	-	-	30 minutes	0.3	100% inactivation (Lothrop and Sproul 1969)
	25-28	7	2 minutes	0.3	≥99.7 inactivation (Kelly and Sanderson 1958)
Total coliforms	-	-	20 minutes	5	99% inactivation (Bergendahl and Stevens 2005)
Vibrio cholera	-	-	20 seconds	0.5	100% mortality (Morris et al. 1996)
		-	5 minutes	8	100% mortality (Sousa et al. 2001)

Phytoplankton:					
Natural population	-	-	48 hours	3.5	Chlorophyll α below detection (Matousek et al. 2006)
Pseudokirchneriella subcapitata	-	-	-	0.05	LC_{90} (Sano et al. 2004)
Zooplankton:					
Acartia tonsa	<28	-	24	0.14-0.56	22.57 ± 2.04% mortality (Bamber and Seaby 2004)
Brachidontes striatulus	-	-	102	1	100% mortality (Rajagopal et al. 1997)
	-	-	468	5	100% mortality (Rajagopal et al. 1997)
Crangon crangon	<28	-	48	0.53	38.44±9.88% mortality (Bamber and Seaby 2004)
Dreissena polymorpha	-	-	588	1	100% mortality (Rajagopal et al. 2003)
	-	-	252	3	100% mortality (Rajagopal et al. 2002)
Homarus gammarus	<28	-	24	0.24-1.0	2.70±3.86% mortality (Bamber and Seaby 2004)
Mytilus edulis	-	-	966	1	100% mortality (Rajagopal et al. 2003)
		-	6	8 (NaOCl)	100% mortality (Thompson et al. 1997)
Mytius leucophaeta	-	-	1104	1	100% mortality (Rajagopal et al. 2003)
Natural population	-	-	-	1	<10 live organisms (Matousek et al. 2006)
Diapausing eggs	-	-	24 hours	1000 (NaOCl)	89% reduction in hatching (Gray et al. 2006)

6.1.3 Factors which affect the use of chlorine

When testing chlorine for a specific application it is vital to determine any problems which could limit its use. These include environmental factors which affect the required dosage, factors which could increase or decrease the time required for decay and conditions under which harmful by-products are produced. Additional factors which specifically impact the effectiveness of chlorine are the contact time, amount of organic material present and the number and type of microorganisms present (www.lenntech.com/water-disinfection/disinfectants-chlorine.htm). In ballast water

research it is necessary to understand how chlorine will perform in this situation to best understand the conditions for optimum production (where necessary), the dose required for high mortality and the breakdown pattern to ensure it is environmentally acceptable. A number of preliminary tests must therefore be performed to assess these factors prior to ballast water application.

The presence of sediment and organic material (including organism density) in seawater affects oxidising biocides by significantly increasing the dose required in comparison to tests using low organic loads (McCracken 2001). This has been observed in studies assessing chlorine ballast water treatments (Sano et al. 2004; Zhang et al. 2004; Gray et al. 2006) and so would need to be assessed in this study. The inclusion of a primary treatment method to physically remove particles and thus lower the presence of organic material would reduce the amount of chlorine required and thus benefit the system.

After discharge from the ship the chlorine is released into the environment along with the ballast water. Biological, chemical and physical processes can increase the rate of decay, although the effects of these processes vary depending on the chemical and the port into which it is discharged. Sunlight is very important for increasing the decay of chemicals, and several factors e.g. day length, season and location of the port will affect the amount and strength of irradiance available for chemical breakdown (Cooper et al. 2007). This will only be a contributing factor for chlorine treatment if high levels of residual chlorine are present after tests, by performing tests which last over a period of days it will be possible to determine whether this will be a problem.

The use of chlorine can cause the production and accumulation of harmful by-products (Vianna da Silva and da Costa Fernandes 2003; Matousek et al. 2006). The by-products produced include bromate which is a possible carcinogen, haloacetic acid (HAAs) and trihalomethanes (THMs) (Matousek et al. 2006). Matousek et al. (2006) assessed the production of chlorine by-products and only observed low concentrations of HAAs and THMs, below the concentrations set for drinking water standards (Matousek et al.

2006). Tests performed by Vianna da Silva and da Costa Fernandes (2003) observed the production of THMs in all experimental tanks and in one case it was above the level permitted by law. THM production varied with chlorine concentration and the number of cells present in samples, but at high cell concentrations relatively low chlorine concentrations, i.e. 3ppm, could result in illegal levels of THMs (Vianna da Silva and da Costa Fernandes 2003). The production of by products is a vital consideration in ballast water treatment as all biocides must be safe for use by the crew and for release into the environment. Any biocide which cannot guarantee this will have serious limitations to its use. While it is beyond the scope of this study to assess any by-products produced, it will be possible to look at the likelihood of this occurring once the required dose has been established.

6.1.4 Aim of this study

The aim of this study was to determine the biological efficiency of the chemical 'AnoFluid' for potential use as a ballast water treatment. AnoFluid is a chlorine based chemical produced by electrolysis. It is formed by adding sodium chloride (NaCl) to water, which is introduced to a cell and an electrochemical reaction produces sodium hypochlorite (NaOCl). The generator produces a 'hypochlorous acid rich' disinfectant at the anode; this is the 'AnoFluid'. The AnoFluid remains active for a number of hours, before degrading back to its original components. The composition of salts in the AnoFluid varies with the current of the electromagnetic cell, and the concentration of chloride is higher at a higher current (www.lvpg-international.com). AnoFluid is considered to be environmentally friendly within its current usage as a sanitizer and disinfectant for drinking and swimming pool water, waste water supplies, as well as removing biofouling organisms from industrial cooling systems (Eguía *et al*., 2007). This was its first application in the marine environment and the first time it has been investigated as a potential ballast water treatment.

Initially tests were performed to look at the conditions required (i.e. current and salinity) to produced the most free-chlorine rich AnoFluid. Subsequently this AnoFluid

was tested on marine plankton in order to determine whether it would kill organisms, and at what dose and exposure time. Determination of the dose is vital for determining the feasibility of a chemical system as preliminary tests must confirm that the chemical is effective at a concentration at which sufficient volume can feasibly be taken on board a vessel for use during the voyage. In order to assess potential environmental risks upon discharge the breakdown of the AnoFluid was monitored firstly over 24 hours, and also during five day tests to ensure it would not be environmentally detrimental. Finally the AnoFluid system was combined with a 40µm stainless steel screen filter in four large scale tests, performed with guidance from the IMO G8 guidelines, to determine its biological efficiency and potential for further development as a ballast water treatment system.

6.2 Methodology

6.2.1 Test series 1

To assess the optimum conditions for AnoFluid production tests were performed at a range of salinities and current. Seawater was collected from Cullercoats Bay on the day of testing for AnoFluid production. Salinity levels were measured using a handheld VWR refractometer. Residual chlorine levels were measured by adding a free chlorine reagent to a 10ml aliquot of AnoFluid. This was then analysed by a Hanna Instruments Free and Total Chlorine Measure (Model HI 96711) and the reading recorded.

6.2.2 Test series 2

Series 2 tests were performed to determine whether AnoFluid is an effective biocide on the test organism *Tetraselmis suecica*, a single celled green alga. In tests seawater was obtained from a storage tank filled with sand-filtered and pumped seawater from Cullercoats Bay. Three 1L replicate 'control' samples and three 10L replicate 'treated' samples were put into storage containers, to which *T.suecica* were added. The AnoFluid was added to the containers as appropriate and stirred. The AnoFluid tested

was produced at a current of 16A and a salinity of 30ppt (as determined in Series 1 tests). The effect of the AnoFluid was tested at a concentration of 1% (1.35mgL^{-1}) and 3% (4.05mgL^{-1}) after 24 hours, and at a concentration of 7% (9.45mgL^{-1}) after 24, 72 and 120 hours. The containers were kept at room temperature, as this is the optimum temperature for the algae and in the dark for the required exposure time. All samples were stained using Evans blue (See Section 5.2.6 for Evans blue staining method). All samples were examined using an Olympus CK X31 microscope at 200-400x magnification.

6.2.3 Test series 3

Series 3 tests were further used to optimise the dose of AnoFluid and exposure time required for high mortality in *T.suecica*. Subsequent to the results of Series 2 these tests looked at residual chlorine levels in samples after mixing, and over a period of 24 hours to assess the breakdown of AnoFluid. Tests were performed during July 2008 at the Dove Marine Laboratory. Seawater was obtained from a storage tank filled with sand-filtered and pumped seawater from Cullercoats Bay. Three 1L replicate 'control' samples and three 10L replicate 'treated' samples were put into storage containers, to which *T.suecica* was added. The AnoFluid was added to the containers as appropriate and stirred to ensure distribution throughout the container. The AnoFluid tested was produced at 16A and had a salinity of 30ppt. The effect of the AnoFluid was tested at a concentration of 1% (1.35mgL^{-1}), 2% (2.70mgL^{-1}) and 3% (4.05mgL^{-1}) after 1, 3-4, 6-7 and 24 hours. The containers were kept at room temperature and in the dark for the required exposure time. All samples were stained with Evans blue using the method described in Section 5.2.6. For analysis samples were made up to a known volume with fresh 0.45µm filtered seawater and a 1ml aliquot was removed and examined using an Olympus CK X31 microscope at 200-400x magnification.

6.2.4 Test series 4

Tests were performed on a combined 40µm stainless steel screen filter and AnoFluid system. Four tests were performed in total: two at the Dove Marine Laboratory in July 2009 and two at a test site in Blyth, UK, in August 2009. In Series 4, tests were performed under guidance from the IMO G8 guidelines "Guidelines for approval of ballast water management systems".

Two test species were used in all experimental runs. The brine shrimp *Artemia salina* was used as a representative for the ≥50µm size class and *T.suecica* as a representative of the ≥10<50µm size class. In all tests seawater was pumped from the sea into a large storage tank to which the test organisms were added.

On Day 0 samples were collected 'Before Filter', 'After Filter' and 'After Anofluid'. Three replicates were collected at each sampling point. For *A.salina* analysis 20L was collected 'Before Filter' and 100L 'After Filter' and 1000L 'After Anofluid. For *T.suecica* analysis 1L was collected 'Before Filter', 1L 'After Filter' and 10L 'After Anofluid'. On the fifth day samples were collected from the control tank and from the treated tank. For *A.salina* analysis 100L was collected from the control tank and 1000L from the treated tank. For *T.suecica* analysis 1L was collected from the control tank and 10L from the treated tank.

A.salina samples were analysed using visual examinations as described in Section 5.2.6. *T.suecica* samples were assessed by FlowCAM using the fluorescent vital stain Fluorescein diacetate (FDA) as described in Section 4.2.2.3.

6.2.5 Statistical analysis

All data were checked for normality using the Anderson-Darling Normality test and equal variance using Levene's Test. If data were found to be non normal they were transformed using common transformation methods. In Series 2 tests a 2-way ANOVA

was used to assess factors Concentration (1, 3, 7%) and Treatment (Control, Treated. A 2-sample T-test was applied to data sets at each of 24, 72 and 120 hours staining time to test the null hypothesis: There is no difference in the number of live organisms due to factor Treatment (Control, Treated). Series 3 data were assessed using a 2-way ANOVA to assess factors Time (1, 3-4, 6-7, 24 hours) and Concentration (Control, 1, 2, 3%) on the number of live *T.suecica* present. A 1-way ANOVA or Kruskal Wallis test was used in Test series 4 analyses to determine the effect of Factor Treatment (Before filter, After filter, After AnoFluid, Control tank, Treated tank) on the number of live organisms. Post hoc tests were applied where required.

6.3 Results

6.3.1 Test Series 1

The AnoFluid with the highest residual chlorine level (135 mgL^{-1}) was produced at a salinity of 30.1ppt and current of 16A, as shown in Table 6.2. In all subsequent tests the AnoFluid used for disinfection was produced under these conditions.

Table 6.2. Conditions required (Salinity and Current) to produce AnoFluid with the highest residual chlorine levels.

Salinity (ppt)	Current (A)	Residual chlorine (mgL^{-1})
17	8	68.6
18.6	12	98.6
30.1	16	135
36.2	16	118
38.9	12	90.4

6.3.2 Test series 2

All data were assessed to look at the effects of 1%, 3% and 7% addition of AnoFluid containing 135mgL^{-1} residual chlorine prior to mixing. A significant difference in the number of live *T.suecica* was observed due to the factor Concentration (2-way ANOVA; p = <0.0001, F = 79.90, d.f. = 2) and the factor Treatment (2-way ANOVA; p = <0.0001, F = 380.52, d.f. = 1). There was no significant interaction between the two factors. These tests showed that the AnoFluid was effective against the *T.suecica* and just 1% addition caused a reduction in live cells (Table 6.3).

Table 6.3. Test conditions and the log inactivation rate of *Tetraselmis suecica* during Series 2 tests.

AnoFluid addition (%)	Residual chlorine (mgL^{-3})	Exposure time (hours)	Log inactivation of *T.suecica*
1	1.35	24	0.9
3	4.05	24	1.2
7	9.45	24	1.8
7	9.45	72	1.9
7	9.45	120	1.7

Tests then looked at the effects of exposure time after 7% addition of AnoFluid containing 135mgL^{-1} residual chlorine prior to mixing. The data showed a significant reduction in the number of live *T.suecica* after AnoFluid treatment at 24 and 120 hours (24 hours: 2-sample T-Test; p = 0.006, T = 12.47, d.f. = 2; 120 hours: 2-sample T-Test; p = 0.007, T = 6.68, d.f. = 3). The reduction at 72 hours was not significant (2-sample T-Test; p = 0.094, T = 3.03, d.f. = 2) but a clear decrease in live *T.suecica* was observed. Although all tests recorded a reduction in live *T.suecica* (Table 6.3) longer exposure to the chlorine did not result in lower numbers of live cells, which could be due to the decay of residual chlorine within the seawater during the testing period.

6.3.3 Test series 3

The test conditions and log inactivation of live *T.suecica* at all exposure times is shown in Table 6.4. The average residual chlorine of the AnoFluid after mixing with the seawater is shown in Table 6.5.

Table 6.4. Test conditions and the log inactivation rate of *Tetraselmis suecica* in Series 3 tests.

AnoFluid addition (%)	Residual chlorine addition (mgL^{-3})	Log inactivation of *T.suecica* after 1 hour	Log inactivation of *T.suecica* after 3-4 hours	Log inactivation of *T.suecica* after 6-7 hours	Log inactivation of *T.suecica* after 24 hours
1	1.35	0.8	0.6	0.7	0.2
2	2.70	1.4	1.5	1.5	0.8
3	4.05	1.8	2.1	1.9	1.7

Table 6.5. The average residual chlorine (mgL^{-1}) in samples after mixing, for each percentage AnoFluid addition and exposure time.

Exposure time (hrs)	**Percentage AnoFluid**		
	1%	**2%**	**3%**
1	0.53	1.59	2.52
3-4	0.38	1.46	2.42
6-7	0.35	1.32	2.32
24	0.12	0.60	1.30

The number of live *T.suecica* was significantly different due to the interaction between factors Time and Concentration (2-way ANOVA; $p = <0.001$, $F = 10.91$, d.f. = 9). Tukey multiple comparisons showed that these differences were between Control and

AnoFluid treated samples at all exposure times. The residual chlorine added in the 2% and 3% samples was ≥1.59mgL^{-1} and so in further tests this concentration should be used as a guide for determining the required dose.

The mapping of residual chlorine over the 24 hour period shows that the concentration decreased by >48% after 24 hours (Table 6.5). In ballast water treatment system testing all experimental runs include a five day holding period, with the rate of decrease of AnoFluid over 24 hours it would be expected to be at negligible levels at the end of the test and so not harmful to the environment upon discharge.

6.3.4 Test series 4

6.3.4.1 Test 1

Test conditions and the log inactivation rate of live *Artemia salina* and *Tetraselmis suecica* are shown in Table 6.6.

Table 6.6. Test conditions and the log inactivation rate of live *Artemia salina* and *Tetraselmis suecica* in Series 4 Test 1.

Flow rate (m^3hr^{-1})	**Residual chlorine concentration (mgL^{-3})**	**AnoFluid addition (%)**	**Log inactivation of live *A.salina***	**Log inactivation of live *T.suecica***
65.5	1.33	2.4	1.8	1.8

There was a significant difference in numbers of live *A.salina* between Treatments (One-way ANOVA; p = <0.001, F = 14.1, d.f. = 4) and the filter significantly reduced *A.salina* numbers from 60,217±11980 individuals in control samples to 50±30 individuals per 1000L after filtration (Least significant difference; p = 0.005). After the five day holding period 1020±107 live *A.salina* per 1000L were present in the treated tank, showing that the treatment system was unable to cause full mortality.

The number of live *T.suecica* present was significantly different between Treatments (One-way ANOVA; p = <0.001, F = 32.4, d.f. = 4). A significant reduction in live *T.suecica* was observed After Filter (Least significant difference; p = <0.001), after AnoFluid addition on Day 0 (Least significant difference; p = <0.001) and in the treated tank after the five day holding period (Least significant difference; p = <0.001). Although the system significantly reduced the number of live organisms the operating conditions needed further improvement, and a higher concentration of residual chlorine required for higher disinfection efficiency.

6.3.4.2 Test 2

Test conditions and the log inactivation rate of live *Artemia salina* and *Tetraselmis suecica* are shown in Table 6.7.

Table 6.7. Test conditions and the log inactivation rate of live *Artemia salina* and *Tetraselmis suecica* in Series 4 Test 2.

Flow rate (m^3hr^{-1})	**Residual chlorine concentration (mgL^{-3})**	**AnoFluid addition (%)**	**Log inactivation of live *A.salina***	**Log inactivation of live *T.suecica***
66.7	1.37	3.9	1.3	0.9

The number of live *A.salina* present was significantly different between Treatments (One-way ANOVA; p = <0.001, F = 26.8, d.f. = 4). The filter removed a significant number of organisms (Least significant difference; p = 0.001) and AnoFluid addition on Day 0 reduced live numbers further. The full 5 day treatment showed a significant reduction from input levels (Least significant difference; p = <0.001), but 2013±148 live *A.salina* individuals per 1000L were present in Day 5 treated samples.

Control samples showed low numbers of live organisms, but this could not be accounted for through any obvious fault in the methods used. The number of live *T.suecica* present was significantly different between Treatments (One-way ANOVA; p

= <0.001, F = 631.2, d.f. = 4) and the control tank showed a significant difference from the treated tank on the fifth day (Least significant difference; p = <0.001). A reduction in organisms was seen but 37±8 live organisms per ml were still present in Day 5 treated samples.

The residual chlorine present in samples on Day 0 was 1.37mgL^{-1}, although a slight increase from Test 1 it was insufficient for reducing live organism numbers as required and addition of a higher concentration of residual chlorine was required.

6.3.4.3 Test 3

Test conditions and the log inactivation rate of live *Artemia salina* and *Tetraselmis suecica* are shown in Table 6.8.

Table 6.8. Test conditions and the log inactivation rate of live *Artemia salina* and *Tetraselmis suecica* in Series 4 Test 3.

Flow rate (m^3hr^{-1})	**Residual chlorine concentration (mgL^{-3})**	**AnoFluid addition (%)**	**Log inactivation of live *A.salina***	**Log inactivation of live *T.suecica***
83.5	1.53	8.4	4.8	1.8

The number of live *A.salina* present was significantly different between treatments (Kruskal wallis; p = 0.014, χ^2= 12.5, d.f. = 4). The filter reduced *A.salina* by 99.8% from 30,017±4042 to 47±15 live individuals per 1000L. After full treatment and the five day holding period 1 live organism remained in samples. The combined system was very effective, however after the high removal by the filter it is not possible to conclude on the effectiveness of the AnoFluid itself on *A.salina*.

The number of live *T.suecica* present was significantly different between Treatments (One-way ANOVA; p = <0.001, F = 35.3, d.f. = 4). The filter showed no removal of *T.suecica*, but there was a significant decrease in live cell numbers due to AnoFluid addition (Least significant difference; p = <0.001). In Day 5 treated tank samples 41±25 live *T.suecica* per ml were observed.

Test 3 recorded 99.8% removal of *A.salina* by the filter and after combined treatment and the holding period this was sufficient to reduce numbers to only 1±0 live organism per 1000L. The filter showed no removal of *T.suecica* and so the AnoFluid treatment must be effective in killing these organisms. At a concentration of 1.53mgL^{-1} residual chlorine mortality is too low. In the final test a higher residual chlorine concentration was applied.

6.3.4.4 Test 4

Test conditions and the log inactivation rate of live *Artemia salina* and *Tetraselmis suecica* are shown in Table 6.9.

Table 6.9. Test conditions and the log inactivation rate of live *Artemia salina* and *Tetraselmis suecica* in Series 4 Test 4.

Flow rate (m^3hr^{-1})	**Residual chlorine concentration (mgL^{-3})**	**AnoFluid addition (%)**	**Log inactivation of live *A.salina***	**Log inactivation of live *T.suecica***
86.9	3.60	11.9	4.7	3.3

The number of live *A.salina* present was significantly different between Treatments (Kruskal wallis; p = 0.026, χ^2= 13.1, d.f. = 4). The filter removed >99.9% organisms and reduced numbers from 48,650±10486 to 40±6 individuals per 1000L. No surviving *A.salina* individuals were found in the chemical tank on the fifth day. As in the previous test the high removal of the filter meant that no conclusions can be drawn on the effect of AnoFluid on *A.salina*.

The number of live *T.suecica* present differed significantly between Treatments (One-way ANOVA; p = 0.002, F = 9.7, d.f. = 4). AnoFluid addition reduced live cell numbers significantly (Least significant difference; p = 0.001). A reduction in organisms throughout the experiment was observed and on Day 5 no live organisms were present in treated samples.

Test 4 used 3.60mgL^{-1} residual chlorine and at this concentration the combined system resulted in no live organisms after the five day treatment. In all Series 4 tests the residual chlorine was measured on Day 5 and negligible levels were present.

6.4 Discussion

AnoFluid treatment was novel to the marine environment and its ability to produce residual chlorine rich AnoFluid with seawater was unknown prior to these tests. This study has confirmed the ability of the system to produce AnoFluid containing 135mgL^{-1} residual chlorine with seawater. It is therefore suitable to be further developed for potential use in marine applications.

The tests conducted show that AnoFluid is an effective biocide on the alga *T.suecica*. Cells were observed to bleach after AnoFluid treatment and chlorophyll pigments could not be distinguished. The tests observed 100% mortality of *T.suecica* at a concentration of 3.6mgL^{-1} free chlorine. In comparison to the literature (Table 6.1) this dose was higher than that required for the alga *P.subcapitata* - LC_{90} 0.05mgL^{-1} free chlorine (Sano et al. 2004), and almost even with the dose by Matousek et al (2006) where chlorophyll α levels in a natural population were reduced below detection by 3.5mgL^{-1}. It could be possible to reduce the AnoFluid dose further, and more tests would need to be performed to assess the efficiency of any lower dose.

In this study the filter performed with an average of <99.8% removal of *A.salina* in three of the Series 4 tests. This high removal made it impossible to make clear

conclusions about the effectiveness of the AnoFluid treatment on *A.salina*. Zhang et al. (2004) observed that 2mgL^{-1} free chlorine was sufficient to kill *A.salina*, while 40mgL^{-1} was required to kill the amphipod *Corophium acherusiem*. This suggests that the *A.salina* may be more susceptible to chlorine treatment and that AnoFluid should be trialled on a range of natural organisms to ensure it is as effective on other organisms. Sano et al (Sano et al. 2004) assessed sodium hypochlorite on three species of zooplankton: the cladoceran *Daphnia magna*, zebra mussel *Dreissena polymorpha*, and brine shrimp *Artemia* spp. cysts. The LC_{90} dosage varied greatly, from 0.7 - 129.7mgL^{-1} for the zooplankton (Sano et al. 2004) and this level shows the variation in tolerance to sodium hypochlorite by the test organisms. Gray et al (2006) assessed the effects of sodium hypochlorite on diapausing eggs and cysts from ballast tank sediment. Hatching was reduced by 24-93% when >500mgL^{-1} was added to samples while exposure to 1000mgL^{-1} for 24 hours reduced hatching by a minimum of 89%. The authors suggested that the high sodium hypochlorite demand could be due to the presence of sediment in the samples. These studies highlight the importance of physical removal of zooplankton and organic material prior to chlorine treatment. This will reduce the dose required, and also limit the effect of varying tolerance as organisms will be removed prior to chlorination.

The production of harmful by-products such as THMs by the AnoFluid was beyond the scope of this study. The production of by-products and persistence of chemicals in the environment are of particular concern when using biocides. The use of one ballast water biocide, Vibrex, has already been found unsafe due to the production of a noxious gas (Gregg and Hallegraeff 2007). The formation of by-products from chlorination is affected by many factors and 22 have been identified to date. These include the chlorine dose, chlorine demand, temperature, pH, TOC, DOC and contact time (See Chowdhury and Champagne 2008 and references therein). While it is not possible for us to establish definitely whether any by-products were produced, the chlorine dose applied is above that which has been shown to result in their formation, >0.3mgL^{-1} (Batjer et al. 1980), and this dose combined with associated factors (listed above) could have led to by-product formation. Matousek et al (2006) assessed the production of trihalomethanes, haloacetic acids and bromate after ballast water

treatment with 3.5mgL^{-1} chlorine, comparable to the dose used in this study (3.6mgL^{-1}). The study assessed discharges against the US EPA drinking water maximum contaminant standards and all were below those stated in the standards (Matousek et al. 2006). At higher chlorine addition levels the concentration of trihalomethane production has been observed to exceed the 100µgL^{-1} level as stated by the US EPA. Vianna da Silva and de Costa Fernandes (2003) observed 430µgL^{-1} trihalomethane productions after application of 10mgL^{-1} chlorine addition. If the system tested in this study was to be further developed for use onboard vessels it would be necessary to monitor the formation of by-products due to the potential for excessive levels to be produced.

To obtain the required dose of AnoFluid from optimum production conditions an addition of 2.7% would be required. There are some ballast water biocides currently on the market which have been assessed in preliminary tests - Seakleen® (Wright and Dawson 2001; Sano et al. 2004; Gregg and Hallegraeff 2007; Wright et al. 2007a) and PERACLEAN® Ocean (Fuchs et al. 2001; Gregg and Hallegraeff 2007; de Lafontaine et al. 2008). In comparison, the recommended dose of Seakleen® is 2mgL^{-1}. This concentration has been found effective on zooplankton (Wright and Dawson 2001; Sano et al. 2004) and phytoplankton (Gregg and Hallegraeff 2007; Wright et al. 2007a). However, a dose of 10mgL^{-1} was ineffective on dinoflagellate cysts, and this shows a limitation to the use of Seakleen® at its suggested dosage (Gregg and Hallegraeff 2007). The effective dose of PERACLEAN® Ocean as determined by preliminary studies is 400mgL^{-1} for zooplankton (Fuchs et al. 2001), 100-200mgL^{-1} for phytoplankton (Fuchs et al. 2001; Gregg and Hallegraeff 2007), 400mgL^{-1} for dinoflagellate cysts (Gregg and Hallegraeff 2007) and 300mgL^{-1} for bacteria, yeasts, moulds and small algae (de Lafontaine et al. 2008). In terms of volume addition AnoFluid is significantly lower than PERACLEAN® Ocean, but greater than the volume of Seakleen®. While this is quite a high volume addition of AnoFluid the chemical is produced onboard as required and so there is no need of space to store the chemical.

AnoFluid does not persist in the environment as it dissociates back to its original salts in a relatively short time period. Table 6.5shows the average residual chlorine present in samples over 24 hours. By the final sampling at 24 hours all chlorine levels had reduced by at least 48% of the original concentration, showing a quick breakdown of the AnoFluid. In all tests in the second series, the residual chlorine level on the fifth day of testing in the treated tank was negligible, and so there would be no risk of chlorine discharge. A previous study has shown that the chemical treatments already available (i.e. PERACLEAN® Ocean and Seakleen®) can take weeks to degrade to low levels (Gregg and Hallegraeff 2007). In many cases this could be longer than the voyage itself, which significantly limits the application of such chemicals. AnoFluid shows an obvious advantage as it will degrade to negligible levels within a 5 day period.

For ballast water purposes the data collected show that with further development the combined filter/AnoFluid treatment system could be an effective treatment system. Increased optimisation at large scale could determine whether a dose between 1.53mgL^{-1} and 3.60mgL^{-1} would be effective and thus potentially reduce the amount of AnoFluid required and the cost involved. A further advantage to the use of AnoFluid is that production by electrolysis means that chlorine can be produced on board the ship and so there are no risks to transporting or storing chemicals. This will be important on large vessels where a substantial volume of ballast water is to be treated, as no space is required for storage of chemicals when using electrolysis.

6.5 Conclusions

This study aimed to determine the biological effectiveness of a chlorine based biocide produced by electrolysis – AnoFluid. The tests were able to determine this and showed that the dose required for 100% mortality of *Tetraselmis suecica* was 3.6mgL^{-1}. This dose is in-line with other studies which have assessed chlorine based biocides for use in ballast water treatment. To obtain this concentration a 2.7% addition of AnoFluid is required, although this is quite a high volume addition the AnoFluid is produced

onboard and so there is no requirement for storage of the chemical. The breakdown of AnoFluid was quick, >48% after 24 hours, and no trace observed after 5 days. Therefore no environmental risks should be posed on discharge. However, the production of by-products through use of AnoFluid was not assessed, and this would be necessary before the chemical could be approved for use. Further testing could also be used to optimise the dose required for disinfection.

Chapter 7

Filtration as a potential ballast water treatment technology

7.1 Introduction

Filtration is a physical treatment used for the removal of particles from water. It is employed in many applications to purify water, e.g. drinking water, swimming pool water and wastewater, as a primary treatment. It is then commonly followed by a disinfection process, e.g. UV radiation or a chemical treatment, as identified in Chapters 5 and 6. One benefit of utilising filtration for the treatment of ballast water is that the technology is already well established and in shore based facilities it is already in use at scales suitable for some ships (Cangelosi et al. 2007).

7.1.1 History and application

Water purification through filtration has been practiced for centuries. Sanskrit writings described the use of sand and charcoal filters to purify water from as early as 2000 BCE. Hippocrates, in around 500 BCE, recognised the need to purify water for medicinal purposes and for this he designed the 'Hippocratic sleeve', a cloth bag through which water was poured before use (Baker and Taras 1981). While early practises such as these continued it was 1627, when interest in developing filtration commenced. Sir Francis Bacon attempted to purify seawater for drinking water by filtering out salt using a sand filter. Although his attempts were unsuccessful water filtration began to be investigated further (Baker and Taras 1981). This was additionally aided by the invention of microscopes in the 16th and 17th centuries which enabled scientists to observe previously unseen organisms within water.

During the 16-18th centuries France was the first county to determine that all houses should be supplied with fresh, clean water as a necessity. In order to achieve this sand filters were installed at treatment works to purify water. In the UK the first municipal water treatment plant opened in Scotland in 1804 (Baker and Taras 1981). Sand filters were again used, however these early designs were large, needed regular cleaning and filtration through the filters was slow. Later, in the 19th century American researchers developed the rapid flow sand filter which enabled much quicker filtration of water (Baker and Taras 1981). This was the beginning of widespread water purification using filtration. In present times filters are used in a wide range of applications, not only to purify water but also to separate liquids and solids in industrial applications.

7.1.2 Types of filters

The most commonly used types of filtration are surface and depth filtration. Surface filters work due to the relationship between particle size and pore size (Sutherland 2008). Screen filters are a commonly used type of surface filter and have been assessed in ballast water studies. They are composed of 'woven' or wire mesh screens and can be single or multilayered (National Research Council 1996). Particles larger than the pores are captured as the water flows through the screen, while smaller particles pass through. The build up of larger particles on the filter screen enhances the effectiveness of the filter by enabling it to trap particles smaller than the pore size. However, this layer will reduce the speed of water flow through the filter due to clogging, and will require cleaning to restore the flow rate (Parsons and Harkins 2002; Sutherland 2008).

Depth filtration includes media filters which can consist of sand, gravel, garnet and anthracite (Riley et al. 2005), and filters which contain irregular pore sizes to trap particles (Sutherland 2008). These filters do not have a standard pore size, but manufacturers can determine a nominal rating (Sutherland 2008). Two types of depth filtration have been assessed for ballast water use: disc filtration and crumb rubber depth filtration.

Disc filtration has been used in a range of industries, particularly for irrigation purposes, for many years (Allhands and Prochaska 1996). Disc filters are made up of a stack of discs with a hollow centre. Both sides of each disc are covered with uniform sized grooves and when the discs are compressed together the grooves form filter 'channels'. The discs are contained in an outer casing (Arkal Filtration Systems). The water entering the filter system must flow down the filter channels and as the water passes through particles become trapped or adhere to the disc surface. Due to the design of these filters and the greater surface area available for particles to become attached it is often possible for the water to flow around trapped particles. This means that disc filters can function for longer periods of time than screen filters before they become clogged and the backwash process needs to be initiated (Parsons and Harkins 2002). Disc filters are run in systems containing a number of filters and to backwash occurs in one filter at a time, meaning that the forward flow of water is little affected (Allhands and Prochaska 1996).

Media filters have been used since 2000 BCE for water purification. Granular media filtration is still commonly used for wastewater treatment (Tang et al. 2006). An environmentally friendly technology has been developed which utilises spare tyres to make granular rubber filters, and these have been assessed for use in ballast water treatment (Tang et al. 2006; Tang et al. 2009). This filter consists of a specific depth of crumb rubber and the filter efficiency depends on the size of crumb rubber particles used and the depth. Preliminary studies have shown that due to the properties of rubber, i.e. its flexibility, filters which incorporate it are lighter, have a higher flow rate, can be used for longer periods of time and require less space than other media filters (Tang et al. 2006).

7.1.3 Factors to address to determine application in ballast water treatment

There are four main factors which will affect the application of filtration when used in ballast water and so will be investigated during this study. These are the filter type, the

effects of clogging, the flow rate and filtration efficiency. The type of filter used will be determined prior to experimentation and only one filter type assessed.

There are three types of filters which have been trialled as ballast water treatments. These are screen filters, disc filters and granular media filters. Screen filters and disc filters have both proved highly effective (Parsons and Harkins 2002; Waite et al. 2003; Anon 2005; Cangelosi et al. 2007). However, the crumb rubber media filter tested by Tang et al (2006) and Tang et al (2009) was not capable of meeting the required flow rate and required too much space for shipboard use. As both screen and disc filters are effective this study assessed a 40µm screen filter.

The major problem associated with filtration is clogging by trapped particles, which leads to a reduction in the flow rate of water through the filter. Different methods have been developed to reduce this problem and the most effective are automatic 'backwashing' filter systems. These systems can be applied to all filter types, potentially requiring some modifications. The backwashing process can be activated by differential pressure across the filter or by a timer (National Research Council 1996). The backwash system opens a valve to the atmosphere which causes a reverse flow of water within the filter unit and through a 'backwash arm'. This flow through the backwash arm is restricted to a small section of the filter screen where it dislodges the trapped particles and discharges them through a backwash pipe. The backwash arm moves across the whole screen slowly, cleaning it gradually and ensuring that the forward flow of water through the filter is not affected (National Research Council 1996). This and other similar automated cleaning methods have meant that both screen and disc filters can be operated at high flow rates continuously and ballasting operation will be minimally affected. The screen filter assessed in this study is self-cleaning and the mechanism is triggered by a pre-defined drop in pressure across the screen.

The flow rate of water through the filter is important for ballast water as high removal efficiency needs to be maintained at the high speeds that will be used onboard vessels.

During testing systems will be assessed at >200m^3hr^{-1} and for shipboard use systems have been built for flow rates up to 1000m^3hr^{-1}. This study assessed a range of flow rates (12-91.8m3hr^{-1}) to determine whether filter efficiency was maintained throughout tests.

Filtration efficiency could also be affected by the organism input density. During ballast water testing the system will be challenged with high organism input loads of a minimum 10^5 organisms ≥50µm (minimum dimension) per m^3 and 10^3 organisms ≥10<50µm (minimum dimension) per ml (MEPC 2005a). It was expected that high organism loads would trigger the self-cleaning mechanism of the filter, which could lead to lower removal efficiency than in tests with fewer organisms which allowed a layer of sediment to build up on the screen. This study assessed the filtration efficiency by using a range of organism input densities to determine whether high removal efficiency was achieved in all conditions.

7.1.4 Aim of this study

This study aimed to determine the biological removal efficacy of filtration for potential as a ballast water treatment. To this end a 40µm stainless steel filter was assessed as a standalone ballast water treatment. The filter consists of 4 sintered layers, for improved strength, working in combination with a primary coarse filter to remove larger particles. The filter design is shown in Figure 7.1. Water flows through the coarse filter (A) before passing through the 40µm screen filter (B). The filter has a self cleaning process which activates when it detects differential pressure at a predefined level. During the screen cleaning clogged particles are sucked from the screen, the filter is then flushed and waste is discharged thorough a discharge pipe (C).

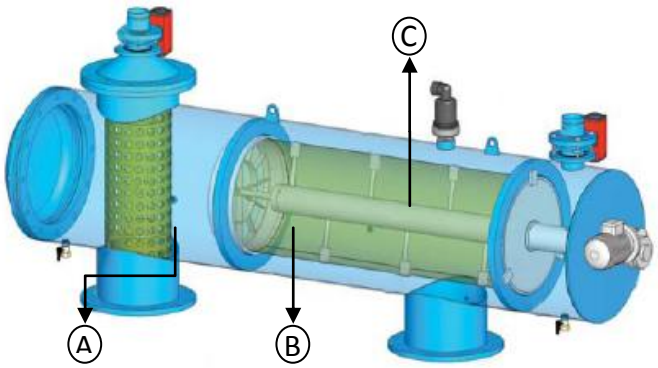

Figure 7.1. The internal design of a FilterSafe filter. Water first flows through the coarse filter (A) and then into the screen filter (B). During backwashing waste is discharged thorough a discharge pipe (C) back to the sea or into storage. (Figure adapted from www.ballastsafe.com).

Tests were carried out at the Dove Marine Laboratory, Newcastle upon Tyne, UK in January and April 2008 and a series of 10 tests was completed from May to September 2009. The removal efficiency of the filter was tested over a range of flow rates and organism input densities to determine whether high efficiency was maintained in all conditions.

7.2 Methodology

7.2.1 Test 1 set up

Test 1 was carried out in January 2008 at a flow rate of $12m^3hr^{-1}$. Three 100L replicates of both 'control' and 'treated' samples were pumped from Cullercoats Bay. 'Control' samples were pumped into storage tanks. 'Treated' samples were passed through the filter and then pumped into storage tanks where they were filtered and taken to the laboratory to be examined. In this test only organisms' ≥50µm were assessed.

7.2.2 Test 2 set up

Test 2 was performed in April 2008 at a flow rate of 12m^3hr^{-1}. 1000L of seawater was pumped from Cullercoats Bay into a storage tank. To increase organism numbers the single celled green alga *Tetraselmis suecica* and natural zooplankton collected from a vertical haul off the north east coast of England were added to the tank. This was then pumped through the filter and three 20L replicate samples of each of 'control' and 'treated' were collected.

7.2.3 Test Series 3 set up

Test series 3 consisted of 10 tests and was carried out from May to September 2009. The flow rates tested are shown in Table 7.1. Natural seawater was pumped into a large storage tank and two test organisms were added to this tank. The test organisms used were the brine shrimp *Artemia salina*, and *T.suecica* as used in the previous test. Three replicates were each collected before and after the filter. For zooplankton analysis 20L replicates were collected 'before filter' and 1000L 'after filter'. For phytoplankton analysis 1L replicates were collected 'before filter' and 10L 'after filter'.

Table 7.1. Flow rate (m^3hr^{-1}) of each test in Series 3.

Test	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Flow rate (m^3hr^{-1})	60.0	27.4	62.9	66.2	65.5	66.7	43.5	91.8	83.5	86.9

7.2.4 Sample analysis

In Test 1 only organisms ≥50µm were collected and counted. In Test 2 organisms from both size classes (≥10<50µm and ≥50µm) were collected and counted. The samples were filtered by 50µm to separate the organisms ≥50µm (zooplankton) and the

organisms ≥10<50µm (phytoplankton). Samples were fixed using 4% formaldehyde and left overnight before analysis.

Zooplankton samples were analysed using a Meiji microscope at 10-40X magnification. Before analysis samples were filtered by 50µm and rinsed using 0.45µm filtered seawater. All organisms in the sample were counted and the total number of organisms per 1000L was calculated.

Phytoplankton samples in Test 2 were analysed using a Brunel inverted microscope at 100-400X magnification. Before analysis samples were filtered using 10µm and rinsed using 0.45µm filtered seawater. The sample was made to a known volume and a 1ml aliquot was analysed in a sedgewick rafter cell. All organisms were counted within the sample and the total number of organisms per ml was calculated.

In Test Series 3 the *A.salina* samples were concentrated by 50µm mesh, rinsed using 0.45µm seawater and visually assessed on the day of testing as described in Test 1. The *T.suecica* samples were assessed by FlowCAM using the 100µm depth flow cell and 10X objective. A 3ml aliquot of each sample was analysed in AutoImage mode and files were assessed manually to determine the number of *T.suecica* imaged. The total number of *T.suecica* cells was determined by FlowCAM using flow cell width, volume of sample imaged and flow cell depth to determine the total number of particles per ml.

In the filtration tests no viability assessment was performed. These tests were used to determine the level of removal of plankton by the filter and therefore the total number of organisms present in each sample was recorded.

7.2.5 Statistics

All data were checked for normality using the Anderson-Darling Normality test and equal variance using Levene's Test. If data were found to be non normal they were

transformed using common transformation methods. For Tests 1 and 2 a 2-sample T-test was applied to all data sets to test the null hypothesis: there is no difference in organism number before or after the filter. For Series 3 tests a Mann Whitney test was applied to the *A.salina* data and a 2-sample T-test to the *T.suecica* data to test the null hypothesis: there is no difference in organism number before or after the filter. A Kruskal-wallis test was applied to percentage removal data to look for significant differences in percentage removal between all tests completed.

7.3 Results

7.3.1 Test 1 results

A significant reduction in the number of organisms ≥50µm was observed after filtration at a flow rate of $12m^3hr^{-1}$ (2-sample T-Test; p = 0.025, T = 6.18). The removal rate of organisms' ≥50µm in this test was 98.2 ± 1.8%, see Figure 7.2.

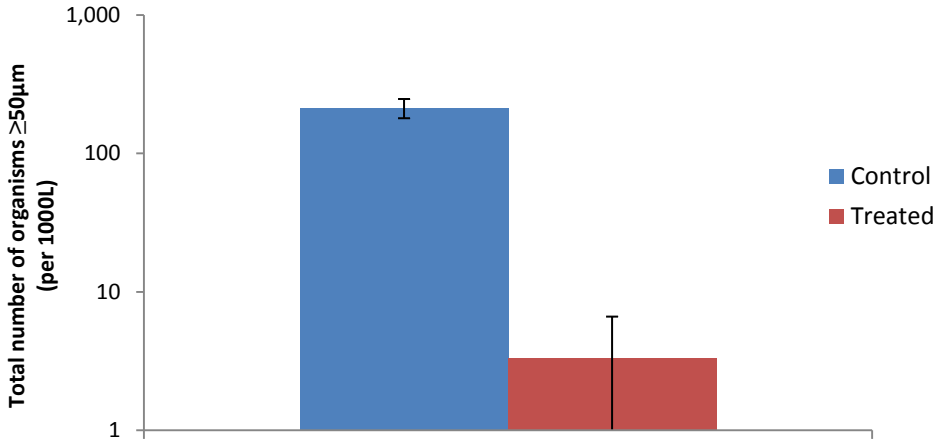

Figure 7.2. The mean number of organisms ≥50µm in 1000L in Control and Treated samples at a flow rate of $12m^3hr^{-1}$. Values are the mean of three 100L replicates ± standard error. (Note: scale is logarithmic).

7.3.2 Test 2 results

A significant reduction in the total number of organisms ≥50µm was recorded after filtration at a flow rate of $12m^3hr^{-1}$ (2-sample T-test; p = 0.041, T = 4.77).The removal rate of organisms' ≥50µm in this test was 98.3 ± 0.38%, see Figure 7.3.

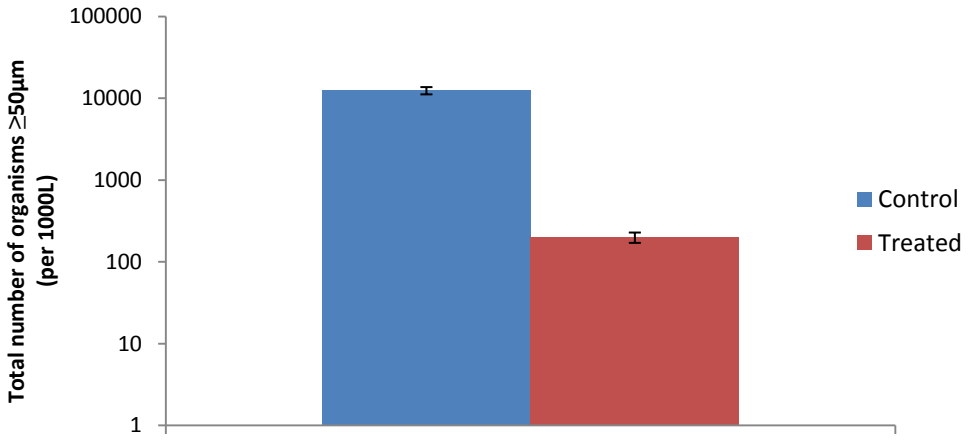

Figure 7.3. The mean number of zooplankton individuals ≥50µm in 1000L in Control and Treated samples at a flow rate of $12m^3hr^{-1}$. Control values are the mean of three 20L replicates ± standard error. Treated values are the mean of three 1000L replicates ± standard error. (Note: scale is logarithmic).

No significant reduction in the number of organisms ≥10<50µm was observed due to filtration (2-sample T-test; p = 0.156, T = 2.23). The removal rate of organisms ≥10<50µm was 44.76 ± 21.51% (Figure 7.4), and this was variable between replicates. Filtration using the 40µm filter could not be relied upon for treatment of phytoplankton. A secondary disinfection treatment would be required to kill organisms which can pass through the filter.

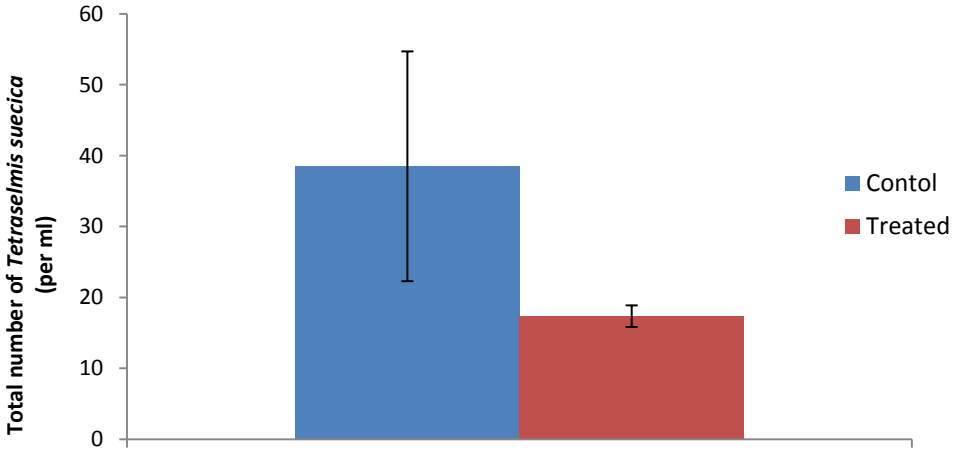

Figure 7.4. The mean number of *Tetraselmis suecica* per ml in Control and Treated samples at a flow rate of 12m^3hr^{-1}. Values are the mean of three 20L replicates ± standard error.

7.3.3 Test Series 3 results

7.3.3.1 *A.salina* results

Test Series 3 data showed a significant reduction in total *A.salina* (≥50µm) number due to filtration (Mann Whitney; p = <0.001, W = 1365.0, d.f. = 1). The filter showed a consistently high removal level of *A.salina* in all tests (Figure 7.5), with >93.5% removal in nine of the tests. A lower percentage removal occurred during Test 6, however this was due to operational difficulties and not due to low performance of the filter.

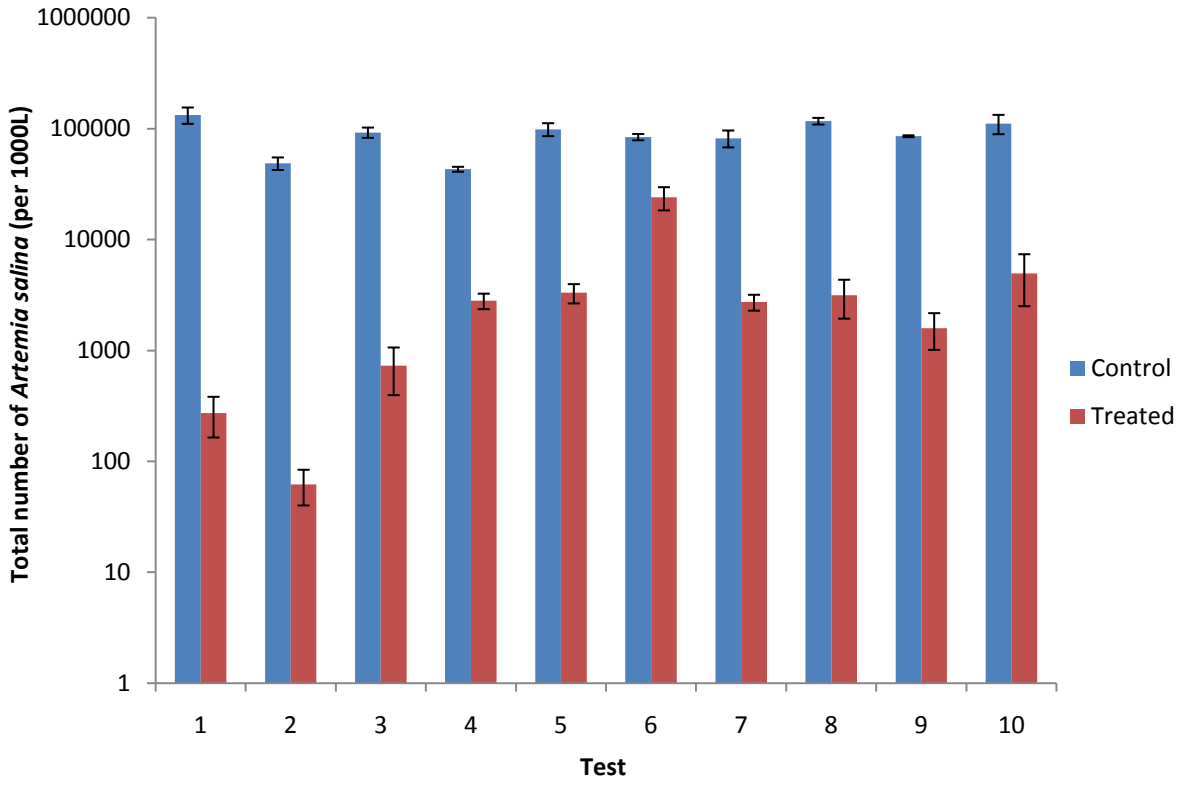

Figure 7.5. The mean number of *Artemia salina* (≥50µm) in 1000L in all tests in Series 3. Control values are the mean of three 20L replicates ± standard error. Treated values are the mean of three 1000L replicates ± standard error. (Note: scale is logarithmic).

7.3.3.2 *T.suecica* results

No significant reduction in *T.suecica* number due to filtration was observed (2-sample T-test; p = 0.982, T = 0.02, d.f. = 56). As the filter pores were 40µm and the *T.suecica* cells ranged in size from 7µm - 15µm low removal would be expected. Figure 7.6 displays the number of *T.suecica* present in all tests before and after the filter.

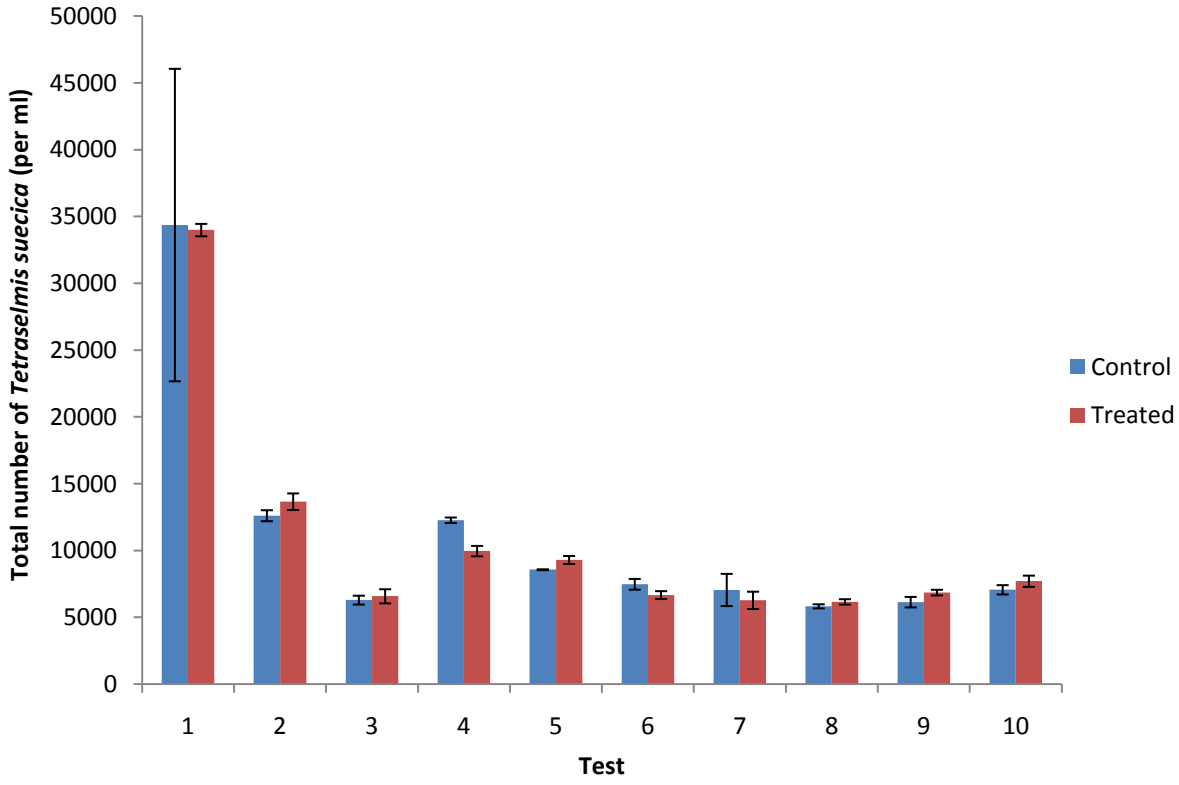

Figure 7.6. The mean number of *Tetraselmis suecica* (≥10<50µm) cells per ml in all tests in Series 3. Control values are the mean of three 1L replicates ± standard error. Treated values are the mean of three 10L replicates ± standard error.

7.3.4 Percentage removal of organisms

7.3.4.1 Effect of flow rate and input density on organisms ≥50µm

The percentage removal of organisms' ≥50µm was determined (Figure 7.7). Test 6 of Series 3 showed a lower removal rate of organisms due to technical problems whilst running the system and so has been omitted from this analysis. It is clear that when challenged with high flow rates the filter was still able to maintain high removal efficiency: a removal rate of 97.2% was observed at the highest flow rate of 91.8m^3hr^{-1}. While this would not be enough to sufficiently treat the ballast water itself, it would remove a substantial number of organisms and enhance conditions for the secondary treatment. The filter also showed high efficiency when challenged with high organism input levels. The highest input density tested was 132,750 *A.salina* per 1000L and the filter performed with 99.8% removal.

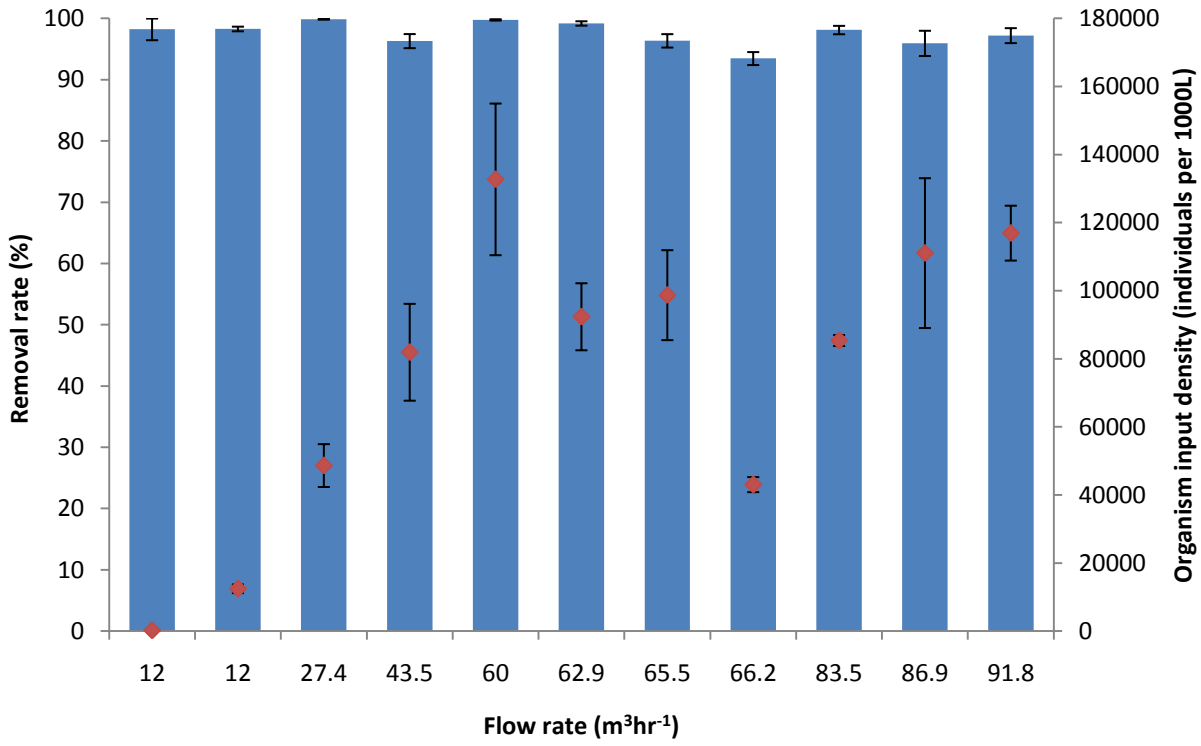

Figure 7.7. The mean percentage removal of organisms ≥50µm in each filter test. Bars show removal rate (%) of organisms ≥10<50µm, the red symbols show mean organism ≥50µm input density (individuals per ml) plotted on a secondary axis. All values are the mean of three replicates ± standard error.

7.3.4.2 Effect of flow rate and input density on organisms ≥10<50µm

The percentage removal of organisms' ≥10<50µm was determined (Figure 7.8). No significant difference in removal rate was found between all filter tests (Kruskal-Wallis; p = 0.094, H = 16.19, d.f. = 10). Samples showed high standard error, there was high variation between replicates and flow rate and input density did not affect filtration. Removal was higher in the 12m^3hr^{-1} test, and this could be due to the effect of build up on the filter screen improving the filters' effectiveness. When higher flow rates were applied the filter's self cleaning process was triggered, particles on the screen were removed and efficiency was reduced.

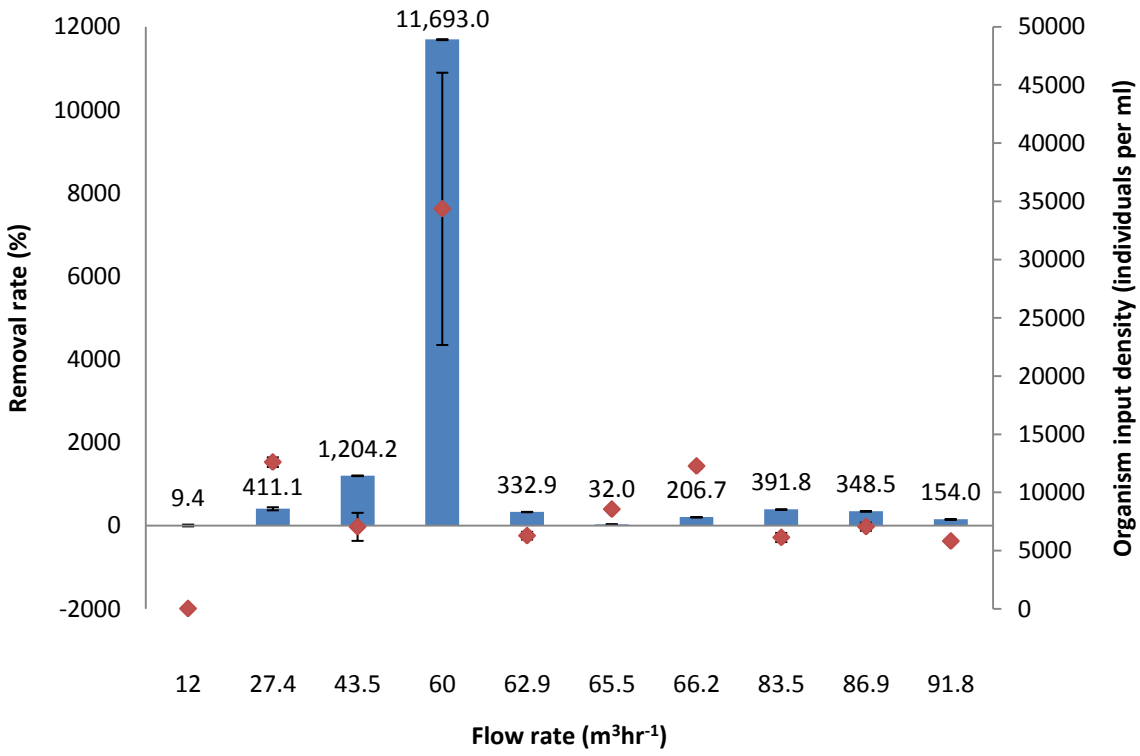

Figure 7.8. The mean percentage removal of organisms ≥10<50µm in each filter test. Bars show removal rate (%) of organisms ≥10<50µm (data labels show values), the red symbols show mean organism ≥10<50µm input density (individuals per ml) plotted on a secondary axis. All values are the mean of three replicates ± standard error.

7.4 Discussion

The filter tested in this study was proven to significantly reduce the number of organisms' ≥50µm present after filtration. Organisms ≥10<50µm were removed in tests at low flow rates in which the self-cleaning mechanism was not triggered. This removal was due to the build up of material on the filter screen which enhanced the filtration efficiency. However, the filter could not be relied upon to remove these organisms (≥10<50µm) and so a secondary treatment would be required to ensure effective disinfection.

The effect of flow rate on the removal rate achieved by the filter was assessed. The highest removal rate of organisms' ≥50µm was 99.9% at a flow rate of 27.4m^3hr^{-1}. The filter was able to maintain this high level of removal at higher flow rates; when trialled

at 91.8m^3hr^{-1}, the highest flow rate tested, the filter performed with 97.2% removal. As the flow rate increases it is possible that the removal efficiency could reduce. Observation by Parsons and Harkins (2002) when assessing a 40µm screen filter showed that 88.7% removal of particles >40µm was achieved at 340m^3hr^{-1}. Further testing would be required to determine the efficiency of this filter at these flow rates.

No significant reduction was observed in organisms ≥10<50µm. The highest mean removal rate observed was 45%, but high variability was observed between replicates and in 7 of the 11 tests no removal of organisms was observed. The results of these tests are similar to those stated in the literature. Waite et al. (2003) observed no reduction in chlorophyll α content after filtration by a 50µm screen filter. Wright et al. (2007b) also monitored phytoplankton reduction using chlorophyll α content and removal ranged from 7.3% to 15.8% with a mean of 10% by a 55µm disc filter. Veldhuis et al (2006) observed no difference in *Phaeocystis* sp. number after treatment with both a hydrocyclone and an automatic self-cleaning 50µm screen filter. This study did not observe any significant reduction in phytoplankton with the 40µm screen filter and thus concludes that for the treatment of phytoplankton filters with smaller pore sizes should be used, if practical, otherwise filtration must be followed by a secondary treatment which is known to be effective against phytoplankton.

Waite et al. (2003) assessed a 50µm screen filter and recorded significant reductions in zooplankton abundance after filtration, a 90% removal rate of gastropod and bivalve larvae and a 60-95% reduction in copepods. It was suggested that there was a lower removal rate of copepods as the larvae were larger in size and so more likely to become trapped by the filter (Waite et al. 2003). In comparison to studies performed on disc filters the screen filter in this study showed higher removal rate of organisms ≥50µm. A 50µm automatic backwash screen filter assessed by Parsons and Harkins (2002) removed 91.9% of particles above 50µm. Cangelosi et al. (2007) found a 89% removal of zooplankton by a 50µm screen filter.

Problems associated with clogging and backwash in both screen and disc filter systems have been observed. During this study a 99.8% removal rate was observed when the system was challenged with a flow rate of $60m^3hr^{-1}$ and with the greatest number of organisms flowing into the system. 132,750 *A.salina* individuals entered the system and only 273 individuals were present after filtration. Due to this high level of organism input to the system the backwash occurred frequently and thus in this test there was no added effect from clogging of the filter. This result demonstrates that the screen filter tested in this study remained efficient even during frequent backwashing. One advantage that has been reported for disc filters is an increase in the time between backwashing cycles, as this was significantly longer in the disc filter than in two screen filters tested (Parsons and Harkins 2002). In addition, the forward flow of water through the filters during backwash was faster through the disc filter than the screen filters: 80% and 67% respectively. Screen filters can continuously backwash after a period of filtering as the screen becomes clogged by fine material. This occurred during tests by Parsons and Harkins (2002) and the filter had to be stopped and steam cleaned before it could be used again. However, in contrast Riley et al. (2005) concluded that the backwash performance of a screen filtration system was superior to that of a disc filter. The authors observed the disc filter stop operating on three occasions due to clogging of the filter which could not be removed by the backwash system. One reason for the lack of removal of phytoplankton during filtration studies is that the size of mesh used by the filters is greater than that of the phytoplankton. To remove smaller organisms these filters rely on clogging by trapped particles to reduce the pore size of the filter enabling smaller organisms to become trapped aswell. Effective backwash systems clear the filter mesh by removing these trapped particles and so smaller organisms flow through the filter.

The size of filter pore also affects the flow rate of water through the system. While using a smaller filter can increase the amount of organisms removed, a compromise between removal and flow rate needs to be met, i.e. is the increased rate of removal by a smaller filter enough to require its use? Cangelosi et al. (2007) tested a 25µm screen filter and a 50µm screen filter. Higher removal of zooplankton was observed with the 25µm filter than the 50µm filter: 91% and 89% respectively. The same study

observed a >90% removal of phytoplankton by the 25µm filter, while phytoplankton removal by the 50µm filter varied and remained below 90%. As can be seen, the 25µm filter was more effective, but in terms of the zooplankton there was only 2% more removal with this filter than the 50µm filter. Filtration has a low removal effect on smaller phytoplankton and it is unable to remove viruses and bacteria from the water, and so for ballast water treatment it must always be used in combination with a secondary treatment option (Veldhuis et al. 2006). It is also true that the inclusion of filtration in a treatment system often enhances the effectiveness of the secondary treatment (Cangelosi et al. 2007) and so a system which uses a larger size filter, e.g. 40µm rather than 25µm when combined with UV or chemical for example, may still provide a treatment system capable of meeting the IMO levels, but which is also more practical in shipboard situations.

The application of filtration in ballast water treatment shows great potential and is a viable treatment option for onboard use (CEPA 2002). Filtration was recommended as the most promising ballast water treatment technology by the National Research Council (1996). Filtered organisms can be discharged back to the point of uptake (Gregg et al. 2009) or could be stored onboard and released into shore facilities depending on the regulations in place (National Research Council 1996). It is an environmentally safe option as no by-products are produced, and it is versatile in use in both freshwater and seawater environments (Cangelosi et al. 2007). Filtration, in ballast water treatment, must be used as water is being pumped on board to ensure that the organisms trapped in the filter are discharged back to their original habitat (Gregg et al. 2009) and are not transported to a new environment (Chase et al. 2009). The high flow rates and volumes of water to be filtered present a challenge to filtration, but technological advances in recent years mean that it is possible (National Research Council 1996). Even at its most effective removal by the filter in this study was not sufficient to reduce the number of organisms below the levels determined by the IMO, and so a secondary treatment step to follow the filter would be required.

7.5 Conclusions

The aim of this chapter was to determine the biological removal efficiency of filtration and this was completed. The highest removal rate of organisms ≥50µm was 99.9% at a flow rate of 27.4m^3hr^{-1}. This high efficiency was also maintained at the highest flow rate of 91.8m^3hr^{-1}, and 97.2% of organisms' ≥50µm were removed. At high organism input densities the self cleaning mechanism on the filter was triggered and the filter was continuously cleaned. This resulted in a lower removal rate of organisms ≥10<50µm as there was no build up of sediment on the screen to enhance removal efficiency. Flow rate was not affected by the cleaning mechanism and the system was run at <91.8m^3hr^{-1}. The filter would be recommended for further use, but a secondary disinfection treatment would be required to kill organisms ≥10<50µm.

Chapter 8

Uncertainties in the assessment of plankton viability

8.1 Introduction

During initial testing of our ballast water treatment (Chapters 5 - 7) it became clear that the viability stains being used had limitations to their application. Under certain conditions ambiguous staining colours were produced which could not be reliably interpreted. The literature contains many studies which have looked at the use of stains (Section 8.1.1), but there is no study which has systematically tested these stains and produced quantitative data to show their effectiveness in terms of staining success under different conditions. There are many observations and suggestions of conditions under which they should not be used and techniques which could improve their application. However, these exist as comments buried in the literature and are not quickly accessible and widely known.

For the biological assessment of any ballast water treatment system it is vital that the method used to assess viability is accurate. The reliability of stains has been questioned, with some researchers not feeling confident with the accuracy of the results obtained using stains, and thus opting for visual observation and 'poking' of organisms instead. This is suitable for the larger zooplanktonic organisms in which external or internal movement can be seen with the aid of a dissecting microscope. But, for smaller organisms, i.e. phytoplankton, where movement cannot always be seen this is not feasible. It now becomes necessary to investigate the limitations regarding the use of these stains and determine whether they can be confidently employed, or finally discarded and new techniques developed.

8.1.1 The history of staining

The earliest known work to involve stains was performed in the sixteenth century by Lassus who observed that animals feeding on a certain plant exhibited staining of their bones and this was used to study bone formation and growth (Conn and Cunningham 1932). In 1858 Gerlach published an extremely important study into the staining of plant tissue cells with carmine solutions. Research on vital stains i.e. those which stain living cells, far preceded that of mortal stains i.e. those which stain dead cells, but yet once mortal stains began to be developed they were used predominantly for many years (Conn and Cunningham 1932). In the early 1900s attention began to return to vital stains, focusing on basic dyes such as neutral red and methylene blue, but since then a large number of different biological stains have been discovered and techniques developed. In 1925 the Stain Commission put together all known research regarding staining techniques into the reference book 'Biological stains' by Harold Conn. The first edition contained information regarding 75 stains and became so important to biologists of many different disciplines that six further editions were written by Harold Conn, each time updating the previous edition with further techniques and stains. Since his death another three editions of the book have been published bringing all research up to date. The most recent version of 'Conn's Biological stains' is the 10th Edition, which was updated by Horobin and Kiernan and published in 2002.

Staining is used in a number of different applications. For example, they can be applied to increase the visibility of objects in both fluorescent and light microscopy, to colour certain cell structures such as proteins, DNA, etc, and to determine the viability of a cell (Kasten 2002). Early work carried out in the field of marine science looked at the effectiveness of stains as viability indicators for plankton. Studies, such as Dressel et al. (1972), Crippen and Perrier (1974), Reynolds et al. (1978) and Seepersad and Crippen (1978) assessed the use of both vital and mortal stains on marine and freshwater planktonic organisms. These studies, in addition to more recent work, will be described in Section 8.1.2 as the commonly used stains are discussed.

8.1.2 Commonly used stains for viability assessment

As already stated there are two types of stains; vital and mortal. Vital stains (e.g. neutral red and fluorescein diacetate) stain living cells, whilst mortal stains (e.g. Evans blue, trypan blue and SYTOX green) stain dead cells. Fluorescent and non fluorescent stains are available, and it has been suggested that tests which use fluorescent microscopy are more sensitive than those using light microscopy (Pouneva 1997).

8.1.2.1 Evans blue

Evans blue is a mortal stain which binds non specifically to proteins and is excluded by the membrane of living cells, but is able to enter and stain dead cells blue (Crippen and Perrier 1974; Horobin 2002b). Evans blue was originally used in histological applications but more recently has been employed as a protein stain and in fluorescence microscopy to mask autofluorescence (Horobin 2002a). Early work studying its efficacy as a mortal stain for plant cells was performed by Gaff and Okong'O-Ogola in the early 1970s, since then more research has looked at its use on phytoplankton.

The use of Evans blue on phytoplankton and plant cells has been found successful in many studies. These include the bean plant *Vicia faba* (Gaff and Okong'O-Ogola 1971), the flowering plant *Rhoeo discolour* (Gaff and Okong'O-Ogola 1971), six cultured algal species (Crippen and Perrier 1974), the single celled green alga *Chlamydomonas reinhardtii* (Crutchfield et al. 1999), the dinoflagellate *Symbiodinium kaguawutii* (Morera and Villanueva 2009), symbiotic dinoflagellates (Bird and Quinn 1986) and six of eight phytoplankton species tested by Reynolds et al (1978). In addition to giving clear distinction between live and dead cells authors have reported Evans blue to stain quickly and uniformly (Reynolds et al. 1978). It allows rapid and easy determination of viability (Taylor and West 1980) and does not leach from cells when observed over a 24 hour period (Crippen and Perrier 1974).

However, limitations have been observed to its use in some organisms: the diatoms *Biddulphia* sp., *Melosira* sp. and the dinoflagellate *Peridinium* sp. showed inconsistent staining (Crippen and Perrier 1974) and in the diatom *Nitzschia closterium* it stained only small regions near the chloroplasts (Reynolds et al. 1978). Cells have been observed to lose their staining if damaged during preparation of sections (Taylor and West 1980) and Li and Song (2007) found that it failed to differentiate between live and dead cells of *Microcystis aeruginosa* which had been killed by heat and $CuSO_4$.

8.1.2.2 Fluorescein diacetate

Fluorescein diacetate (FDA) freely enters cells with intact cell membranes and once inside is hydrolysed by non-specific esterases to produce free fluorescent fluorescein. This stains live cells green under blue light excitation (Jochem 1999; Buttino et al. 2004; Franklin and Berges 2004). The accumulation of FDA is a result of cell activity and therefore is an indicator of cell viability (Franklin and Berges 2004). Non-fluorescing cells are considered to be dead (Garvey et al. 2007).

Li and Song (2007) concluded that FDA staining was a reliable method of assessing viability of the phytoplankton *Thalassiosira* sp., *Dunaliella* sp., *Emiliania* sp. and *Chlorella* sp.. Jansen and Bathmann (2007) employed FDA to determine the viability of phytoplankton in copepod faecal pellets and concluded that FDA staining is a valid, simple and rapid method to determine viability. Jochem (1999) used FDA successfully to assess viability of the chlorophyte *Brachiomonas submarina*, the prymnesiophytes *Pavlova lutheri*, *Chrysochromulina hirta* and *Prymnesium parvum* and the diatoms *Bacteriastrum* sp. and a *Nitzschia*-like pennate diatom.

Problems have been reported by studies using FDA as a vital stain. Murphy and Cowles (1997) obtained highly variable results and concluded FDA was unreliable on the diatom *Thalassiosira weissflogii*. Other problems include the masking of FDA fluorescence by red autofluorescence, which has been reported by Garvey et al (2007) and Pouneva (1997). Taghi-Kilani (1996) found FDA to be ineffective on cysts which had been inactivated by chemical disinfectants, this was thought to be due to the

prevention of FDA diffusion through the cell by the lipid membrane and cyst wall. Variance in the strength of fluorescence produced by live cells is also a problem. Gilbert et al (1992) observed a variance in fluorescence produced by the green alga *Tetraselmis suecica*, fluorescence strength was high before the exponential phase and dropped during the rapid growth phase. It was suggested that the cells prioritised other metabolic pathways during the stage of rapid division and so the fluorescence differed. These factors could restrict the use of FDA.

8.1.2.3 Neutral red

Neutral red is commonly employed as a vital stain in many different applications, e.g. red blood cells, chick embryos and in marine sciences for plankton research. The first recorded use of neutral red was by Galleotti in 1894 where it was tested alongside a number of other stains on the blood of a salamander (Conn and Cunningham 1932). Much of the early work focused on its use for viability detection of blood cells, but it has since been employed to assess the mortality of whole organisms, such as copepods.

Neutral red stain enters cells in a molecular form. Once inside the cells the stain is converted into an ionic form which is unable to permeate the cell membrane (Levitt 1969). It therefore enters lysosomes, endocytotic vesicles and vacuoles where it remains unless the cell is subsequently killed as the cell membranes then become freely permeable and it can leach out into the surrounding medium (Levitt 1969). Neutral red is able to stain the cell wall of both live and dead cells, however, the stain is absorbed only by live organisms (Levitt 1969). Dead organisms have been observed to remain light pink-white, while live organisms are turned magenta-red by the stain (Dressel et al. 1972).

Neutral red has been tested on a range of zooplankton. It has been found to be effective on adult and larval stages of the marine copepod *Eurytemora hermani* polychaete larvae *Polydora ligni* and *Streblospio benedicti* (Crippen and Perrier 1974)

and the harpacticoid copepod *Scottolana canadensis* (Dressel et al. 1972). Positive results were also observed on an estuarine zooplankton population containing adults and larval stages of calanoid copepods, polychaete eggs and larvae, eggs of *Littorina littorea*, hydrozoan larvae, rotifers and chaetognaths (Crippen and Perrier 1974).

Dressel et al (1972) assessed neutral red on a range of zooplankton and found it was ineffective on two unidentified species of cyclopoid copepods, a gammarid amphipod, the cladoceran *Podon polyphemoides* and when used on the cladoceran *Bosmina* sp. it produced ambiguous results. Dressel et al (1972) reported inconsistent staining of copepod eggs. Sarvel et al (2006) found it ineffective on eggs of the trematode worm *Schistosoma mansoni* as it penetrated both the live and dead eggs. Crippen and Perrier (1974) observed cyprid and naupliar stages of *Balanus balanoides* and zoea of the crab *Cancer irroratus* to show varying results and only partial success.

A common problem encountered when using neutral red is leaching of the stain from cells as some organisms show lower retention times than others. The amount of leaching from cells was decreased by Dressel et al (1972) by experimenting with different staining times and concentrations to find the optimum for each type of organism. In order to increase the retention period of the stain during storage Dressel et al (1972) also discovered that storing samples to 4°C or freezing them increased retention by many days. Further improvements were made by Crippen and Perrier (1974) who observed that fixing samples with methylated ethanol, instead of formalin which is often used, could increase storage time to over a month when combined with cooling.

Acidification of samples has been found to improve neutral red staining techniques as it enhances the colour of the stain in organisms which were live prior to staining. This is because at <pH8 neutral red stain is red, but at >pH8 it is yellow. Acidification also increases the difference in colour between stained and unstained organisms, making assessment of samples quicker and easier. Dressel et al (1972) determined that sodium acetate should be applied to samples to acidify preserved samples and more recent techniques include the addition of glacial acetic acid to samples before assessment.

The application of neutral red for live/dead determination of phytoplankton has produced varied results. Reynolds et al (1978) used neutral red on eight species of algae and its application was successful in four of these species; *Nitzchia closterium*, *Navicula* sp., *Chaetoceros septentrionalis* and *Prorocentrum minimum* all stained successfully. Crippen and Perrier (1974) successfully used neutral red on the diatoms *Biddulphia* sp. and *Melosira* sp, and the dinoflagellate *Peridinium* sp.. However, neutral red has most often been found to be ineffective for use with phytoplankton. Toxic effects of the stain have been observed in *Skeletonema costatum* and *Olisthodiscus luteus*. Within the 30 minute staining period cells were killed, causing them to lyse and the stain to leach (Dressel et al. 1972; Crippen and Perrier 1974; Reynolds et al. 1978). Distortion and contraction of cell membranes has been observed during neutral red uptake and the diatom *Dunaliella tertiolecta* changed shape from oval to rounded and became immobilized after the addition of the stain (Reynolds et al. 1978).

Rinsing, preservation and acidification of phytoplankton samples stained with neutral red was not recommended by Crippen and Perrier (1974). Leaching of the stain from cells was observed during rinsing and preservation. Acidification caused a red tint to the cell walls which was strong enough to obscure the colour of the cell contents (Crippen and Perrier 1974). However, Reynolds et al (1978) assessed the effects of fixation on stained samples and observed no deleterious effects, and in one species it actually reduced leaching. Acidification was observed by Reynolds et al (1978) to enhance the colour of the stained organisms making it easier to determine live from dead organisms.

8.1.2.4 SYTOX green

SYTOX green is a fluorescent mortal stain, which enters cells with damaged plasma membranes causing the nuclei to become fluorescent and stained cells to appear green (Buttino et al. 2004). Cells stained by SYTOX green, i.e. those which have compromised membranes, are considered to be ‘dead’. Live cells have been observed

to show a weak, but detectable, fluorescent signal as the SYTOX green stain binds to their surface (Lebaron et al. 1998).

Franklin et al (2004) successfully used SYTOX green to assess the viability of the dinoflagellate *Symbiodinium* sp. and the stain clearly labelled the nucleus of cells with degraded plasma membranes. It has been successfully used on cysts of the dinoflagellates *Alexandrium catenella* (Binet and Stauber 2006; Gregg and Hallegraeff 2007) and *Gymnodinium catenatum* and *Protoceratium reticulatum* (Gregg and Hallegraeff 2007) and Veldhuis et al (1997; 2001; 2006) employed SYTOX green to determine phytoplankton viability.

Lebaron *et al* (1998) used SYTOX green on *Escherichia coli* and *Salmonella typhimurium,* however the results obtained suggest that SYTOX green should be used with caution and restricted to specific applications e.g. the analysis of antibiotic susceptibility of bacteria with undamaged nucleic acids. This restriction is necessary due to DNA degradation which can occurs after the membrane of a cell becomes compromised. These cells show a weaker fluorescence than that of cells which have compromised membranes but intact DNA and appear in the same region as the fluorescent signal from live cells. This led to an underestimation of the number of dead cells by Lebaron et al (1998).

8.1.2.5 Trypan blue

Trypan blue is a mortal stain similar in chemical structure to Evans blue. It is prevented from entering cells with intact cell membranes but is able to penetrate damaged membranes. It is rarely used in plankton studies and has been mainly employed on tissue from mammals, lower vertebrates and insects (Conn 1946). Trypan blue has been extensively used in human tissue viability testing and was the most widely used viability stain until the development of fluorescent viability probes (Altman et al. 1993). However, trypan blue has been observed to overestimate viability and it has been suggested that this could be due to quick assessment of samples after addition of the stain (within 3-5 minutes) which could enable dying cells to partially exclude the

stain and appear unstained. Its use has more recently been replaced by more effective fluorescent stains, e.g. FDA and Propodium iodide (Altman et al. 1993).

In its limited use on phytoplankton Miron et al (2003) successfully used Trypan blue for viability assessment in the marine diatom *Phaeodactylum tricornutum.* No studies have employed Trypan blue for zooplankton assessment and so this will be its first recorded use for viability detection in zooplankton.

8.1.2.6 Other commonly used fluorescent stains

A range of other fluorescent stains which can be used for viability detection are available and these are described in the Molecular Probes handbook of fluorescent dyes and probes (Haugland 1992). They vary from stains which detect live cell functions, e.g. esterase activity, respiratory activity, and ion pump activity across cell membranes, to stains which detect dead cells by entering only cells which have damaged of compromised membranes (Haugland 1992). Of the fluorochromes available, some of the more commonly used stains are Propodium Iodide, Calcein AM and the FDA derivatives.

Propodium iodide (PI) is a nucleic acid stain which is unable to cross intact cell membranes, but which can pass through damaged or broken cell membranes (Taghi-Kilani et al. 1996; Chitarra et al. 2006). Stained cells appear red and are assumed to be non-viable (Komen et al. 2008). PI can be used as a counter stain, for example, with FDA or Calcein AM (Taghi-Kilani et al. 1996; Chitarra et al. 2006). Calcein AM is a fluorescent stain which enters viable cells and is hydrolyzed by intracellular esterases into the fluorescent anion calcein which produces a bright green (530 nm) emission signal (Bratosin et al. 2005). It has better cell retention in viable cells than FDA and CFDA as it is more negatively charged (Chitarra et al. 2006).

Chitarra et al (2006) employed PI, both on its own and in combination with Calcein AM, to assess the viability of the plant bacterium *Clavibacter michiganensis*. When used alone it was found to be unreliable as it was able to stain live cells, but when used in

combination with the live cell stain Calcein AM accurate counts were obtained. One limitation to the use of PI is that it is not applicable to phytoplankton as its emission spectrum overlaps that of chlorophyll α (Veldhuis et al. 2001).

8.1.3 Alternatives to staining

8.1.3.1 Visual observations

Visual observations of organisms are favoured by some researchers over stains to determine mortality as the results can be more easily 'trusted', as shown in a study by Perrins et al (2006) which used visual observations to determine the amount of mortality caused to mesozooplankton by ozone treatment. However, the drawback to this process is that samples cannot be stored for any period of time after treatment and in large scale tests many biologists will be required to process the results quickly. Lahdes (1995) assessed the mortality of two copepod species *Calanoides acutus* and *Calanus propinquus* by visually assessing individuals for movement. The author found this to be problematic when assessing *C.acutus* individuals as they often remained immobile for long periods and so were classed as dead. However, on occasion an individual which had been assessed as dead began moving rapidly, and so counting had to be restarted.

Visual observations have been used to assess the mortality of cultured mussels, oysters, clams, brine shrimp and a natural zooplankton population after treatment with a UV system. The organisms were poked and viability was assessed by their response to the physical stimuli, i.e. swimming, a heartbeat or visible evidence of internal (Sutherland et al. 2003). The assessment of the bivalve larvae was more difficult and so processing of these samples had to be delayed and was not completed immediately after the treatment when the main processing was performed (Sutherland et al. 2003). Veldhuis et al (2006) visually assessed the viability of zooplankton after being pumped through a ballast water treatment system. Organisms were considered to be dead if there was visible damaged e.g. body parts were absent,

discolouration due to loss of body pigments or if they did not respond to physical stimuli (Veldhuis et al. 2006).

There are limitations to the organisms which can be visually observed, for example, larger organisms such as copepods can be assessed by watching for external or internal movement. This movement can be induced by gentle 'probing' by the assessor, but smaller organisms, such as diatoms, do not always show any visible sign of movement and yet this is not confirmation that they are dead. Therefore for these organisms movement cannot be used as a sign of viability and alternate methods must be employed.

8.1.3.2 Chlorophyll α measurement

Due to the previous lack of a suitable measure of phytoplankton viability chlorophyll α measurements have been taken in many studies to monitor the change in biomass of phytoplankton in response to ballast water treatments (McCollin et al. 2007a; Wright et al. 2007a; Wright et al. 2007b; Quilez-Badia et al. 2008). Waite et al (2003) used chlorophyll α levels to monitor the biomass of viable phytoplankton when assessing the efficacy of a ballast water treatment system. In addition phaeophytin was measured as it is composed of the degradation products from chlorophyll breakdown (Waite et al. 2003). No changes in chlorophyll α levels were observed after UV exposure and it was suggested that this could be because there was insufficient time left between obtaining samples and processing to allow the chlorophyll α to degrade as the breakdown process can take from hours to days (Waite et al. 2003; Gavand et al. 2007). This could mean that over estimates of viability are made if measurements are taken immediately after treatment. It has been observed that long term incubation studies should also be completed if chlorophyll α is to be used as the measure of phytoplankton viability (Gavand et al. 2007).

8.1.3.3 Post-treatment germination

Research looking at the effectiveness of chemical ballast water treatments on dinoflagellates cysts has used germination experiments in addition to vital stains to determine viability. Binet and Stauber (2006) used this method successfully on cysts of the dinoflagellate *A.catenella* and Gregg and Hallegraeff (2007) on cysts of the dinoflagellates *G. catenatum*, *P.reticulatum* and *A.catenella*. This method of assessment can give accurate results but data cannot be obtained for many weeks due to the time taken for the cysts to germinate. This is a drawback to this method and thus it could not be employed in situations where decisions have to be made quickly, e.g. dockside assessment of a ships ballast water discharge.

8.1.3.4 The cell digestion method

The cell digestion method tests the permeability of a cell membrane and thus can determine viability in natural plankton communities without the requirement of a stain (Agusti and Sanchez 2002). It exposes the cells to the enzymes DNAse and Trypsin and those cells with damaged membranes are unable to prevent the enzymes from entering. The cell's DNA is fragmented and hydrolysed by the DNAse, and the trypsin hydrolyses the phospholipid cell membrane. These processes result in the loss of fluorescence, both autofluorescence and that produced by stains, and the cells are not visible by microscopic examination. This method effectively removes the dead cells from the population leaving only cells with intact membranes, which are therefore assumed viable, to be counted (Agusti and Sanchez 2002).

Agusti and Sanchez (2002) used this method successfully on nine species of cultured phytoplankton and a natural coastal population from the North West Mediterranean Sea. Six of the cultured phytoplankton species were also assessed using FDA and the percentage viability obtained by both methods did not differ significantly. The authors did recommend further testing on a broader range of organisms to ensure this method can be widely applied, but concluded that this method is superior to staining methods as it does not produce ambiguous results; the live cells are present and the dead cells

are removed from samples (Agusti and Sanchez 2002). This method was subsequently successfully used on the phytoplankton *Prochlorococcus* sp. and *Synechococcus* sp. (Llabres and Agusti 2006; Agusti and Llabres 2007) and has also been successfully applied to freshwater phytoplankton populations (Agusti et al. 2006).

8.1.4 Problems associated with assessing the viability of ballast water treated samples

The various techniques used by researchers to determine live from dead organisms have been described above. These methods are not limited to ballast water research and are used in a wide variety of applications. However, it is evident that none of these methods work in all situations. All stains have proven ineffective on some organisms and before being used, each stain should be tested to determine the optimum concentration and time required for the situation in which it is being applied. The main problems observed with the non-fluorescing stains are inconsistent staining and cell retention. Neutral red has been observed to produce staining colours which are difficult to interpret. The author has observed orange stained organisms and it was unclear whether or not these organisms were showing positive staining. Unstained organisms have been observed to show movement (Gollasch pers. comm.) and due to these inconsistencies in staining behaviour other researchers have carried out morphological examinations of all organisms within samples (Quilez-Badia pers. comm.). This increases the processing time and can lead to doubt in the results obtained. A major issue when using fluorescent stains is ensuring there is no confusion between the staining colour and autofluorescence. Masking of the staining colour has been observed and fluorescent mortal stains often produce red fluorescence, for example propodium iodide, and this can be confused with the natural red autofluorescence produced by phytoplankton (Agusti and Sanchez 2002).

Measuring chlorophyll α levels is quicker than performing cell counts but it does not provide data of the number of viable cells present in samples, which is the information required by the IMO. There is also the potential to overestimate viability if insufficient

time is left between obtaining samples and measuring chlorophyll α to allow it to degrade (Waite et al. 2003; Gavand et al. 2007). In practice this method is not ideal. When used by Quilez-Badia et al (2008) on shipboard samples much of the data could not be used since few samples contained any detectable chlorophyll α.

Germination experiments using phytoplankton cysts gives accurate viability data but these experiments can take a minimum of four weeks to complete, and so rapid results cannot be obtained (Binet and Stauber 2006). Viable and non viable cysts cannot be distinguished by size, natural fluorescence or internal cell structure and so research using cysts has often relied on germination experiments to obtain numbers of viable organisms. An alternative method using SYTOX green was trialed by Binet and Stauber (2006) on cysts of the dinoflagellate *A.catenella* and compared with a germination experiment. The results of both tests gave significantly similar results and so they concluded that SYTOX green gave rapid and accurate results. The staining method was able to assess a larger number of cysts than were manually counted in the germination experiments, increasing accuracy (Binet and Stauber, 2006). Using cysts as test organisms is important as they are more robust than 'active' phytoplankton stages and a system which is able to kill cysts is likely to be effective on a wide range of organisms (Gregg and Hallegraeff 2007).

For the purpose of testing a ballast water treatment system it is only necessary for the viability assessment method used to work on the test species chosen. *A.salina* and *T.suecica* are commonly used test species in ballast water testing and therefore were identified for this study. It is necessary to determine any limitations to the use of viability stains, and when these restrictions are known it is then possible to employ the most appropriate assessment method for the intended use.

8.1.5 Aim of this study

The aim of this chapter was to identify the staining success of five viability stains on test species and natural plankton populations (live and dead organisms) to identify effective staining techniques. The five stains tested in this study were Evans blue, FDA,

neutral red, SYTOX green and trypan blue. Sections 8.1.2.1-5 described the previous use of each stain, and from this it is apparent that there has been no recorded use of Evans blue, FDA, SYTOX green and trypan blue with zooplankton. This study investigated the staining success of the five viability stains with the test species *Artemia salina* and subsequently a wild caught multi species zooplankton assemblage (hereafter referred to as a 'natural population'). All of the stains have been tested previously with phytoplankton and it is clear that in the right conditions and with the right organism they can be used successfully. This study determined the optimum staining conditions with the test organism *Tetraselmis suecica* and subsequently a 'natural population'.

8.2 Methodology

The viability stains Evans blue, Fluorescein diacetate, Neutral red, SYTOX green and Trypan blue were assessed on two test organisms: the single celled green alga *Tetraselmis suecica* and the brine shrimp *Artemia salina*. During the literature search on each stain the previously used staining conditions were collated. From this a range of staining concentrations and times was determined for each stain for this study. After initial tests were performed the optimum staining conditions were determined for each stain on each test organism and those which showed highest staining success were applied to 'natural populations'. All data are reported as percentage staining success i.e. the percentage of test organisms showing the expected response to the stain applied.

8.2.1 Artemia salina

A.salina is commonly known as the brine shrimp. Cysts were obtained from Brine Shrimp Direct, stored at -20°C before use and hatched in the laboratory 24 hours prior to experimentation. To hatch the cysts H_2Ocean aquarium salt was added to tap water to obtain a salinity of 25ppu and a pH >8. 1g of cysts was added per litre of water and

this was placed in an incubator at 28°C with constant light and aeration. Before testing the A.salina were filtered using a 63µm filter and rinsed into GF/F fresh filtered seawater. All A.salina samples were assessed using a Leica microscope at 125x magnification.

Heat killed and chlorine treated dead controls were used in experiments. Heat killed organisms were obtained by heating rapidly to 80°C for 30 minutes. 1ml sodium hypochlorite was added per 5ml sample to obtain chlorine killed individuals. Samples killed with chlorine were rinsed with GF/F filtered fresh seawater before use. In all tests control, heat killed and chlorine killed organisms were assessed.

8.2.1.1. Evans blue

Evans blue powder was obtained from Applichem (Catalogue number – A4388) and stored at room temperature throughout use. Five replicates for each of three staining concentrations were tested: 1:1000, 1:25000 and 1:50000. In each replicate 100 individuals were counted after 30 minutes staining time (stored in the light) and the number of stained, partially stained and unstained individuals was recorded.

8.2.1.2. Fluorescein diacetate (FDA)

FDA powder was obtained from Sigma-Aldrich (Catalogue number – F7378) and stored at -20°C prior to use. A stock solution of FDA was prepared by dissolving 5mg FDA in 1ml dimethylsulfoxide (DMSO). This was stored at 4°C and thawed only for preparation of working solutions. The stock solution was diluted 100 fold in distilled water as required for the working stock, this was stored on ice for up to 3 hours (Jochem 1999). Two concentrations of FDA were assessed: 1:30 as Jochem (1999) and 1:40 as Selvin (1989). After the stain was added to samples they were stored in the dark until assessment. Samples were assessed after 5 and 15 minutes staining time. Five replicates of each treatment (concentration and staining time) were completed. All samples were examined using a Brunel epi-fluorescent microscope with blue light

excitation (420-485nm) at 20-200x magnification. Organisms were assessed using micro-well plates and the number of stained and unstained individuals was recorded.

During preliminary tests it became apparent that the age of the nauplii affected staining and so a follow up experiment was performed to determine the extent to which this was valid. Nauplii which had been hatching for 15-16 hours and 25-26 hours were stained at 1:30 concentration for 15 minutes and then the number of stained organisms was recorded. Five replicate for each condition were completed.

8.2.1.3. Neutral red

Neutral red powder was obtained from BDH (Catalogue number – 340564A) and stored at room temperature throughout use. Five replicates of three concentrations of neutral red were tested: 1:15000, 1:33333 and 1:80000. 100 individuals were counted after each of 30 minutes and 60 minutes staining time (stored in the light) and the number of stained and unstained individuals was recorded. In this test positive staining was only recorded if seen clearly in the gut. Assessment of these samples showed that neutral red stain was not taken up by dead organisms. To further optimise the staining efficiency five replicates of each of two concentrations (1:24,000 and 1:56,000) were tested solely on live culture species.

In the first series of experiments the most effective staining conditions were observed to be 1:33333 for 60 minutes. The effects of preservation and storage in buffered formalin, acidification using glacial acetic acid, storage temperature and duration of storage were assessed. After staining (1:33,333 concentration for 60 minutes) samples were preserved by adding 0.2µl NaAc and 5ml 10% buffered formalin per 5ml sample. The effects of storing the samples at different temperatures were investigated. Samples were stored at 4°C and 19°C (room temperature). To assess the effect of acidifying samples using glacial acetic acid 'Control' and 'Acidified' samples were assessed. A drop of glacial acetic acid was added to 'Acidified' samples immediately prior to observation. All samples were kept in the dark and assessed on days 1, 7 and

14 after preparation. Five replicates were prepared for each condition (Temperature: 4°C and 19°C, acidification: acidified vs. non acidified, and storage duration: 1, 7 and 14 days).

8.2.1.4 SYTOX green

The SYTOX green primary stock was obtained from Invitrogen (Catalogue number – S7020) as 5mM in DMSO and stored at ≤ -20°C prior to use. A working stock of 100µM was made by adding 1µl of primary stock to 50µl distilled water and was stored on ice for the duration of use. This working stock was added to obtain the final concentration 5µM. Five replicates of 100 cells were counted after each of 5 and 15 minutes staining time (stored in the dark) and the number of stained and unstained individuals was recorded. All samples were assessed using a Brunel epi-fluorescent microscope with blue light excitation (420-485nm) at 20-200x magnification.

8.2.1.5 Trypan blue

Trypan blue powder was obtained from Sigma-Aldrich (Catalogue number – 302643) and stored at room temperature. A primary stock solution of 0.4% Trypan blue was made using Trypan blue powder and distilled water and was stored at room temperature. This primary stock was added to 5ml samples to obtain the final concentrations 0.1% and 0.2%. For each concentration five replicates of 100 individuals were counted after 15 and 30 minutes staining time (stored in the light) and the number of stained and unstained individuals was recorded.

8.2.2 Tetraselmis suecica

Tetraselmis suecica is a single celled green alga commonly used as a test species in ballast water treatment research. Cells were grown in F2 enriched seawater and experiments were performed during the exponential growth phase. Heat and chlorine killed cells were obtained in the same way for *T.suecica* as for *A.salina.* All *T.suecica*

samples were assessed using an Olympus CKX31 inverted microscope at 400x magnification. In all tests control, heat killed and chlorine killed samples were assessed.

8.2.2.1. Evans blue

Three staining concentrations were tested: 1:1000, 1:25000 and 1:50000. For each concentration five replicates of 100 cells were counted after 30 minutes and the number of stained, partially stained and unstained individuals was recorded.

8.2.2.2. Fluorescein diacetate (FDA)

FDA was prepared as described in Section 8.2.1.2. Two concentrations of FDA were assessed: 1:30 and 1:40. After the stain was added to samples they were kept in the dark until assessment. Control, heat killed and chlorine killed cells were stained at 1:30 to determine the effect of the stain on the dead cells. Only control samples were assessed at 1:40 concentration as no positive staining was observed in heat killed and chlorine killed cells at the higher staining concentration of 1:30.

Samples were examined after 5 and 15 minutes staining time. All samples were assessed using a Brunel epi-fluorescent microscope with blue light excitation (420-485nm) at 400x magnification. For each concentration five replicates of 100 cells were counted after 5 and 15 minutes and the number of stained and unstained individuals was recorded.

8.2.2.3. Neutral red

Three staining concentrations were tested: 1:15000, 1:33333 and 1:80000. Preliminary tests were performed to look at the uptake of neutral red by the *T.*suecica and it was observed that 20 minutes was sufficient for cells to take up the stain. For each

concentration five replicates of 100 cells were counted after 20 minutes and the number of stained, partially stained and unstained individuals was recorded.

8.2.2.4 SYTOX green

A working stock was prepared as described in Section 8.2.1.4. Final staining concentrations of 0.2, 1, 3, 4 and 5µM were assessed. To determine the optimum staining concentration only heat killed cells were tested at all 5 concentrations. Chlorine killed and live cells were used in addition to heat killed cells for final concentration 5µM tests. For each concentration five replicates of 100 cells were counted after 5 and 15 minutes staining time and the number of stained and unstained individuals was recorded. All samples were assessed using a Brunel epi-fluorescent microscope with blue light excitation (420-485nm) at 400x magnification.

8.2.2.5 Trypan blue

A primary stock solution of 0.4% Trypan blue was prepared as described in Section 8.2.1.5. This primary stock was added to 1ml samples to obtain the final concentrations 0.1% and 0.2%. For each concentration 100 cells were counted after 15 and 30 minutes staining time and the number of stained and unstained individuals was recorded. Five replicates of each condition were completed.

8.2.3 'Natural plankton population' testing

After preliminary experiments the optimum conditions found for each of the five stains on the test organisms were then applied to 'natural populations' of zooplankton and phytoplankton (Table 8.1). The organisms used were collected from the North Sea on the day of or the day prior to experimentation. Three replicates of each control, heat killed and chlorine killed were performed. The number of stained and unstained organisms was recorded and the success of the stain was calculated.

Table 8.1. Staining conditions for natural population tests.

	Stain	Concentration	Staining time (mins)
Phytoplankton	Evans Blue	1:1000	60
	Fluorescein diacetate	1:30	15
	Neutral red	1:33,000	20
	SYTOX green	5µM	15
	Trypan blue	0.1%	30
Zooplankton	Evans Blue	1:1,000	60
	Fluorescein diacetate	1:30	15
	Neutral red	1:24,000	60
	SYTOX green	5µM	15
	Trypan blue	0.2%	30

8.2.4 Statistical analysis

All data were checked for normality using the Anderson-Darling Normality test and equal variance using Levene's Test. If data were non-normal common transformation methods were applied. If unsuccessful in tests were three or four Factor analysis was required ANOVA was proceeded with as it is deemed robust enough to cope with such aberrations from ANOVA assumptions (Underwood, 1997). This enabled post hoc tests to be performed. Kruskal Wallis tests were used where appropriate. Where post hoc tests were applied the significant interactions at the highest levels were first considered, if these were insignificant lower level interactions were reported.

8.2.4.1. Zooplankton data

To determine the effect of Evans blue staining concentration on staining success for each Treatment (control, heat killed or control, chlorine killed) and at each Concentration (1:1,000, 1:25,000, 1:50,000) data were assessed using a 2-way ANOVA. For FDA data a 3-way ANOVA was applied to assess factors Concentration (1:30, 1:40),

Time (5, 15 minutes) for each Treatment (control, heat killed or control, chlorine killed). To assess factors Treatment (Control, Heat killed) and Age (15, 25 hours) on FDA staining success a Kruskal Wallis test was applied. A 2-way ANOVA was applied to factors Concentration (1:15,000, 1:24,000, 1:33,333, 1:56,000, 1:80,000) and Time (30, 60 minutes) to determine effective neutral red staining. To assess neutral red Concentration (1:15,000, 1:33,333, 1:80,000), Time (30, 60 minutes) and Treatment (Control, Heat killed, Chlorine killed) a 3-way ANOVA was used. Preservation of neutral red was assessed using a 4-way ANOVA on the factors Time (1, 7, 14 days), Temperature (4, 19°C), Acidification (Acidified, Non-acidified) and Treatment (Control, Heat killed, Chlorine killed). SYTOX green data were assessed using a Kruskal Wallis test to look at the effect of staining Time (5, 15 minutes) on success in different Treatments (Control, Heat killed, Chlorine killed). The effect of the factors Trypan blue Concentration (0.1, 0.2%), exposure Time (5, 15 minutes) and Treatment (Control, Heat killed, Chlorine killed) were analysed using a 3-way ANOVA. Post hoc Tukey tests were applied to all ANOVA tests where required.

8.2.4.2 Phytoplankton data

To determine the effect of Evans blue staining concentration on staining success for each Treatment (control, heat killed or control, chlorine killed) and at each Concentration (1:1,000, 1:25,000, 1:50,000) data were assessed using a 2-way ANOVA. FDA data showed only 100% success and so statistical analysis was not performed. Neutral red data were assessed using a 2-way ANOVA to determine the effect of factors Concentration (1:15,000, 1:33,333, 1:80,000) and Treatment (Control, Heat killed, Chlorine killed). Preservation of neutral red was assessed using a 4-way ANOVA on the factors Time (1, 7, 14 days), Temperature (4, 19°C), Acidification (Acidified, Non-acidified) and Treatment (Control, Heat killed, Chlorine killed). The effect of factors Concentration (0.2, 1, 3, 4, 5µM) and exposure Time (5, 15 minutes) on SYTOX green staining success in live *T.suecica* was examined using a 2-way ANOVA. The staining success at a concentration of SYTOX green 5µM was assessed using a 2-way ANOVA to look at the effect of staining Time (5, 15 minutes) and Treatment (Control, Heat killed, Chlorine killed) on success. The effect of the factors Trypan blue Concentration (0.1,

0.2%), exposure Time (5, 15 minutes) and Treatment (Control, Heat killed, Chlorine killed) were analysed using a 3-way ANOVA. Post hoc Tukey tests were applied to all ANOVA tests where required.

8.3 Results

8.3.1 Artemia salina

8.3.1.1 Evans blue

Staining of *A.salina* with Evans blue showed a mix of stained, unstained and partially stained organisms. Partial staining ranged from the staining of a single appendage, small areas of the exoskeleton to the majority of the organism. These partially stained organisms were grouped with fully stained organisms when determining the success of the stain.

Three concentrations of Evans blue stain were tested with live and heat killed *A.salina* and the most effective concentration observed was 1:1,000 (Figure 8.1). Lower concentrations were less effective on heat killed organisms and live organisms remained unstained. The data showed a significant interaction in staining success between the factors Treatment and Concentration (2-way ANOVA, $p = 0.003$, $F = 7.63$, d.f. = 2). Tukey multiple comparisons showed that this was due to differences between Control and heat killed *A.salina* within certain concentrations.

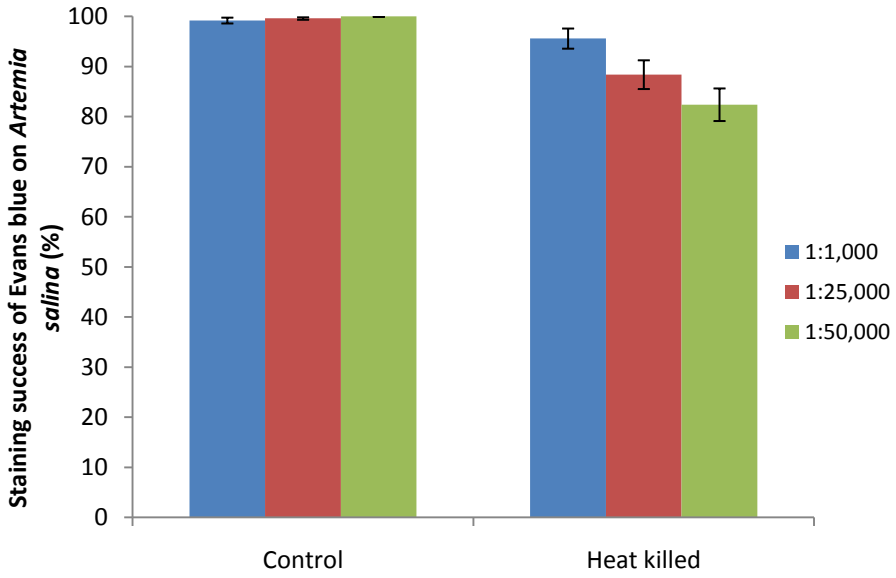

Figure 8.1. Staining success (%) of Evans blue at 3 concentrations in live and heat killed *Artemia salina*. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

A significant interaction was present between the factors Treatment and Concentration (2-way ANOVA, p = <0.001, F = 28.91, d.f. = 2). Tukey multiple comparisons showed that this was due to differences between Control and chlorine killed *A.salina* at all concentrations. The use of Evans blue with chlorine killed *A.salina* proved ineffective with its highest success 15.2% at a concentration of 1:1,000, and 0% success at 1:25,000 and 1:50,000 (Figure 8.2).

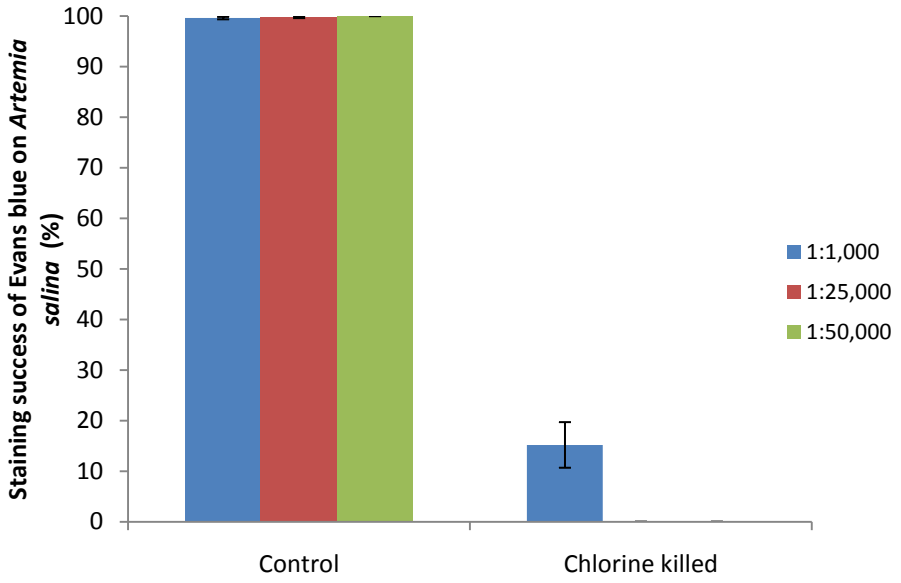

Figure 8.2. Staining success (%) of Evans blue at 3 concentrations in live and chlorine killed *Artemia salina*. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

Overall, Evans blue showed good potential for use on live and heat killed *A.salina,* but not for chlorine treated individuals. The amount of staining did vary between organisms and so for a high success partially stained organisms must be counted as positively stained.

8.3.1.2 Fluorescein diacetate (FDA)

Both of the staining concentrations showed high staining success (Figure 8.3 and Figure 8.4). The stain was clearly visible in the gut after 15 minutes and remained so while assessment of the sample took place. Dead organisms, both heat killed and chlorine killed, did not show staining in the gut, but some did show slight tissue staining and so positive staining was only recorded if the gut showed bright fluorescence.

No positive staining was observed in either heat or chlorine killed organisms. In the control and heat killed organisms a significant interaction was present between the factors Time and Treatment (3-way ANOVA; $p = <0.001$, $F = 27.80$, d.f. = 1) and 15

minutes exposure increased staining success. Tukey multiple comparisons showed that this was due to differences between Control and heat killed *A.salina* at both concentrations. For the control and chlorine killed data there was a significant interaction between the factors Time and Concentration (3-way ANOVA; p = 0.006, F = 8.81, d.f. = 1) and 15 minutes exposure time again showed higher staining success. Tukey multiple comparisons showed that this was due to differences between Time periods at 1:40 concentration.

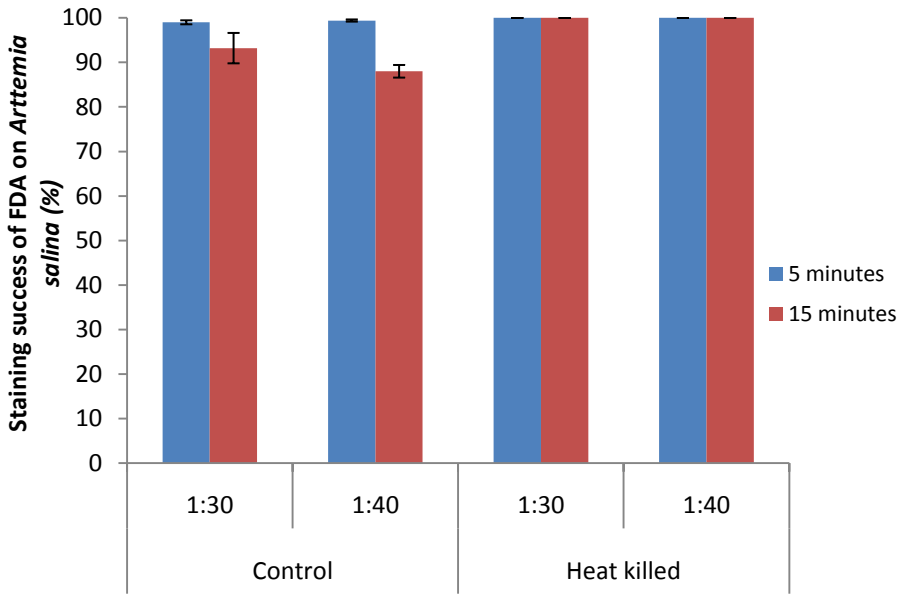

Figure 8.3. Staining success (%) of two concentrations of Fluorescein diacetate (FDA) on control and heat killed *Artemia salina* at 5 and 15 minutes exposure. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

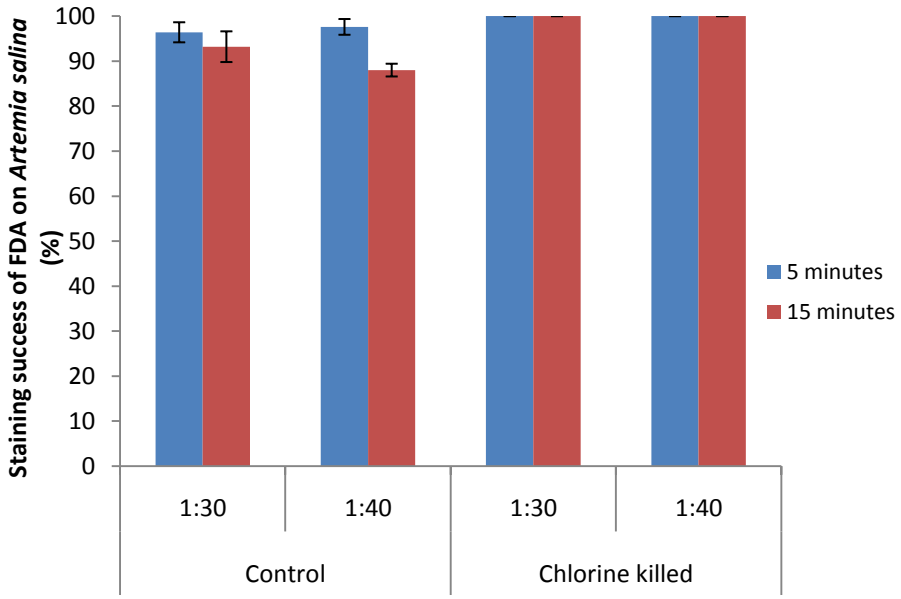

Figure 8.4. Staining success (%) of 2 concentrations of Fluorescein diacetate (FDA) on control and chlorine killed *Artemia salina* at 5 and 15 minutes. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

During the preliminary tests it was observed that some live organisms within the control samples were not showing positive staining but showed clear external movement. This was determined to be related to the development stage of the individuals and their morphology, i.e. the presence of a formed gut. Two different stages of organisms were observed in the samples (Figure 8.5 and Figure 8.6).

Figure 8.5. Early stage nauplii which do not yet have a fully formed gut.

Figure 8.6. Later stage nauplii with clearly visible gut.

Tests which looked at the staining success of FDA in these organisms showed a significant difference in staining success between the early and later stage nauplii (Kruskal Wallis; p = 0.012, H = 6.33, d.f. = 1), with 100% success in the older nauplii and 0% success in the younger nauplii, as shown in Figure 8.7.

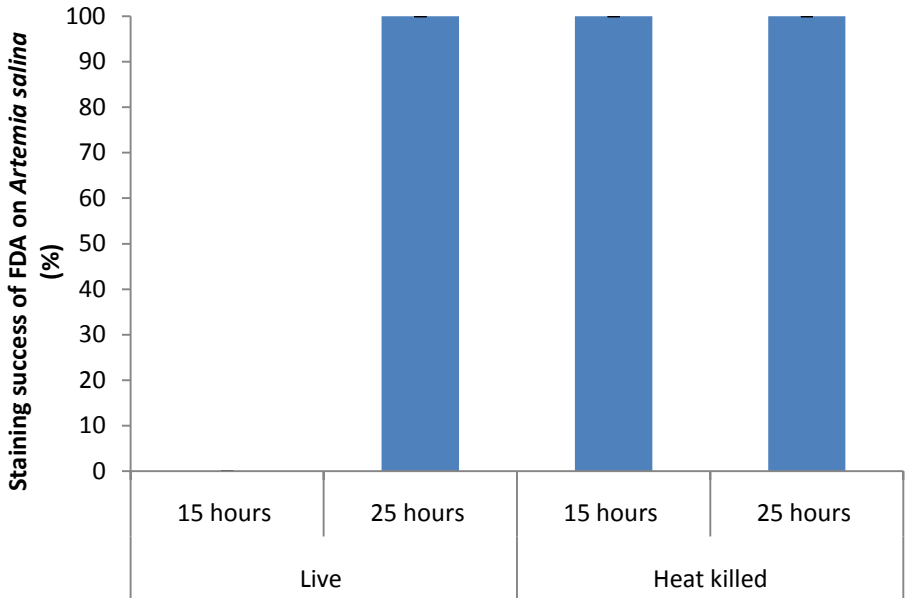

Figure 8.7. Staining success (%) of Fluorescein diacetate (FDA) in 15 hour old and 25 hour old *Artemia salina* individuals. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

8.3.1.3. Neutral red

The neutral red stain was taken up by live *A.salina* individuals only in their gut and was not apparent in any other part of the organism. The concentration of neutral red had a significant effect on the overall effectiveness of the stain (2-way ANOVA p = <0.001, F =

377.51, d.f. = 4), whereas staining time did not (2-way ANOVA p = 0.574, F = 0.32, d.f. 1). A post hoc Tukey Test showed differences between all staining concentrations except 1:24,000 and 1:33,333. These concentrations gave the highest staining efficiency, however this was still below 80% effective. The staining success of neutral red on *A.salina* is shown in Figure 8.8.

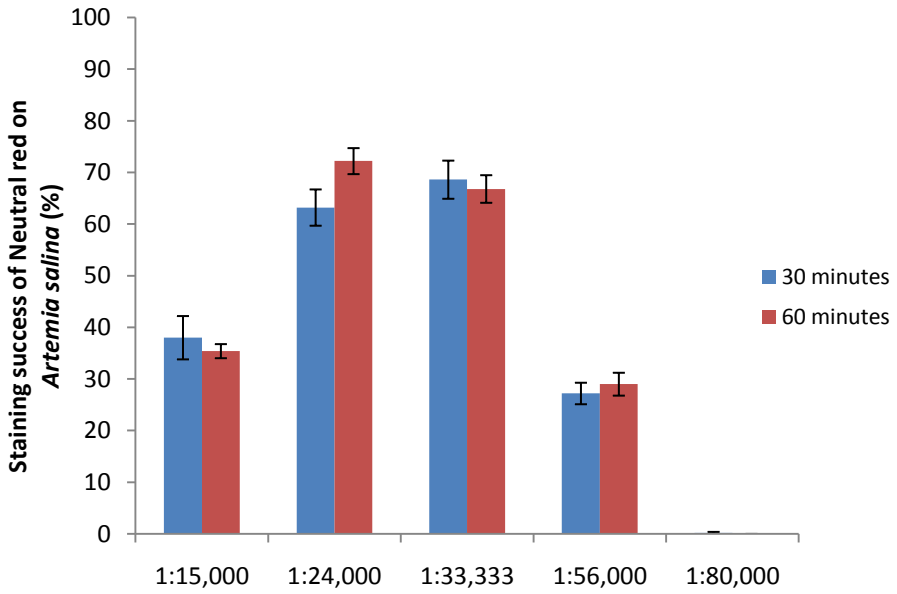

Figure 8.8. The staining success (%) of neutral red with live *Artemia salina* individuals at five staining concentrations and at two exposure times. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

Neutral red was ≥99.8% effective on heat and chlorine killed organisms at all concentrations tested (Figure 8.9). Statistical analysis showed there was a significant 2 way interaction between the factors Concentration and Treatment (3-way ANOVA; p = <0.001, F = 584.34, d.f. = 4). Tukey multiple comparisons showed that this was due to differences between Treatments within certain concentrations.

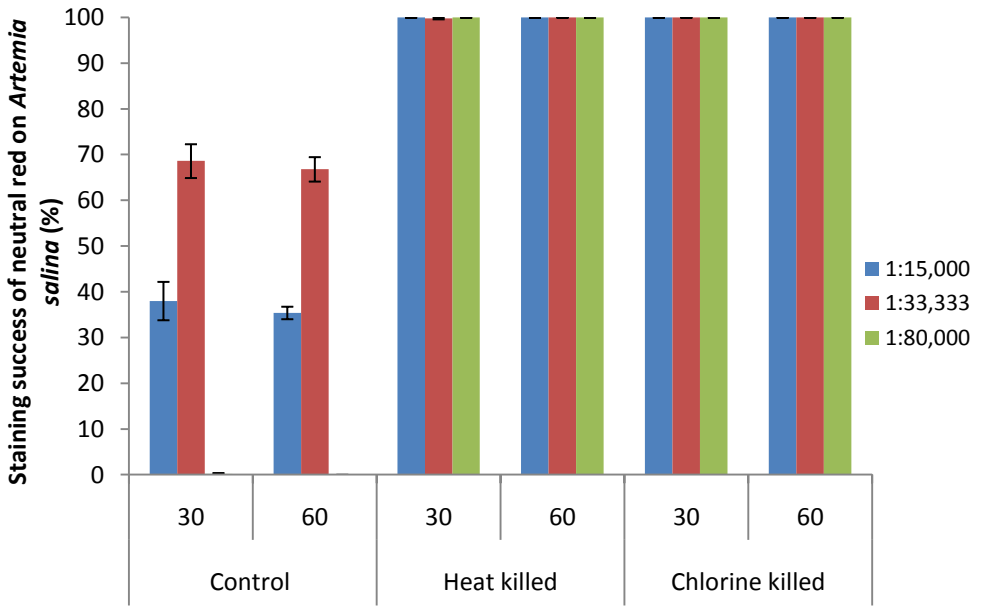

Figure 8.9. The staining success (%) of neutral red at three concentrations with control, heat killed and chlorine killed *Artemia salina*. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

The effect of fixation, preservation and acidification on neutral red staining success was assessed over a two week period (Figure 8.10-12). The staining effectiveness in the preserved control samples increased from the preliminary tests which assessed the live organisms immediately after the 60 minute staining period. However, the heat killed organisms began to take up the neutral red stain in their tissue after being preserved, reducing the effectiveness of neutral red on these organisms as the gut became obscured and accurate viability determination could not be made. This uptake of the stain by heat killed organisms increased over the two week period. The effect of preservation for two weeks at two temperatures was assessed. A significant three way interaction was observed between the factors Treatment, Day and Temperature (4-way ANOVA; p=<0.001, F=45.59, d.f. = 4). Tukey multiple comparisons showed that this was due to differences between Day and Temperature within Treatments. A significant interaction was present between the factors Sample, Day and Acidification (4-way ANOVA; p = 0.022, F = 2.96, d.f. = 4). Tukey multiple comparisons showed this to be due to differences between Treatment and Days, and Acidification within certain Treatments. A significant interaction was present between the factors Treatment, Temperature and Acidification (4-way ANOVA; p = 0.035, F = 3.43, d.f. = 2). Tukey

multiple comparisons showed this to be due to differences between Treatment and Days, and Acidification within certain Treatments. Staining success was still high for chlorine killed and control organisms after the 2 week period and storage at lower temperature did not increase the staining success. Storage of heat killed organisms would not be recommended. Acidification did not increase the staining success, but it did increase the visibility of the stain and made assessment of samples quicker and easier.

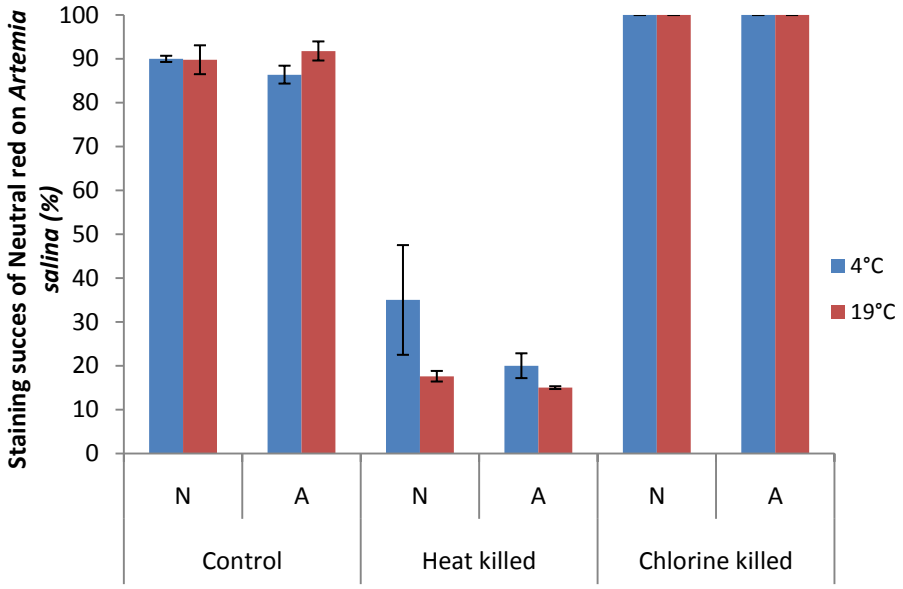

Figure 8.10. Day 1 *Artemia salina* samples preserved with 4% buffered formalin with sodium acetate. (N = non-acidified, A=acidified with glacial acetic acid). Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

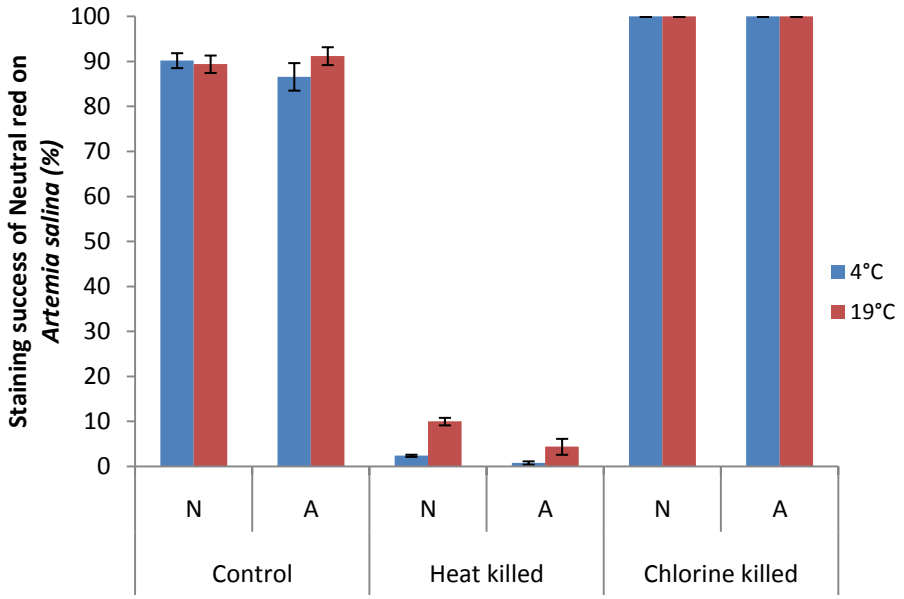

Figure 8.11. Day 7 *Artemia salina* samples preserved with 4% buffered formalin with sodium acetate. (N = non-acidified, A=acidified with glacial acetic acid). Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

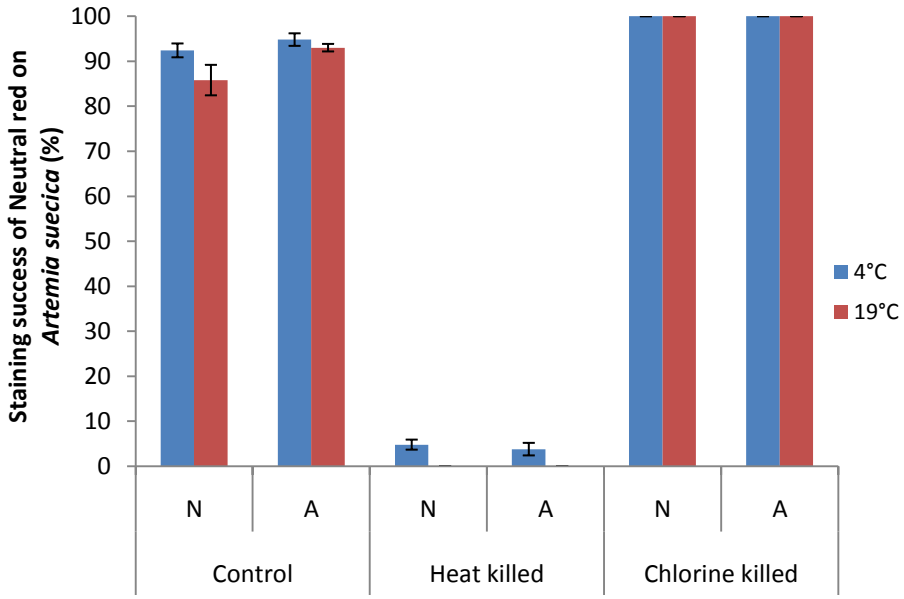

Figure 8.12. Day 14 *Artemia salina* samples preserved with 4% buffered formalin with sodium acetate. (N = non-acidified, A=acidified with glacial acetic acid). Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

8.3.1.4 SYTOX green

SYTOX green fluorescent stain was tested at 5µM on *A.salina* and the staining success was assessed after 5 and 15 minutes exposure, as shown in Figure 8.13. There was no significant difference in success between the staining times (Kruskal Wallis; p = 0.966, H = 0.00, d.f. = 1) although there was a noticeable increase in the visibility of the stain after 15 minutes. The staining success of the stain on control, heat killed and chlorine killed organisms did show a significant difference (Kruskal Wallis; p = <0.001, H = 27.86, d.f. = 2). No live organisms exhibited staining. Heat killed organisms showed >77% staining success, although staining was inconsistent, and SYTOX green was entirely ineffective on chlorine killed *A.salina*.

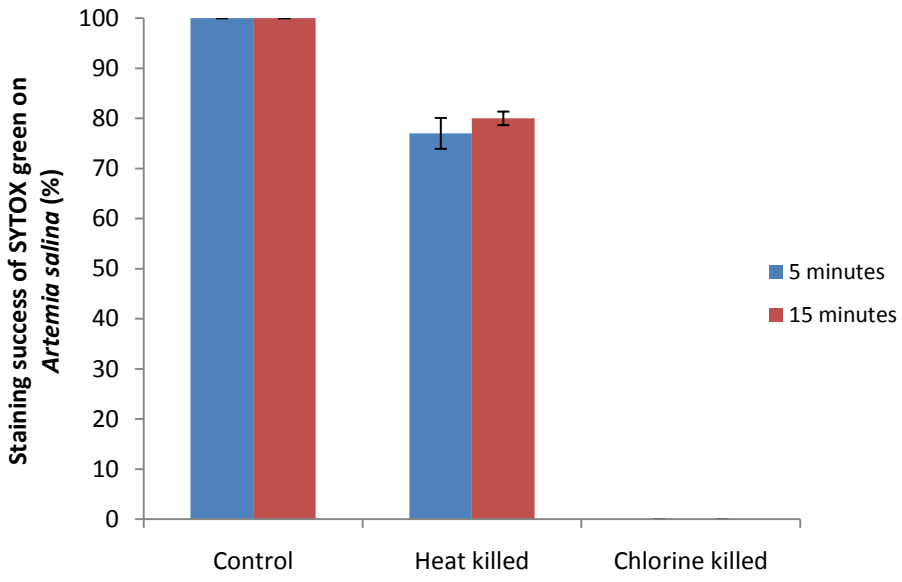

Figure 8.13. Staining success (%) of SYTOX green on control, heat killed and chlorine killed *Artemia salina*. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

8.3.1.5 Trypan blue

The effectiveness of Trypan blue on *A.salina* was assessed at two concentrations and two staining times (Figure 8.14). Live individuals remained unstained. Staining success

in heat and chlorine killed samples included full and partially stained organisms. There was a significant interaction between the factors Concentration and Treatment (3-way ANOVA; p = <0.001, F = 31.15, d.f. = 2). Tukey multiple comparisons showed that this was due to differences between Concentrations within Treatments. A significant interaction was present between the factors Time and Concentration (3-way ANOVA; p = 0.005, F = 5.87, d.f. = 2). Tukey multiple comparisons showed this to be due to differences between Treatments at both Concentrations. The best staining conditions were 0.2% and 30 minutes exposure, but this does include the partially stained organisms which exhibited a wide variation in staining pattern.

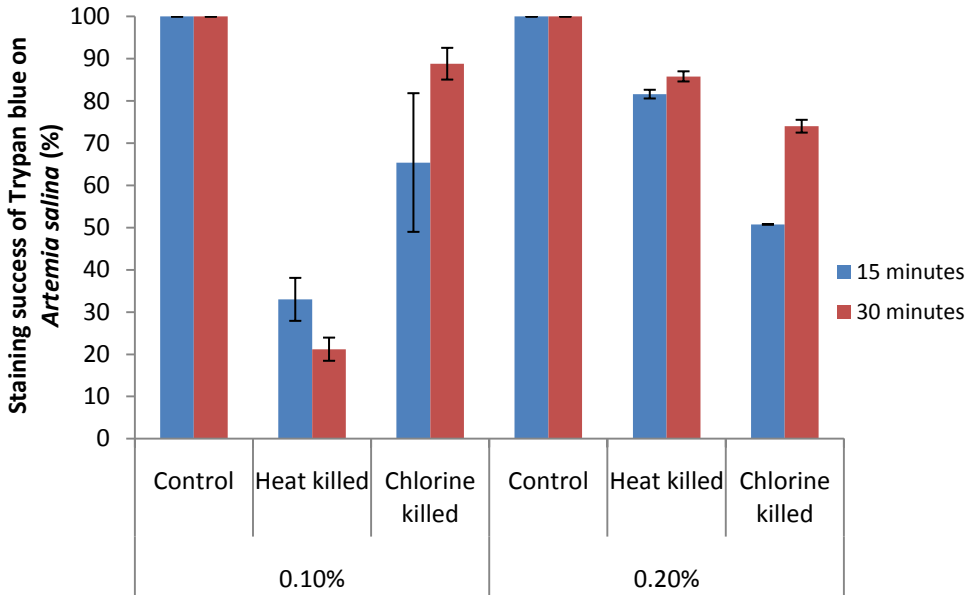

Figure 8.14. Staining success (%) of Trypan blue on live, heat killed and chlorine killed *Artemia salina* at two concentrations. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

8.3.2 Tetraselmis suecica

8.3.2.1. Evans blue

Evans blue showed high staining success in the test algal species at a concentration of 1:1000: 98% of heat killed cells were stained successfully and 99.6% of chlorine killed cells (Figure 8.15). A significant interaction between the factors Treatment and

Concentration was observed (2-way ANOVA p = <0.001, F = 6.68, d.f. = 4). Tukey multiple comparisons showed this to be due to a significant difference between the Heat killed *T.suecica* at 1:50,000 concentration value and all other data. Chlorine was observed to affect the Evans blue stain in that the cells were left without green pigmentation but did not show Evans blue staining clearly like the heat killed cells (Figure 8.16). Instead cells had a blue tint and individuals were marked as stained if they exhibited this.

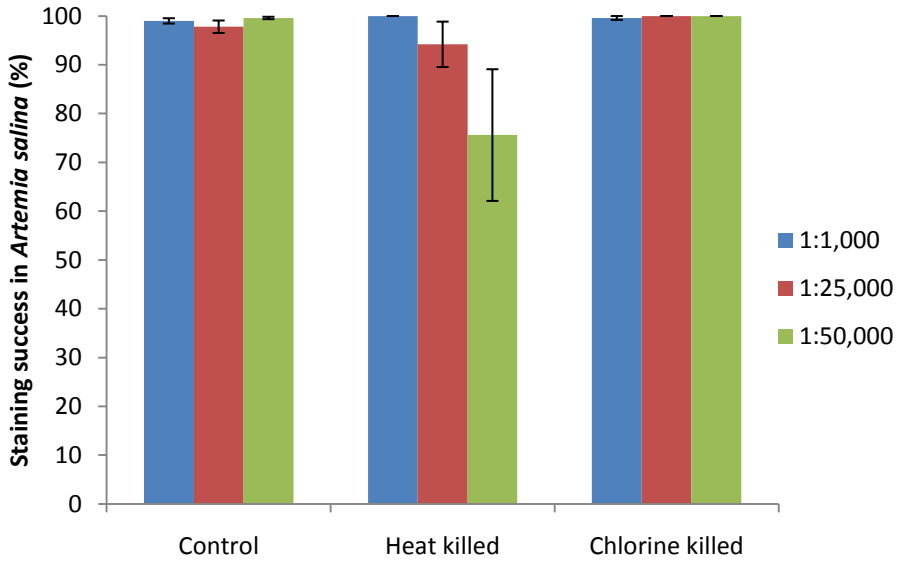

Figure 8.15. The staining success (%) of Evans blue with *Tetraselmis suecica*. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

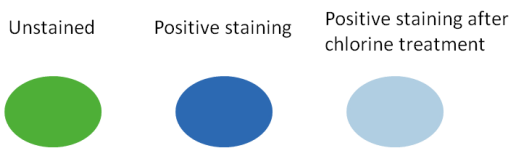

Figure 8.16. Staining colour observed after chlorine treatment of *Tetraselmis suecica* cells.

8.3.2.2 Fluorescein diacetate (FDA)

FDA was 100% effective at both staining concentrations, both staining times and in all treatments (Figure 8.17 and Figure 8.18) so statistical analysis was not performed. The visibility of the stain was best after 15 minutes making assessment quicker and easier.

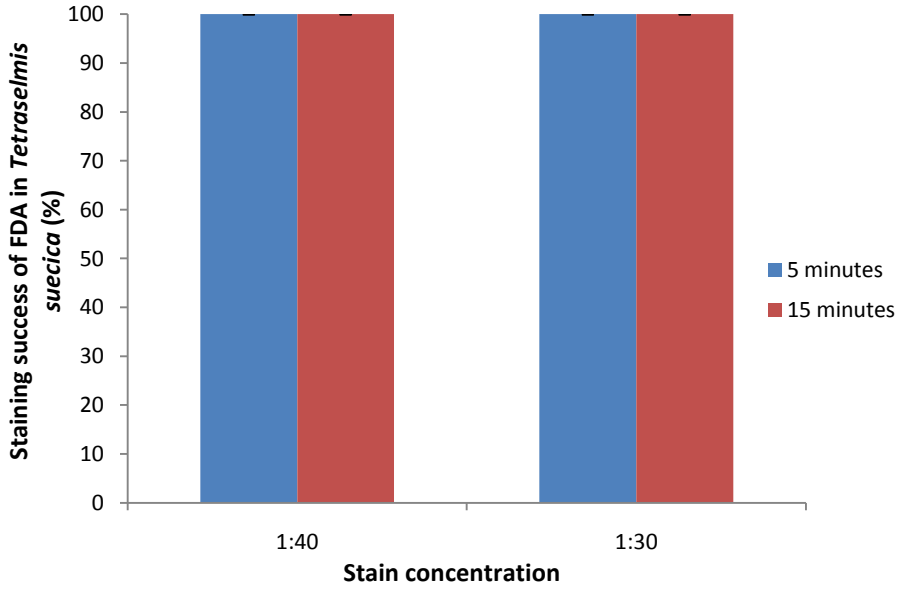

Figure 8.17. Staining success (%) of two concentrations of Fluorescein diacetate (FDA) on *Tetraselmis suecica* after 5 and 15 minutes stain exposure. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

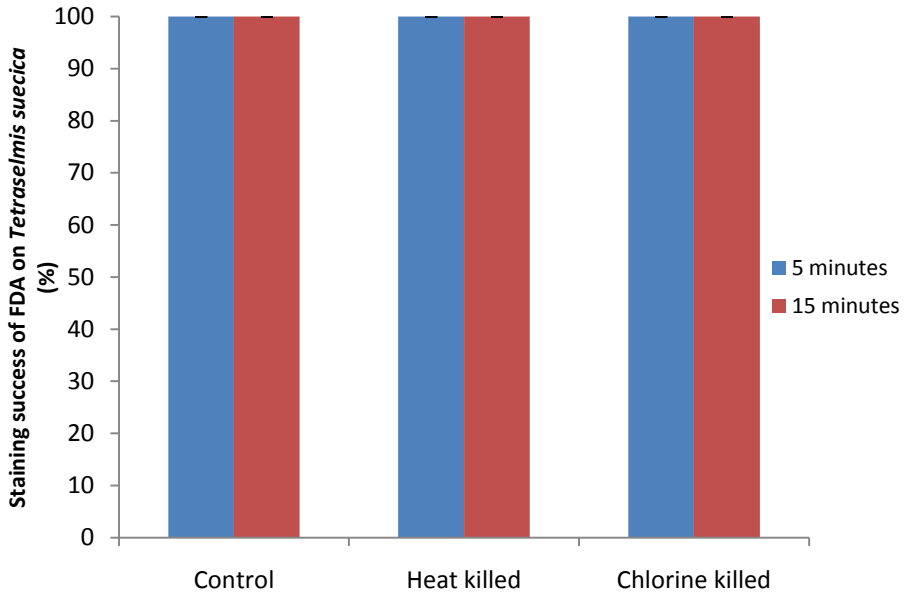

Figure 8.18. Staining success (%) of Fluorescein diacetate (FDA) on control, heat killed and chlorine killed *Tetraselmis suecica* after 5 and 15 minutes stain exposure at 1:30 concentration. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

8.3.2.3. Neutral red

The neutral red stain was taken up by live *T.suecica* individuals. Positive staining was observed to be in red 'patches' and was not visible as fully red cells. No red staining was observed in dead cells and so this could be differentiated easily from the live staining.

Neutral red showed >97.8 staining success at all concentrations (Figure 8.19). There was a significant interaction between the factors Treatment and Concentration (2-way ANOVA; $p = 0.002$, $F = 5.26$, d.f. = 4). Tukey multiple comparisons showed this to be due to the lower success of the Control samples at a concentration of 1:80,000. At concentrations 1:15,000 and 1:33,333 cells showed reduced movement after 5 minutes, but at 1:80,000 cells were still moving. This suggests that the stain was toxic at the higher concentrations, but as the stain was taken up into cells prior to cells

showing immobility and did not leach after death then toxicity does not appear to affect the staining success.

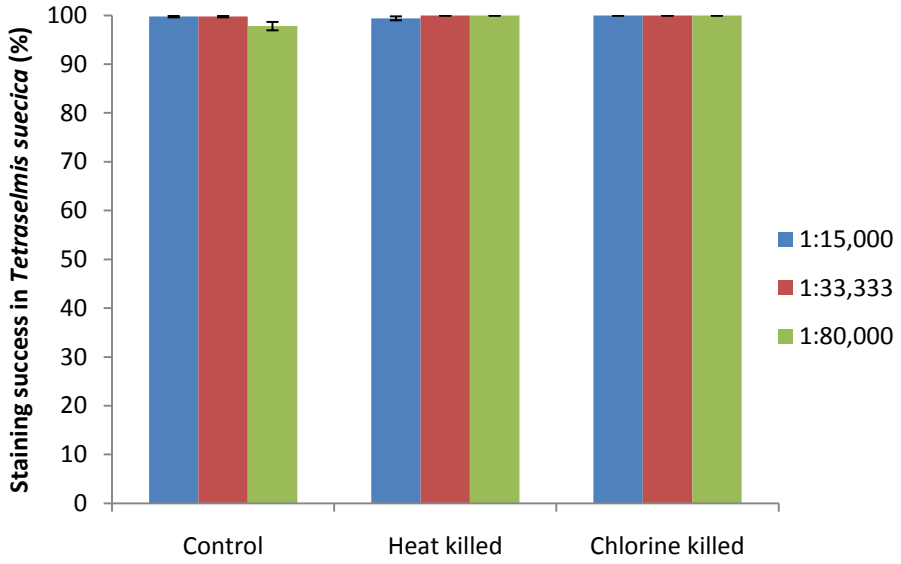

Figure 8.19. Staining success (%) of Neutral red on live, heat killed and chlorine killed *Tetraselmis suecica* at three concentrations. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

Preservation of *T.suecica* samples resulted in >93% staining success after 2 weeks, as shown in Figure 8.20-23. Significant interactions were observed between the factors Treatment and Temperature (4-way ANOVA; p = 0.001, F = 7.43, d.f. = 2), Day and Temperature (4-way ANOVA; p = 0.008, F = 5.01, d.f. = 2) and Day and Acidification (4-way ANOVA; p = 0.034, F = 3.47, d.f. = 2). Storing samples in cool conditions did not increase staining success. Acidification enhanced the colour of the stain prior to sample assessment, making examination quicker and easier.

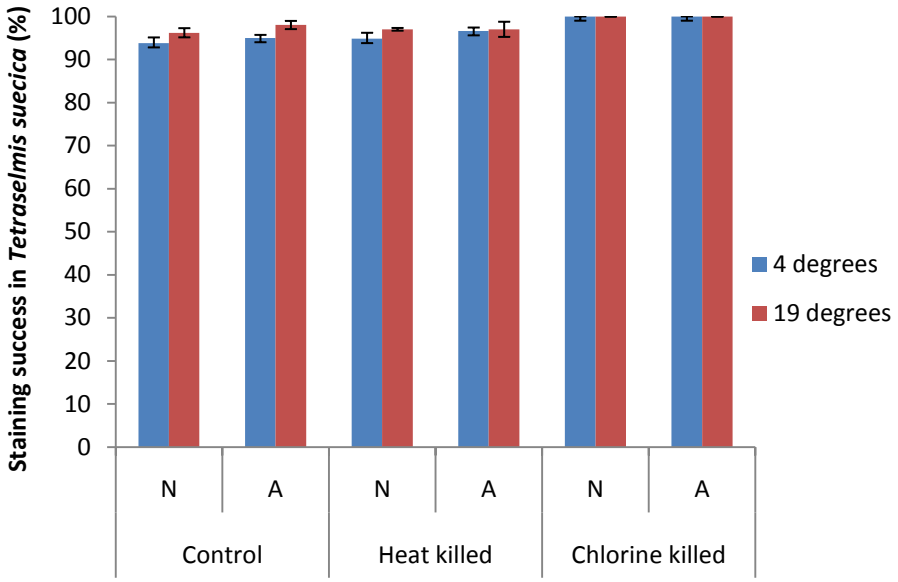

Figure 8.20. Staining success (%) of Neutral red in *Tetraselmis suecica* 1 day after preservation. Values are the mean of 5 replicates SE. (N = non-acidified, A=acidified with glacial acetic acid). (Staining success = the percentage of test organisms showing the expected response to the stain applied).

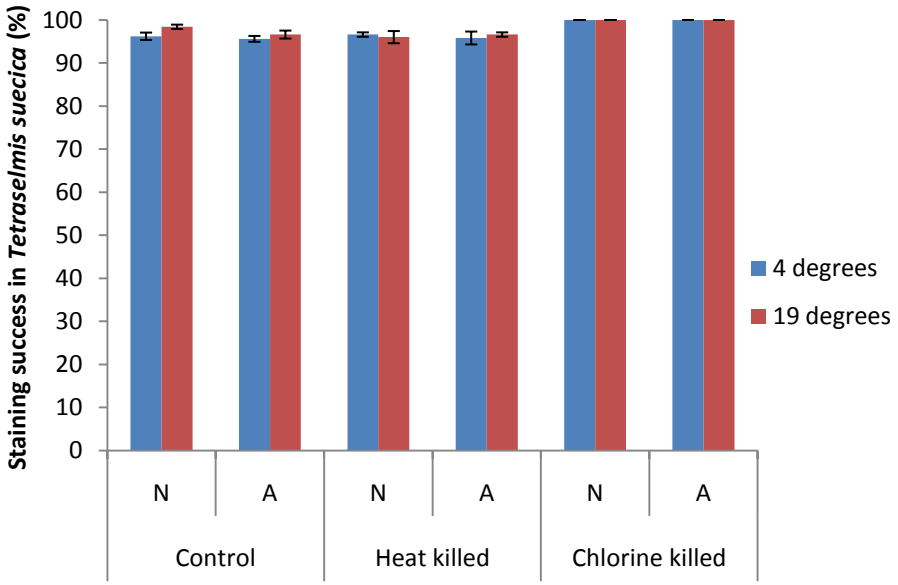

Figure 8.21. Staining success of Neutral red in *Tetraselmis suecica* 7 days after fixation. Values are the mean of 5 replicates ± SE. (N = non-acidified, A=acidified with glacial acetic acid). (Staining success = the percentage of test organisms showing the expected response to the stain applied).

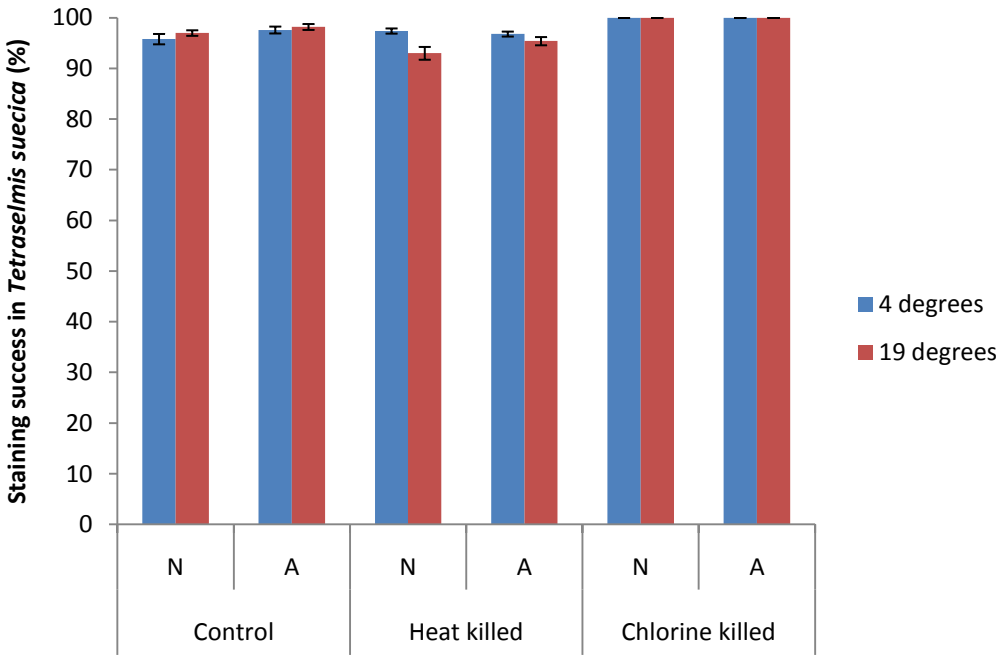

Figure 8.22. Staining success (%) of Neutral red in *Tetraselmis suecica* 14 days after fixation. Values are the mean of 5 replicates ± SE. (N = non-acidified, A=acidified with glacial acetic acid). (Staining success = the percentage of test organisms showing the expected response to the stain applied).

8.3.2.4 SYTOX green

A concentration of 5µM SYTOX green was most effective for heat killed cells. When tested on chlorine killed cells none showed positive staining, this was thought to be due to the bleaching effect of chlorine, and so lower concentrations of stain were not tested. There was a significant interaction between the factors Concentration and Time (2-way ANOVA, $p = 0.008$, $F = 4.04$, d.f. = 4). Tukey multiple comparisons showed this to be due to differences between staining Concentrations. 5µM had a much higher staining success than the other concentrations tested, as shown in Figure 8.23. Increased exposure time increased staining success and the fluorescent signal was more visible after 15 minutes.

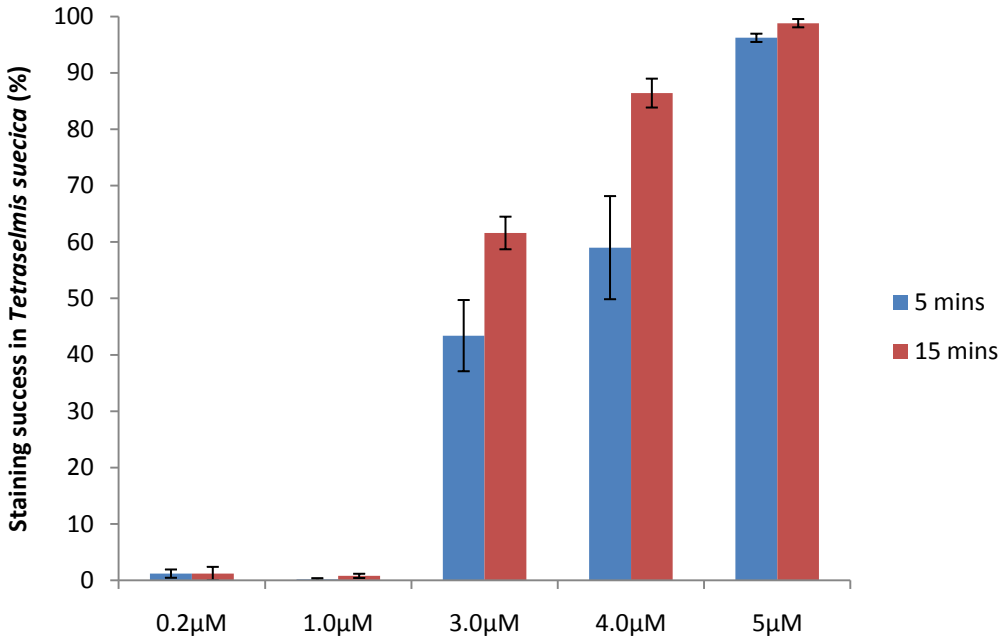

Figure 8.23. Staining success (%) of SYTOX green with heat killed *Tetraselmis suecica* cells at five concentrations after 5 and 15 minutes staining time. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

The success of SYTOX green on live, heat killed and chlorine killed *T.suecica* cells was assessed at 5µM (Figure 8.24). There was a significant interaction between the factors Treatment and Time (2-way ANOVA, $p = 0.004$, $F = 7.06$, d.f. = 2). Tukey multiple comparisons showed this to be due to differences between Treatments at both Time periods. Chlorine treated cells showed no staining and this is a limitation to the use of SYTOX green. However, when used with heat killed cells it was 98.8% effective after 15 minutes and would be recommended for use in this application.

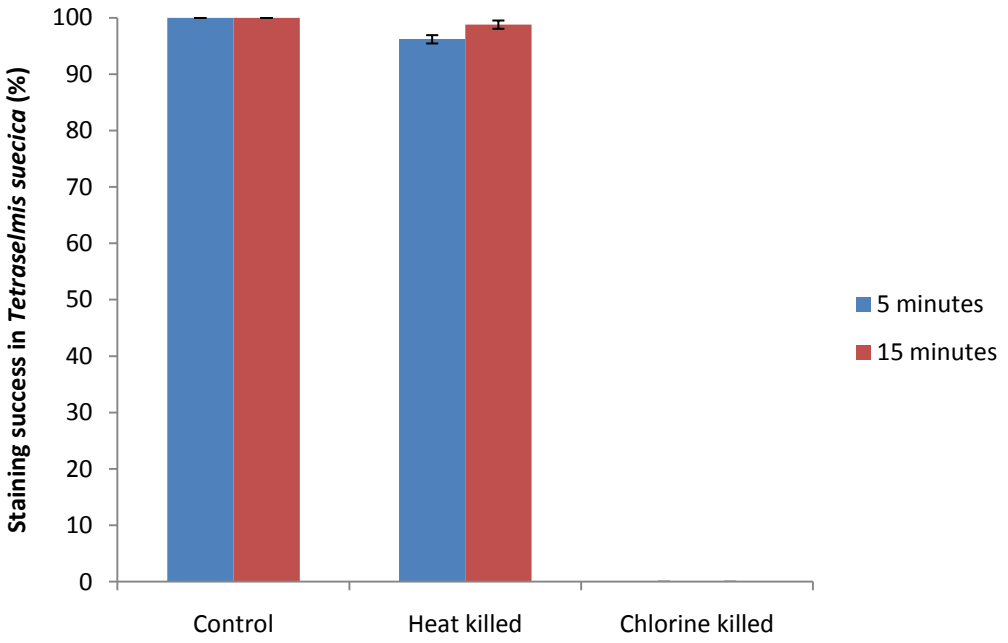

Figure 8.24. Staining success of SYTOX green in control, heat killed and chlorine killed *Tetraselmis suecica* cells at 5µM staining concentration after 5 and 15 minutes staining time. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

8.3.2.5 Trypan blue

The staining success of Trypan blue on *T.suecica* was assessed at two concentrations and two staining times (Figure 8.25). There was a significant interaction between the factors Concentration and Treatment (3-way ANOVA; $p = 0.043$, $F = 3.37$, d.f. = 2). Tukey multiple comparisons showed this to be due to differences between Heat killed and other Treatments at both Concentrations. Live cells did not take up the stain. Heat killed cells showed very low staining success (<0.8%). As observed with the Evans blue chlorine killed cells bleached and after addition of the stain cells were observed to possess a blue tint (see Figure 8.16). All cells which exhibited this were classed as stained. This resulted in 100% staining success for Trypan blue with chlorine treated cells.

Figure 8.25. Staining success (%) of Trypan blue with control, heat killed and chlorine killed *Tetraselmis suecica*. Values are the mean of 5 replicates ± SE. (Staining success = the percentage of test organisms showing the expected response to the stain applied).

8.3.3 Natural population testing

All five stains were assessed on natural phytoplankton and zooplankton populations at the optimum working conditions determined from the *A.salina* and *T.suecica* tests. The organisms present in samples varied between replicates and between stains and statistical analysis could not be performed. As observed in preliminary tests FDA, Evans blue and neutral red stains showed highest staining success. Trypan blue and SYTOX green showed varying results in both zooplankton and phytoplankton populations. Neutral red stained zooplankton individuals exhibited reduced movement when assessed after the 60 minute staining period. The results of these tests are shown in Tables 8.2 and 8.3.

Table 8.2. Staining success (%) of all stains on natural phytoplankton populations. Values are the mean of 3 replicates ± SE (NP = not present, * = present in 2 samples. Where SE is not shown organisms were present in 1 sample). (Staining success = the percentage of test organisms showing the expected response to the stain applied).

	Evans blue			**Fluorescein diacetate**			**Neutral red**			**SYTOX green**			**Trypan blue**		
Organism	**Chlorine killed**	**Heat killed**	**Live**	**Chlorine killed**	**Heat killed**	**Live**	**Chlorine killed**	**Heat killed**	**Live**	**Chlorine killed**	**Heat killed**	**Live**	**Chlorine killed**	**Heat killed**	**Live**
Asterionella japonica	NP	NP	NP	NP	NP	NP	NP	NP	NP	0	0±0	100	NP	NP	NP
***Ceratium* spp.**	NP	100±0	0	NP	NP	100±0*	100±0	100±0	100±0	NP	NP	NP	100	NP	NP
***Coscinodiscus* sp.**	NP	NP	100	NP	NP	NP	NP	NP	NP	0±0	100	NP	NP	NP	100±0*
Cylindrotheca closterium	NP	100±0*	0±0	NP	NP	NP	NP	100±0*	25±25*	N/P	NP	100±0	NP	NP	NP
***Dinophysis* sp.**	NP	NP	NP	NP	NP	NP	100±0	100±0	67±33	NP	NP	NP	NP	NP	100
Odontella mobilensis	34±24	100±0	100±0	100±0	100±0	100±0	100±0	100±0	99.6±0.4	0±0	6±6	100	0±0	92±2	100±0
Paralia sulcata	NP	NP	NP	100	100±0	100±0	100±0*	NP	NP	0±0	0±0	100±0	0±0*	19±19	100
***Pseudo-nitzschia* spp.**	9±5	100±0	98±2	100±0	100±0	80±11	100±0	100±0	96.7±3.3	0±0	7±1	100±0	0±0	20±15	100±0
***Rhizosolenia* sp.**	NP	NP	NP	NP	100	0	100±0	NP	50±50*	0±0	0±0	100	NP	NP	100
Skeletonema costatum	NP	NP	NP	100±0*	100±0*	NP	NP	100	100±0*	0±0	0±0	100±0	NP	NP	NP
Thalassionema nitzschiodes	NP	NP	NP	100±0*	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP

Table 8.3. Staining success (%) of all stains on natural zooplankton populations. Values are the mean of 3 replicates ± SE (NP = not present, * = present in 2 samples. Where SE is not shown organisms were present in 1 sample). (Staining success = the percentage of test organisms showing the expected response to the stain applied).

	Evans blue			Fluorescein diacetate			Neutral red			SYTOX green			Trypan blue		
Organism	**Chlorine killed**	**Heat killed**	**Live**	**Chlorine killed**	**Heat killed**	**Live**	**Chlorine killed**	**Heat killed**	**Live**	**Chlorine killed**	**Heat killed**	**Live**	**Chlorine killed**	**Heat killed**	**Live**
Barnacle cyprid	70±29	87±4	19±2	100±0	100±0	100±0	100±0	100±0	90±6	0±0*	23±13	8±5	0±0	22±6	100±0
Calanoid copepod	NP	NP	NP	100±0	100±0	100±0	100±0	100±0	42±14	0	NP	67±33	0±0	67±33	67
Copepodite	60±30	96±4	66±9	NP	100±0	NP	NP	NP	NP	0±0	92±5	6±0.5	0±0	90±2	100±0
Echinoderm larvae	NP	NP	NP	NP	100	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
Gastropod larvae	56±17*	100±0	NP	NP	NP	100±0	100	100	NP	41±10	87±3	78±13	19±10	13±12	0±0
Mite	NP	0±0	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
Oithona similis	73±27	100±0	0±0	100	100±0	100±0	100±0	98.6±3.9	27±4	0±0*	50±50*	0	0	83±17*	0
Oligotroph	NP	NP	0±0	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	100±0	92±8
Ostracod	NP	56±29	50±50*	100	100±0	100±0	67±0*	100	75±20*	NP	NP	NP	NP	NP	NP
Benthic polychaete	100±0	100±0*	0±0*	100±0	NP	100	NP	NP	100	NP	100±0*	0±0	NP	100±0*	92±8
Polychaete larvae	100±0	100±0	0±0	NP	NP	NP	NP	NP	100	NP	100±0	0±0	0	95±5	86±7
***Podon* sp.**	NP	NP	NP	NP	NP	100	NP	NP	NP	NP	NP	NP	NP	NP	NP
Temora longicornis	NP	NP	0±0	100±0	100±0	100±0	100±0	100±0	67±6	NP	NP	NP	NP	NP	NP
Tintinnid	NP	NP	NP	NP	NP	NP	NP	NP	NP	0±0*	4±4	0±0	NP	NP	NP
Unidentified copepod	59±24	64±19	75±14	NP	100±0	100±0	100	100±0	NP	0±0	0±0*	NP	11±11	41±5	100±0*

8.4 Discussion

The viability assessment methods tested in this study showed variability and limitations to their use, but when properly optimised some stains were able to work accurately and reliably. For phytoplankton assessment Evans blue, neutral red and FDA showed >99% staining success on all *T.suecica* samples. SYTOX green and trypan blue showed some success under limited conditions but would only be recommended for use if other stains were unavailable.

FDA gave highly accurate results on *A.salina* and natural zooplankton and would be recommended for future application. This is the first published use of FDA with zooplankton and shows that there is potential for an effective assessment method of live zooplankton using FDA to be developed. Neutral red would also be recommended for zooplankton, although the staining behaviour differed between organisms and so preliminary testing would be required before use. Evans blue, SYTOX green and trypan blue showed partial and inconsistent staining and would not be recommended for use on zooplankton.

The killing method used on organisms did have an effect on the staining success, especially when organisms were killed using chlorine. If using a chlorine based biocide SYTOX green would not be recommended as staining was completely inhibited in all tests. Evans blue and trypan blue could be used, but preliminary experiments to assess staining behaviour would be required. Neutral red and FDA would be suitable for use, although in the case of neutral red preservation of samples would not be recommended due to the absorption of neutral red stain by heat killed organisms.

8.4.1 Staining observations

8.4.1.1 Evans blue

Evans blue showed a bleaching effect due to chlorine treatment in both phytoplankton and zooplankton tests. Positive staining was affected as the colour produced by each

stain was altered. However, as this was observed during preliminary tests positive staining behaviour was determined and high staining success was obtained. Evans blue has been successfully used in previous studies (see Section 8.1.2.1) and would be recommended for use with phytoplankton. However, it would first be necessary to ensure that any chemical treatment does not interfere with the normal appearance of positive staining.

8.4.1.2 Fluorescein diacetate

One interesting observation made during the *A.salina* tests was the effect of age on positive FDA fluorescence in live organisms. Live stained *A.salina* exhibited fluorescence only in their gut, but individuals of a young nauplii stage without a fully developed gut did not exhibit any fluorescence even though they were observed to show movement. Further experimentation showed that none of the younger individuals stained with FDA, whereas 100% of the older organisms were stained. FDA staining was clear and accurate in live *A.salina*, but if employed in tests then cultures would have to be grown for >24 hours to ensure all individuals were in a suitable development stage to show positive FDA fluorescence.

During initial tests FDA showed 100% staining success in all *T.suecica* tests. Gilbert et al (1992) successfully used FDA with *T.suecica* and other authors have found FDA effective on marine phytoplankton (Murphy and Cowles 1997; Pouneva 1997; Jochem 2005). Jochem (1999) performed preliminary experiments to assess the staining behaviour of FDA and observed that after 5 minutes stain exposure accurate readings could be made, but if left for 15 minutes before starting to assess samples the FDA began to leach from cells. This study did not observe leaching from cells after 15 minutes FDA exposure, and in contrast to Jochem would advise a 15 minute staining period as the FDA stain showed much stronger fluorescence after this staining period and could be more easily distinguished in cells. FDA would be recommended for use with phytoplankton, and the development of methods to apply this stain with

automated detection and counting techniques would be useful for future ballast water research.

The masking of FDA fluorescence by autofluorescence has been reported by previous studies, e.g. it was observed in the diatom *Thalassiosira weissflogii* by Garvey et al (2007). When used on natural phytoplankton in this study some cells still showed red autofluorescence, however, those cells showing autofluorescence also exhibited patches of green FDA staining and so could be detected. If FDA were to be used in conjunction with automated counting techniques preliminary tests would need to be carried out to ensure that the FDA signal could be detected above the autofluorescence for accurate counts to be obtained. If not this would limit the use of FDA to manual counting by microscopy which can be a time consuming and laborious process.

8.4.1.3 Neutral red

The application of neutral red with phytoplankton has not been widely used or recommended by previous studies, see Section 8.1.2.4. However, this study observed neutral red to give high staining success with *T.suecica* within a 20 minute staining period. This agrees with Onji (2000) who observed uptake of neutral red stain only in live cells of *Tetraselmis* sp. and concluded that it was a good stain to use to distinguish metabolically active cells. When applied to natural phytoplankton neutral red staining was clearly visible in live cells, and no dead individuals took up the stain. *Odontella mobilensis*, *Pseudo-nitzschia* spp., *Ceratium* spp. and *Skeletonema costatum* all showed >95% staining success in live organisms. Crippen and Perrier (1974) also observed clear staining of *Odontella* sp., and Onji (2000) observed successful staining of *Skeletonema* costatum with neutral red. If used with *T.suecica* neutral red would be recommended for samples which need to be analysed quickly, i.e. without preservation. The results of this study would also recommend neutral red for use with natural phytoplankton populations, although if this stain is used preliminary experiments would be necessary to ensure the stain is suitable for the species present.

A toxic effect of neutral red has been observed by various authors in natural phytoplankton (Crippen and Perrier 1974; Reynolds et al. 1978). This study observed reduced mobility in *T.suecica* and natural zooplankton after exposure to the neutral red stain showing that it could be toxic to organisms. Crippen and Perrier (1974) and Reynolds et al (1978) both reported leaching of neutral red from phytoplankton before the end of the staining period due to the toxic effect of the stain. This study did not observe any leaching of stain from cells and accurate counts of live cell numbers were made.

Leaching was not observed on samples assessed immediately, and this could be due to the shorter staining time used than that of the previous studies, which were 60 minutes and 30 minutes (Crippen and Perrier 1974; Reynolds et al. 1978). When applied to natural populations leaching was not observed after the 20 minute staining time. Reynolds et al (1978) observed *Skeletonema costatum* to die and the stain to leach from cells after 30 minutes, whereas this study observed clearly stained cells, thus recommending the reduced staining period for accurate viability determination.

However when applied to natural plankton populations the success of neutral red on copepods varied: calanoid copepods 40%, *Oithona helgolandica* 25% and *Temora longicornis* 70%, and all showed variability between replicates. This result contrasts with Dressel (1972), who found neutral red to show clear distinction between live and dead copepods, *Acartia tonsa* and *Eurytemora affinis*, with no problems reported for either species. These results highlight the need for specific optimisation of the staining technique for the species present.

The process of preserving samples stained with neutral red has been discussed in previous literature as a hindrance to phytoplankton assessment (Crippen and Perrier 1974) but a benefit to zooplankton assessment (Dressel et al. 1972). Although varying results have been observed in zooplankton, and Waite et al (2003) found neutral red to be ineffective as both live and dead zooplankton absorbed the stain after preservation, leaving no indication which had been alive prior to fixation. Dressel (1972) reported

that when used at high concentrations (1.5:100,000) neutral red could be fixed and left at 5°C or 30°C and zooplankton samples could be accurately assessed after 7 days. This study also observed no advantage to storing *A.salina* samples at low temperatures (4°C) and when left at room temperature (19°C) high staining success was still observed in all samples after 14 days. In regards to onboard testing of ballast water treatments these results mean that no special facilities would be required for storage of samples for up to 14 days, giving ample time for samples to be taken to a laboratory for assessment. Crippen and Perrier (1974) observed reduced staining success when neutral red samples were preserved in buffered formalin, and this study also showed reduced staining success in *T.suecica* samples with preservation suggesting that the stain leached from cells over time.

Heat killed organisms preserved in formaldehyde began to absorb the neutral red stain, this has previously been reported by Crippen and Perrier (1974). Stain absorption caused the gut of organisms to become obscured and thus if assessment was performed on preserved samples overestimations of the number of live organisms could be made. This is a limitation to the use of neutral red with high temperature ballast water treatment systems.

Acidification enhanced the colour of stained *A.salina* enabling easier assessment, agreeing with Dressel (1972) who observed enhancement of staining colour in copepods after acidification. Acidification was observed to produce a reddish tint to the frustules which obscured the colour of the cell contents and thus the stain could not be seen (Crippen and Perrier 1974), but when used on *T.suecica* in this study acidification enhanced the staining colour, enabling quicker and easier assessment of samples.

8.4.1.4 SYTOX green

SYTOX green was very effective when used on heat killed and live *T.suecica*, and it has been successfully used in previous studies on various diatoms, dinoflagellates and bacteria (Lebaron et al. 1998; Brussaard et al. 2001; Franklin and Berges 2004; Franklin

et al. 2004; Binet and Stauber 2006; Veldhuis et al. 2006; Gregg and Hallegraeff 2007). However, when applied to natural phytoplankton populations in this study staining success in heat killed cells was very low (see Table 8.2) and chlorine killed cells remained unstained in both *T.suecica* and natural population tests. SYTOX green would be recommended for use with phytoplankton, but initial experiments to optimise the staining procedure and ensure the stain is effective with the killing treatment being applied and the organisms present are vital to ensure viability can be accurately determined.

When applied to zooplankton live organisms did not show staining, as expected. However, inconsistent staining was observed in heat killed organisms and chlorine killed organisms did not exhibit any staining. From the observations made in this study SYTOX green would not be recommended for further use with zooplankton.

8.4.1.5 Trypan blue

Trypan blue was effective on live cells in the natural phytoplankton and *T.suecica* tests, however, when applied to dead cells staining success was reduced and varied between species (see Table 8.2). As described in Section 8.4.1.1 Trypan blue also showed a bleaching effect due to chlorine addition. The colour produced for positive staining was affected, but when noted during preliminary tests it was recorded and so numbers of dead cells could be determined. The use of Trypan blue with phytoplankton in the literature is limited and in the one study found in which it was used with phytoplankton no comments were made about the effectiveness of the stain (Miron et al. 2003). The results of this study would suggest that Trypan blue is an ineffective viability stain with phytoplankton and further use would not be recommended in this application.

When applied to live zooplankton no staining was observed. However, when applied to heat and chlorine killed individuals partial staining was observed in many organisms. When partial and complete staining were combined to determine staining success the stain was quite effective (see Figure 8.14), but the partial staining observed was

inconsistent between organisms. This stain would not be recommended for further development with zooplankton.

8.5 Conclusions

This study aimed to determine the staining success of the five viability stains and identify accurate viability assessment methods. The tests completed can confirm the accuracy of commonly used viability stains through their staining success under known conditions on frequently used test organisms. Overall, FDA was the most effective stain on phytoplankton. Neutral red and Evans blue would also be recommended for use with phytoplankton, although within the limitations previously discussed. This study describes the first observation of successful application of FDA with zooplankton. The stain was clearly visible in organisms and did not leach during the examination period. With further development FDA could be combined with an automated counting technique, e.g. FlowCam, into a rapid and accurate data collection method for zooplankton.

The difference in staining success for the test organisms and natural populations was vast in three of the stains. This highlights the importance of optimising the stain being used for the organisms present in samples when performing ballast water treatment tests. If this is not performed prior to experimentation it will impact on the accuracy of the data obtained.

Chapter 9

General Discussion

This thesis began with the idea of the Earth as 'Gaia' – a living system comprising of all organisms and materials in the Earth's surface capable of self-regulating conditions for the maintenance of life. Due to our scientific and technological progression we have crossed the boundaries at which Gaia is capable of self-regulating. We have entered a time during which we need to be responsible for our own activities and manage these for the benefit of both mankind and the Earth. The transportation of organisms to new environments can have detrimental effects to the environment, economy and human health. The vectors responsible for this were identified (Chapter 2) and the transport mechanism, ballast water, was addressed in this study.

The first aspects of the ballast water transportation cycle addressed in this thesis were the 'unintentional treatments' i.e. the passing of organisms through a centrifugal pump and their prolonged storage in dark, confined conditions. These unintentional treatments are forced upon organisms on every ballast water journey and pose the possibility of reducing the health of individuals. Understanding the impacts of these processes will aid future management strategies by increasing our ability to predict likely scenarios resulting from ballast water transportation.

Travelling though a centrifugal pump could result in damage to individuals through collisions with the pumps impellers. To the best of the author's knowledge the effects of the centrifugal pump itself have not previously been looked at in isolation and supported by the collection of quantitative data of plankton mortality before and after the pump. This study did not observe a significant increase in phytoplankton mortality due to the pump tested. While zooplankton data was limited and could not be statistically analysed, the study did not show an increase in mortality due to the effects of the pump tested. The data collected by this study supports findings published in

previous studies (Mohlenberg 1987; Nayar et al. 2002; Veldhuis et al. 2006) and does not show that being pumped into ballast tanks will kill organisms. These results can only be accurately applied to the pump tested.

Once organisms have entered the ballast tanks for storage during the voyage a set of artificial conditions are imposed upon them. These conditions vary between different vessels and different journeys. Factors such as temperature, pH, oxygen and salinity levels of the seawater all vary depending on the conditions at the location of uptake (Carlton 1985), and all of these factors will influence the survival of organisms within the tanks. This study observed phytoplankton from both tropical and temperate locations to survive a 28 day dark period and show the capability for growth when returned to light. One of the findings of most concern was the high level of growth of temperate Nitzschioid pennate diatoms when returned to light and exposed to fresh seawater after surviving the 28 day dark period. Pennate diatoms were also demonstrated by Kang et al (2010) to successfully establish in foreign ports after ballast water transportation. There are many strategies employed by organisms to endure prolonged dark conditions and this study observed that organisms from very different geographical locations are capable of utilising these to survive. In addition one of the major deficiencies from this area of research is continuous monitoring of both the dark period and the regrowth period. Further work which addressed both of these aspects would give relevant information to increase our understanding of how organisms survive dark periods and respond to subsequent reintroduction to light. The application of the information obtained in this study and similar studies e.g. Kang et al (2010), when assessing the potential risk of ballast water transport routes could aid the reduction of discharge in areas likely to result in successful transportation.

After addressing the ‘unintentional treatments’ this study looked at ‘intentional treatment’ technologies which can be employed in ballast water treatment systems to prevent the discharge of live organisms. These treatments can be employed before organisms enter tanks, during the journey or upon discharge. Three treatment

technologies were assessed: a UV light system, the chlorine based chemical AnoFluid and a 40µm screen filter.

UV light is a well established water treatment technology and has been employed in ballast water treatment. Three combined filter and UV systems have already gained IMO Type approval with more expected to be developed (Lloyds Register 2010). UV based systems are reliable, require little maintenance and last for many years which makes them good candidates for further development. The UV system tested in this study was effective on *T.suecica* at a dose suitable for shipboard use, at high flow rates and using a treatment protocol which could be applied onboard a vessel, i.e. filtration and UV irradiation on uptake and UV irradiation on discharge. The reapplication of UV irradiation prior to discharge can kill surviving organisms, or act to reverse repair completed by organisms when in the ballast tanks. In the large scale tests performed the combined filter and UV system passed the IMO D-2 discharge standard and proved high biological effectiveness in ballast water treatment.

Application of the chlorine based biocide AnoFluid was successful and caused 100% mortality of *T.suecica* at a dose feasible for shipboard use. An advantage to AnoFluid is its production via electrolysis and direct application to ballast water as this eliminates the requirement for storage space on board ships and the risk of large chemical spills (White 1999). The breakdown of AnoFluid was monitored to determine any environmental risk upon discharge. The level of free chlorine present after 120 hours residence time was negligible although further tests would need to be performed to assess any production of harmful by-products during use. The combined filter and AnoFluid system tested in this study passed the IMO D-2 discharge standard in the large scale tests, and if application does not result in the formation of harmful by-products the system could be suitable for use in ballast water treatment.

The filter showed extremely high removal efficiencies for zooplankton. The inclusion of an initial treatment such as screen filtration, which physically removes larger organisms from the water, would be recommended for ballast water treatment

systems. The removal of these larger organisms and particles from the water subsequently enhances the effectiveness of the secondary treatment by increasing the clarity of the water and reducing the number of particles present. For example, in terms of a UV treatment, this increased clarity means that there are fewer non-target particles to absorb the UV light, and that the UV transmission through the water is increased, thus the overall effectiveness of the UV system is optimised. The filter tested in this system was effective at flow rates >91.8m^3hr^{-1} and high organism input density did not reduce efficiency. The self-cleaning mechanism did not affect the flow of water through the filter and so would not reduce efficiency if in use onboard a vessel. Filtration would be recommended for use in ballast water treatment, but the inclusion of a secondary disinfection treatment would be necessary for treatment to meet the D-2 discharge standard.

The final aspect investigated in this thesis was the identification of effective viability assessment methods which are vital for accurate assessment of biological effectiveness of any ballast water treatment system. The work completed shows that the stains currently used to assess viability, often in research not related to ballast water, can be optimised to the specific organisms being used in order to gain >99% success. When applied to natural populations the success in some stains decreased, highlighting the importance of pilot tests to optimise the method to be employed to ensure high staining success. The stains used in this study do not require specialist equipment and could easily be applied in any laboratory. Some could be utilised when performing shipboard tests, e.g. neutral red and Evans blue. In regards to zooplankton assessment visually observing organisms for movement is also an accurate examination method and has been employed in various ballast water treatment system testing studies (Sutherland et al. 2003; Veldhuis et al. 2006), but may have limitations if used during onboard testing due to the motion of the ship. The identification and application of accurate assessment methods is vital to ensure that accurate viability assessments are made when testing treatment systems.

This thesis has addressed one mechanism by which anthropogenic activity detrimentally impacts the Earth: the transportation of organisms across the globe in ballast water. Before working to effectively reduce the impacts of ballast water transportation it is vital to address the scale of the problems caused. After surviving transportation invasive species affect the environment, economy and pose threats to human health (E.g. Hallegraeff and Bolch 1991; Ruiz et al. 1997; Hall and Mills 2000; Herborg et al. 2003; Lewis et al. 2003; Bolch and de Salas 2007) affecting both marine and freshwater ecosystems. Ballast water is required by ships and as such this transportation mechanism cannot be stopped, more so the volume of ballast water transported is increasing as global shipping trade increases. Management is the only option available. Firstly this requires an understanding of the transportation conditions experienced within ballast and the effects they have on organisms present. Secondly, the best management option available is to 'treat' the ballast water to kill the organisms present.

This thesis enhances our understanding of the effects of ballast water transportation conditions on organism survival. When added to the current literature it will aid improved risk management for the prevention of new invasions via ballast water. Management cannot rely simply upon treatment systems, especially as these are not currently present on all vessels. The 'unintentional' processes described in this thesis play a major part in the successful transportation of organisms. With increased understanding of how uptake and storage affect plankton, as has been described in this thesis, we can better predict high risk transportation routes. This, combined with further research and development of effective treatment systems, will lead to the minimisation of successful invasions via ballast water.

The introduction to this thesis described the complexity of interactions within ecosystems and the necessity for consideration of all aspects to be incorporated in effective management techniques. The example used in this thesis was ballast water transportation; a vital process which cannot be halted. This is true of many practices which need management, e.g. the fishing industry, the use of chemicals in agriculture

and power generation by nuclear radiation. These practices can pose threats to both the environment and human health, but the benefits gained necessitate their continued use. Only by addressing all aspects, i.e. benefits and costs, can we implement effective measures to prevent further detrimental impacts. Effective management is paramount for reducing the impacts of human activity and protecting the Earth for a sustainable future.

References

Agusti S, Alou E, Hoyer MV, Frazer TK, Canfield DE (2006) Cell death in lake phytoplankton communities. Freshwater Biology 51: 1496-1506

Agusti S, Llabres M (2007) Solar radiation induced mortality of marine pico-phytoplankton in the oligotrophic ocean. Photochemistry and Photobiology 83: 793-801

Agusti S, Sanchez CM (2002) Cell viability in natural phytoplankton communities quantified by a membrane permeability probe. Limnology and Oceanography 47: 818-828

Akin EW, Hoff JC, Lippy EC (1982) Waterborne outbreak control: which disinfectant? Environmental Health Perspectives. 46: 7-12

Alam MD, Otaki M, Furumai H, Ohgaki S (2000) Direct and indirect inactivation of *Microcystis aeruginosa* by UV radiation. Water Research 35: 1008-1014

Allhands M, Prochaska J (1996) Disc filtration: Something old and something new. In: Schernier B (ed) Proceedings of the American Filtration and Seperation Society Meeting, Valley Forge, VA, pp 496-500

Altman SA, Randers L, Rao G (1993) Comparisons of Trypan Blue dye exclusion and fluorometric assays for mammalian cell viability determinations. . Biotechnology Progress. 9: 671-674

Andrewartha H (1952) Diapause in relation to the ecology of insects. Biological Reviews. 27: 50-107.

Anon (1997a) Heat treatment solution for infected ballast water. Mer-Marine Engineers Review: 15-15

Anon (1997b) Out with the old - A new method of exchanging infected ballast water. Mer-Marine Engineers Review: 16-17

Anon (2005) Filter tests complete on new ballast water system. The Naval Architect January 24

Anon (2006a) Ballast water management - the green solution. The Motor Ship: 12

Anon (2006b) Ballast water management system for sediment removal. Naval Architect: 26-26

Antia N (1976) Effects of temperature on the darkness survival of marine microplanktonic algae. Microbial Ecology. 3: 41-54.

Antopol SC, Ellner PD (1979) Susceptibility of *Legionella pnuemophila* to ultraviolet radiation. Applied and Environmental Microbiology 38: 347-348

Arkal Filtration Systems Disc Filtration Technology, pp Technical description

Arnold W (1933) The effects of ultraviolet light on photosynthesis. Journal of General Physiology 17: 135-143

Bailey S, Duggan I, van Overdijk C, Jenkins P, MacIsaac H (2003) Viability of invertebrate diapausing eggs collected from residual ballast sediment. Limnology and Oceanography 48: 1701-1710

Baker M, Taras M (1981) The quest for pure water: a history of the twentieth century. AWWA, Denver

Bamber RN, Seaby RMH (2004) The effects of power station entrainment passage on three species of marine planktonic crustacean, *Acartia tonsa* (Copepoda), *Crangon crangon* (Decapoda) and *Homarus gammarus* (Decapoda). Marine Environmental Research 57: 281-294

Barnard JE, Morgan HR (1903) The physical factors in phototherapy. The British Medical Journal 14: 1269-1271

Barry SC, Hayes KR, Hewitt CL, Behrens HL, Dragsund E, Bakke SM (2008) Ballast water risk assessment: principles, processes, and methods. . ICES Journal of Marine Science 66: 121-131

Batjer K, Gabel B, Koschorrek M, Lahl U, Lierse KW, Stachel B, Thiemann W (1980) Drinking water in Bremen: trihalomethanes and social costs. A case study of bromoform formation during chlorination of river water highly contaminated with bromide ions. . The Science of the Total Environment. 14: 287-291

Bax N, Williamson A, Aguero M, Gonzalez E, Geeves W (2003) Marine invasive alien species: a threat to global biodiversity. Marine Policy 27: 313-323

Behrens HL, Leppakoski E, Olenin S (2005) Ballast water risk assessment guidelines for the North Sea and Baltic Sea. Nordic Innovation Centre, TR 587

Bergendahl JA, Stevens L (2005) Oxidation reduction potential as a measure of disinfection effectiveness for chlorination of wastewater. . Environmental Progress 24: 214-222

Binet MT, Stauber JL (2006) Rapid flow cytometric method for the assessment of toxic dinoflagellate cyst viability. Marine Environmental Research 62: 247-260

Bird A, Quinn R (1986) Isolation of symbiotic dinoflagellates by centrifugal elutriation. Limnology and Oceanography 31: 225-228

Bitton G, Henis Y, Lahav N (1972) Effect of several clay minerals and humic acid on the survival of *Klebsiella aerogenes* exposed to ultraviolet irradiation. Applied Microbiology 23: 870-874

Björn LO (1996) Effects of ozone depletion and increased UV-B on terrestrial ecosystems. International Journal of Environmental Studies 51: 217-243

Bohrerova Z, Linden KG (2006) Assessment of DNA damage and repair in *Microbacterium terrae* after exposure to UV irradiation. Journal of Applied Microbiology 101: 995-1001

Bolch CJ, Hallegraeff GM (1993) Chemical and Physical options to kill toxic dinoflagellate cysts in ships' ballast water. Journal of Marine Environmental Engineering 1: 23-29

Bolch CJS, de Salas MF (2007) A review of the molecular evidence for ballast water introduction of the toxic dinoflagellates Gymnodinium catenatum and the Alexandrium "tamarensis complex" to Australasia. Harmful Algae 6: 465-485

Bratosin D, Mirtrofan L, Palii C, Estaquier J, Montreuil J (2005) Novel fluorescence assay usng Calcein-AM for the determination of human erythrocyte viability and aging. Cytometry Part A 66A: 78-84

Browning WJJ, Davis JP, Browning WJ (2003) Progress report on the AquaHabiStat deoxygenation system. In: Matheickal JT, Raaymakers S (eds) 2nd International Ballast Water Treatment R&D Symposium, IMO, London, pp 26-33

Brungs WA (1973) Effects if residual chlorine on aquatic life. Journal of the Water Pollution Control Federation. 45: 2180-2193

Brussaard CPD, Marie D, Thyrhaug R, Bratbak G (2001) Flow cytometric analysis of phytoplankton viability following viral infection. Aquatic Microbial Ecology 26: 157-166

Bukhari Z, Hardy TM, Bolton JR, Dussert B, Clancy JL (1999) Medium pressure UV light for oocyst inactivation. Journal of the American Water Works Association 91: 86-94

Bunt JS, Lee CC (1972) Data on the composition and dark survival of four sea-ice microalgae. Limnology and Oceanography 17: 458-461

Buttino I, Santo MD, Ianora A, Miralto A (2004) Rapid assessment of copepod (Calanus helgolandicus) embryo viability using fluorescent probes. Marine Biology 145: 393-399

Calkins J, Thordardottir T (1980) The ecological significance of solar UV radiation on aquatic organisms. Nature 283: 563-566

Caminiti IM, Palgan I, Munoz A, Noei F, Whyte P, Morgan DJ, Cronin DA, Lyng JG (2009) The effect of ultraviolet light on microbial inactivation and quality attributes of apple juice. Food and Bioprocessing Technology Publised online

Campbell M, Gould B, Hewitt C (2007) Survey evaluations to assess marine bioinvasions. Marine Pollution Bulletin 55: 360-378

Cangelosi A, Mays N, Balcer M, Reavie E, Reid D, Sturtevant R, Gao X (2007) The response of zooplankton and phytoplankton from the North American Great Lakes to filtration. Harmful Algae 6: 547-566

Carlson DA, Seabloom RW, DeWalle FB, Wetzler TF, Engeset J, Butler R, Wangsuphachart S, Wang S (1985) Ultraviolet disinfection of water for small water supplies. United Stated Environmental Protection Agency, Cincinnati

Carlton JT (1985) Trans-oceanic and interoceanic dispersal of coastal marine organisms - The biology of ballast water. Oceanography and Marine Biology 23: 313-371

Carlton JT (1996) Pattern, process, and prediction in marine invasion ecology. Biological Conservation. 78: 97-106.

Carlton JT, Geller JB (1993) Ecological roulette - the global transport of nonindigenous marine organisms. Science 261: 78-82.

Carnimeo D, Contini E, Di Marino R, Donadio F, Liberti L, Ranieri E (1994) Wastewater disinfection by UV at Trani municipal plant. Water Science and Technology 30: 125-132

Carpenter C, Fayer R, Trout J, Beach (1999) Chlorine disinfection of recreational water for *Cryptosporidium parvum*. Emerging Infectious Diseases 5: 579-584

Carson R (1962) Silent spring. Penguin in association with Hamilton, London

Castilla JC, Neill PE (2008) Marine bioinvasions in the Southeastern Pacific: status, ecology, economic impacts, conservation and management. . In: Caldwell M, Heldmaier G, Jackson R, Lange O, Mooney H, Schulze E, Sommer U (eds) Biological invasions in marine ecosystems: ecological, management, and geographic perspectives. Springer, Dordrecht, pp 439-458.

CEPA (2002) Evaluation of ballast water treatment technology for control of nonindigenous aquatic organisms. California Environmental Protection Agency

Champ MA (2002) Marine Testing Board for certification of ballast water treatment technologies. Marine Pollution Bulletin 44: 1327-1335

Chase C, Reilly C, Pederson J (2009) Marine Bioinvasions fact sheet: Ballast water treatment options. Sea Grant, pp 1-4

Chelossi E, Faimali M (2006) Comparative assessment of antimicrobial efficacy of new potential biocides for treatment of cooling and ballast waters. Science of the Total Environment 356: 1-10

Chitarra LG, Breeuwer P, Abee T, Bulk RW (2006) The use of fluorescent probes to assess viability of the plant pathogenic bacterium *Clavibacter michinganensis* subsp. *michiganensis* by flow cytometry. Fitopatologia Brasileira 31: 349-356

Cho M, Kim J, Kim JY, Yoon J, Kin JH (2010) Mechanisms of *Escherichia coli* inactivation by several disinfectants. Water Research 44: 3410-3418

Chowdhury S, Champagne P (2008) An investigation on parameters for modeling THMs formation. Global NEST Journal 10: 80-91

Christensen NL, Bartuska A, Brown JH, Carpenter S, D'Antonio C, Francis R, Franklin JF, MacMahon JA, Noss RF, Parsons DJ, Peterson CH, Turner MG, Moodmansee RG (1996) The report of the Ecological Society of America Committee on the scientific basis for ecosystem management. Ecological Applications. 6: 665-691

Chrtek S, Popp W (1991) UV disinfection of secondary effluents from sewage treatment plants. Water Science and Technology 24: 343-346

Clancy JL, Bukhari Z, Hardy TM, Bolton JR, Dussert B, Marshall MM (2000) Using UV to inactivate *Cryptosporidium*. Journal of the American Water Works Association 92: 97-104

Clemence W (1911) The water supply of marseilles, and trials of filtering and sterilising apparatus. . Engineering 91: 106, 139-142

Cohen AN, Calder DR, Carlton JT, Chapman JW, Harris LH, Kitayama T, Lambert CC, Lambert G, Piotrowski C, Shouse M, Solorzano LA (2005) Rapid assessment shore surve for exotic species in San Francisco Bay - May 2004. Final report for the California State Coastal Conservancy, Association of Bay Area Governments/San Francisco Bay - Delta Science Consortium, National Geographic Society and Rose Foundation. . San Francisco Estuary Institute, Oakland, CA

Cohen AN, Carlton JT (1997) Transoceanic transport mechanisms: Introduction of the Chinese Mitten Crab, *Eriocheir sinensis*, to California. Pacific Science 51: 1-11

Conn H, Cunningham R (1932) History of staining: The use of dyes as vital stains. Stain Technology 7: 81-90

Conn HJ (1946) Biological stains: a handbook on the nature and uses of the dyes employed in the biological laboratory. Biotech Publication, Geneva, U.S.A

Conover RJ, Siferd TD (1993) Dark season survival strategies of coastal zone zooplankton in the Canadian Arctic. Arctic. 46: 303-311.

Cooper IR, Hanlon GW (2010) Resistance of *Legionella pneumophila* serotype 1 biofilms to chlorine-based disinfection. Journal of Hospital Infection 74: 152-159

Cooper W, Jones A, Whitehead R, Zika R (2007) Sunlight-induced photochemical decay of oxidants in natural waters: implications in ballast water treatment. Environmental Science and Technology 41: 3728-3733

Coutts A, Moore K, Hewitt C (2003) Ships' sea chests: an overlooked transfer mechanism for non-indigenous marine species? Marine Pollution Bulletin 46: 1504-1515

Coutts ADM, Dodgshun TJ (2007) The nature and extent of organisms in vessel sea chests: A protected mechanism for marine bioinvasions. Marine Pollution Bulletin 54: 875-886

Craik SA, Weldon D, Finch GR, Bolton JR, Belosevic M (2001) Inactivation of *Cryptosporidium parvum* oocysts using medium- and low-pressure ultraviolet radiation. Water Research 35: 1387-1398

Crippen R, Perrier J (1974) The use of neutral red and Evans blue for live dead determinations of marine plankton (with comments on the use of Rotenone for inhibition of grazing). Stain Technology 49: 97-104

Crutchfield ALM, Diller KR, Brand JJ (1999) Cryopreservation of Chlamydomonas reinhardtii (Chlorophyta). European Journal of Phycology 34: 43-52

Cutler SJ, Cutler HG, Glinski J, Wright DA, Dawson R, Lauren D (2004) SeaKleen, a potential product for controlling aquatic pests in ships' ballast water. In: Matheickal JT, Raaymakers S (eds) 2nd International Ballast Water Treatment R&D Symposium, IMO, London, pp 164-174

David M, Gollasch S, Cabrini M, Perkovic M, Bosnjak D, Virgilio D (2007) Results from the first ballast water sampling study in the Mediterranean Sea - the Port of Koper study. Marine Pollution Bulletin. 54: 53-65.

de Lafontaine Y, Despatie S, Wiley C (2008) Effectiveness and potential toxicological impact of PERACLEAN Ocean ballast water treatment technology. Ecotoxicology and Environmental Safety 71: 355-369

Demers S, Roy S, Gagnon R, Vignault C (1991) Rapid light-induced changes in cell fluorescence and in xanthophyll-cycle pigments of *Alexandrium excavatum* (Dinophyceae) and *Thalassiosira pseudonana* (Bacillariophyceae): a photo-protective mechanism. Marine Ecology Progress Series. 76: 185-193

Dibdin WJ (1903) The purification of sewage and water. The Sanitary Publishing Company, London, New York

Dickman M, Zhang FZ (1999) Mid-ocean exchange of container vessel ballast water. 2: Effects of vessel type in the transport of diatoms and dinoflagellates from Manzanillo, Mexico, to Hong Kong, China. Marine Ecology Progress Series. 176: 253-262.

Dittel AI, Epifanio CE (2009) Invasion biology of the Chinese mitten crab *Eriochier sinensis*: a brief review. Journal of Experimental Marine Biology and Ecology 374: 79-92

Downes MD, Blunt TP (1877) Researches on the effect of light upon Bacteria and other organisms. Proceedings of the Royal Society of London 26: 488-500

Drake LA, Doblin MA, Dobbs FC (2007) Potential microbial bioinvasions via ships' ballast water, sediment, and biofilm. Marine Pollution Bulletin. 55: 333-341.

Drake LA, Ruiz GM, Galil BS, Mullady TL, Friedman DO, Dobbs FC (2002) Microbial ecology of ballast water during a transoceanic voyage and the effects of open-ocean exchange. Marine Ecology-Progress Series. 233: 13-20

Dressel DM, Heinle DR, Grote MC (1972) Vital staining to sort dead and live copepods. Chesapeake Science 13: 156-159

Elliott M (2003) Biological pollutants and biological pollution - an increasing cause for concern. Marine Pollution Bulletin 46: 275-280

Elton C (1958) The ecology of invasions by animals and plants. Chapman and Hall, London

Emerick RW, Loge FJ, Thompson D, Darby JL (1999) Factors influencing ultraviolet disinfection performance Part II: association of coliform bacteria with wastewater particles. Water Environment Research 71: 1178-1187

Endresen O, Behrens HL, Brynestad S, Andersen AB, Skjong R (2004) Challenges in global ballast water management. Marine Pollution Bulletin 48: 615-623

Eno NC (1996) Non-native marine species in British waters: effects and controls. Aquatic Conservation Marine and Freshwater Ecosystems 6: 215-228

Eno NC, Clark RA, Sanderson WG (1997) Non-native marine species in British waters: a review and directory. Joint Nature Conservation Committee

Faimali M, Garaventa F, Chelossi E, Piazza V, Saracino O, Rubino F, Mariottini G, Pane L (2006) A new photodegradable molecule as a low impact ballast water biocide: efficacy screening on marine organisms from different trophic levels. Marine Biology 149: 7-16

Farr BM, Gratz JC, Tartaglino JC, Getchell-White SI, Groschell DHM (1988) Evaluation of ultraviolet light for disinfection of hospital water contaminated with *Legionella*. . The Lancet: 669-672

Ferrato C, Ward L, Roles A, Ceri H, Drinkwater D, Louie T, Louie M (2009) P317 Impact of ultraviole light sterilization on microbiological water quality in a newly designed hospital water distribution system (WDS). International Journal of Antimicrobial Agents. 34: S125

Ferreira CEL, Junqueira AOR, Villac MC, Lopes RM (2008) Marine bioinvasions in the Brazilian coast: brief report on history of events, vectors, ecology, impacts and management of non-indigenous species. In: Caldwell M, Heldmaier G, Jackson R, Lange O, Mooney H, Schulze E, Sommer U (eds) Biological invasions in marine ecosystems: ecological, management, and geographic perspectives. Springer, Dordrecht, pp 459-478.

Fofonoff PW, Ruiz GM, Hines AH, Steves BD, Carlton JT (2008) Four centuries of biological invasions in tidal waters of the Chesapeake Bay region. In: Caldwell M, Heldmaier G, Jackson R, Lange O, Mooney H, Schulze E, Sommer U (eds) Biological invasions in marine ecosystems: ecological, management, and geographic perspectives. Springer, Dordrecht, pp 479-506

Franklin D, Berges J (2004) Mortality in cultures of the dinoflagellate *Amphidinium carterae* during culture senescence and darkness. Proceedings of the Royal Society B: Biological Sciences. 271: 2099-2107.

Franklin DJ, Hoegh-Guldberg O, Jones RJ, Berges JA (2004) Cell death and degeneration in the symbiotic dinoflagellates of the coral *Stylophora pistillata* during bleaching. Marine Ecology-Progress Series 272: 117-130

Fritz L, Quilliam MA, Wright JLC, Beale AM, Work TM (1992) An outbreak of domoic acid poisoning attributed to the pennate diatom *Pseudonitzschia australis*. Journal of Phycology 28: 439-442.

Fuchs R, de Wilde I (2004) Peraclean Ocean - a potential environmentally friendly and effective treatment option for ballast water. In: Matheickal JT, Raaymakers S (eds) 2nd International Ballast Water Treatment R&D Symposium, IMO, London, pp 175-180

Fuchs R, Steiner N, de Wilde I, Voigt M (2001) Peraclean Ocean - a potential ballast water treatment option. In: Raaymakers S (ed) 1st International Ballast Water Treatment R&D Symposium, International Maritime Organisation, London, pp 76-80

Furusato E, Asaeda T, Manatunge J (2004) Tolerance for prolonged darkess of three phytoplankton species, *Microcystis aeruginosa* (Cyanophyceae), *Scenedesmus*

quadricauda (Chlorophyceae) and *Melosira ambigua* (Bacillariophyceae). Hydrobiologia. 527: 153-162.

Gaff DF, Okong'O-Ogola O (1971) The use of non-permeating pigments for testing the survival of cells. Journal of Experimental Botany 22: 756-758

Gala WR, Giesy JP (1991) Effects of ultraviolet radiation on the primary production of natural phytoplankton assemblages in Lake Michigan. Ecotoxicology and Environmental Safety 22: 345-361

Galil BS (2008) Alien species in the Mediterranean Sea - which, when, where, why? Hydrobiologia 606: 105-116.

Garvey M, Moriceau B, Pasow U (2007) Applicability of the FDA assay to determine the viability of marine phytoplankton under different environmental conditions. Marine Ecology Progress Series 352: 17-26

Gavand MR, McClintock JB, Amsler CD, Peters RW, Angus RA (2007) Effects of sonication and advanced chemical oxidants on the unicellular green alga *Dunaliella tertiolecta* and cysts, larvae and adults of the brine shrimp *Artemia salina*: A prospective treatment to eradicate invasive organisms from ballast water. Marine Pollution Bulletin 54: 1777-1788

Gehr R, Wagner M, Veerasubramanian P, Payment P (2003) Disinfection efficiency of peracetic acid, UV and ozone after enhanced primary treatment of municipal wastewater. Water Research 37: 4573-4586

Gilbert F, Galgani F, Cadiou Y (1992) Rapid assessment of metabolic activty in marine microalgae: application in ecotoxicological tests and evaluation of water quality. Marine Biology 112: 199-205

Gollasch S (2002) The importance of ship hull fouling as a vector of species introductions into the North Sea. Biofouling 18: 105-121

Gollasch S (2007) International collaboration on marine bioinvasions - The ICES response. Marine Pollution Bulletin 55: 353-359

Gollasch S, Haydar D, Minchin D, Wolff WJ, Reise K (2008) Introduced aquatic species of the North Sea coasts and adjacent brackish waters. In: Caldwell M, Heldmaier G, Jackson R, Lange O, Mooney H, Schulze E, Sommer U (eds) Biological invasions in marine ecosystems: ecological, management and geographic perspectives. Springer, Dordrecht, pp 507-548

Gollasch S, Lenz J, Dammer M, Andres HG (2000a) Survival of tropical ballast water organisms during a cruise from the Indian Ocean to the North Sea. Journal of Plankton Research. 22: 923-937.

Gollasch S, Leppakoski E (1999) Initial risk assessment of alien species in Nordic coastal waters. Nordic Council of Ministers, Copenhagen

Gollasch S, Leppakoski E (2007) Risk assessment and management scenarios for ballast water mediated species introductions into the Baltic Sea. Aquatic Invasions 2: 313-340

Gollasch S, Rosenthal H, Botnen H, Hamer J, Laing I, Leppakoski E, Macdonald E, Minchin D, Nauke M, Olenin S, Utting S, Voigt M, Wallentinus I (2000b) Fluctuations of zooplankton taxa in ballast water during short-term and long-term ocean-going voyages. International Review of Hydrobiology. 85: 597-608.

Gomoiu M, Alexandrov B, Shadrin N, Zaitsev Y (2002) The Black Sea - a recipient, donor and transit area for alien species. In: Leppakoski E, Gollasch S, Olenin S (eds) Invasive aquatic species of Europe. Distribution, impacts and management. Kluwer Academic Publishers, Dordrecht, pp 341-350

Goodsell D (2001) The molecular perspective: Ultraviolet light and pyrimidine dimers. The Oncologist 6: 298-299

Goulletquer P, Bachelet G, Sauriau P, Noel P (2002) Open Atlantic coasts of Europe - a century of introduced species into French waters. In: Leppakoski E, Gollasch S, Olenin S (eds) Invasive aquatic species of Europe. Distribution, impacts and management. Kluwer Academic Publishers, Dordrecht, pp 276-290

Gray D, Duggan I, MacIsaac H (2006) Can sodium hypochlorite reduce the risk of species introductions from diapausing invertebrate eggs in non-ballasted ships? Marine Pollution Bulletin 52: 689-695

Gregg M, Rigby G, Hallegraeff G (2009) Review of two decades of progress in the development of management options for reducing or eradicating phytoplankton, zooplankton and bacteria in ship's ballast water. Aquatic Invasions. 4: 521-565

Gregg MD, Hallegraeff GM (2007) Efficacy of three commercially available ballast water biocides against vegetative microalgae, dinoflagellate cysts and bacteria. Harmful Algae 6: 567-584

Griffiths CL, Robinson TB, Mead A (2008) The status and distribution of marine alien species in South Africa. In: Caldwell M, Heldmaier G, Jackson R, Lange O, Mooney H, Schulze E, Sommer U (eds) Biological invasions in marine ecosystems:ecological, management and geographic perspectives. Springer, Dordrecht, pp 393-408

Guerrero-Beltran JA, Barbosa-Canovas GV (2004) Advantages and limitations on processing foods by UV light. Food Science and Technology International, pp 137-147

Guo M, Hu H, Bolton JR, El-Din MG (2009) Comparison of low- and medium-pressure ultraviolet lamps: photoreactivation of *Escerichia coli* and total coliforms in secondary effluents of municipal wastewater treatment plants. . Water Research 43: 815-821

Hader DP, Worrest RC (1991) Effects of enhanced solar ultraviolet radiation on aquatic ecosystems. Photochemistry and Photobiology 53: 717-725

Hall SR, Mills EL (2000) Exotic species in large lakes of the world. Aquatic Ecosystem Health and Management 3: 105-135

Hallegraeff GM (2007) Ballast water - Foreword. Harmful Algae 6: 461-464

Hallegraeff GM, Bolch CJ (1991) Transport of toxic dinoflagellates in cysts via ships ballast water. Marine Pollution Bulletin 22: 27-30.

Hallegraeff GM, Bolch CJ (1992) Transport of diatom and dinoflagellate resting spores in ships ballast water - implications for plankton biogeography and aquaculture. Journal of Plankton Research 14: 1067-1084.

Hallegraeff GM, Valentine JP, Marshall JA, Bolch CJ (1997) Temperature tolerances of toxic dinoflagellate cysts: application to the treatment of ships' ballast water. Aquatic Ecology 31: 47-52

Hamer J, McCollin T, Lucas IAN (2000) Dinoflagellate cysts in ballast tank sediments: between tank variability. Marine Pollution Bulletin. 40: 731-733.

Hanes DE, Worobo RW, Orlandi PA, Burr DH, Miliotis MD, Robl MG, Bier JW, Arrowood MJ, Churey JJ, Jackson GJ (2002) Inactivation of *Cryptosporidium parvum* oocysts in fresh apple cider by UV irradiation. Applied and Environmental Microbiology 68: 4168-4172

Harrington WO, Hills CH (1968) Reduction of the microbial population of apple cider by ultraviolet radiation. Food Technology 22: 117-120

Harris ASD, Medlin LK, Lewis J, Jones KJ (1995) *Thalassiosira* species (Bacillariophyceae) from a Scottish sea loch. European Journal of Phycology 30: 117-131

Harris GD, Adams VD, Sorensen DL, Dupont RR (1987) The influence of photoreactivation and water quality on ultraviolet disinfection of secondary municipal wastewater. Journal of Water Pollution Control Federation 59: 781-787

Harris RP, Fortier L, Young RK (1986) A large vertical pump system for the studies of the vertical distribution of fish larvae under open sea conditions. Journal of the Marine Biological Association of the United Kingdom 66: 845-854

Haugland RP (1992) Molecular Probes: Handbook of fluorescent probes and research chemicals

Hayden B, Inglis G, Schiel D (2008) Marine invasions in New Zealand: a history of complex supply side dynamics. In: Caldwell M, Heldmaier G, Jackson R, Lange O, Mooney H, Schulze E, Sommer U (eds) Biological invasions in marine ecosystems: ecological, management and geographic perspectives. Springer, Dordrecht, pp 409-424.

Hayes K, Sliwa C (2003) Identifying marine pests - a deductive approach applied to Australia. Marine Pollution Bulletin 46: 91-98

Hayes KR (1998) Ecological risk assessment for ballast water introductions:A suggested approach. ICES Journal of Marine Science 55: 201-212

Hayes KR, Hewitt CL (1998) Risk assessment framework for ballast water introductions. CRIMP Technical Report no. 14.

Hayes KR, Hewitt CL (2000) Risk assessment framework for ballast water introductions - Volume 2. CSIRO, Centre for research on introduced marine pests. Hobart, Australia.

Haynes P (1989) The recurring silent spring. Pergamon Press, New York

Herbert G (2002) Full scale design studies of ballast water treatment systems. Northeast Midwest Institute

Herborg LM, Rushton SP, Clare AS, Bentley MG (2003) Spread of the Chinese mitten crab (Eriocheir sinensis H. Milne Edwards) in Continental Europe: analysis of a historical data set. Hydrobiologia 503: 21-28.

Herwig R, Cordell J, Perrins J, Dinnel P, Gensemer R, Stubblefield W, Ruiz G, Kopp J, House M, Cooper W (2006) Ozone treatment of ballast water on the oil tanker *S/T Tonsina*:chemistry, biology and toxicity. Marine Ecology Progress Series 324: 37-55

Hess-Erga OK, Attramadal KJK, Vadstein O (2008) Biotic and abiotic particles protect marine heterotrophic bacteria during UV and ozone disinfection. Aquatic Biology 4: 147-154

Hess-Erga OK, Blomvagnes-Bakke B, Vadstein O (2010) Recolonization by heterotrophic bacteria after UV irradiation or ozonation of seawater; a simulation of ballast water treatment. Water Research doi:10.1016/j.watres.2010.06.059

Hockberger PE (2002) A history of ultraviolet photobiology for humans, animals and microorganisms. Photochemistry and Photobiology 76: 561-569

Holt AS, Brooks IA, Arnold WA (1951) Some effects of 2537Å on green algae and chloroplast preparations. Journal of General Physiology 34: 627-645

Hopkins C (2002) Introduced marine organisms in Norwegian waters, including Svalbard. In: Leppakoski E, Gollasch S, Olenin S (eds) Invasive aquatic species of Europe: distribution, impacts and management. Kluwer Academic Publishers, Dordrecht, pp 240-252

Horobin R (2002a) Dis-, tris- and polyazo dyes. In: Horobin R, Kiernan J (eds) Conns biological stains: a handbook of dyes, stains and fluorochromes for use in biology and medicine. BIOS Scientific Publishers, Oxford, UK

Horobin R (2002b) Mechanisms of biological staining. In: Horobin R, Kiernan J (eds) Conns biological stains: a handbook of dyes, stains and fluorochromes for use in biology and medicine. BIOS Scientific Publishers, Oxford, UK

Hoyer O (1998) Testing performance and monitoring of UV systems for drinking water disinfection. Water Supply 16: 419-424

Hu J, Quek PH (2008) Effects of UV radiation on photoyase and implications with regards to photoreactivation following low and medium pressure Uv disinfection. Applied and Environmental Microbiology 74: 327-328

IMO (2004) International Convention for the Control and Management of Ships' Ballast Water and Sediments

Iseri Y, Kawabata Z, Sasaki M (1993) Development of a boat equipped with UV lamps for suppression of freshwater red tide in a reservoir. Japanese Journal of Water Treatment Biology 29: 61-70

Iseri Y, Nakamichi M, Miyagawa S, Maeda T (2004) Development of a red tide control ship equipped with a UV irradiation system - preliminary and field experiments on harmful marine algae. Oceans MTS/Ieee Techno-Ocean, pp 1787-1792

Jansen S, Bathmann U (2007) Algae viability within copepod faecal pellets: evidence from microscopic examinations. Marine Ecology Progress Series 337: 145-153

Jarroll EL, Bingham AK, Meyer EA (1981) Effect of chlorine on *Giardia lamblia* cyst viability. Applied and Environmental Microbiology 41: 483-487

Jelmert A (1999) Testing the effectiveness of an integrated hydrocyclone/UV-treatment system for ballast water. Consultancy report, Institute of Marine Research, Austevoll Aquaculture Research Station

Jitts HR, Morel A, Saijo Y (1976) The relation of oceanic primary production to available photosynthetic irrandiance. Australian Journal of Marine and Freshwater Research 27: 441-454

Jochem FJ (1999) Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate. Marine Biology. 135: 721-728.

Jochem FJ (2005) Short-term physiologic effects of mechanical flow sorting and the Becton-Dickinson cell concentrator in cultures of the marine phytoflagellata Emiliania huxleyi and Micromonas pusilla. Cytometry Part A 65A: 77-83

Johnston EL, Piola RF, Clark GF (2009) The role of propagule pressure in invasion success. In: Rilov G, Crooks JA (eds) Biological invasions in marine ecosystems. Springer-Verlag, Berlin

Kang J, Hyun B, Shin K (2010) Phytoplankton viability in ballast water from international commercial ships berthed at ports in Korea. Marine Pollution Bulletin. 60: 230-237.

Kasten F (2002) Introduction to dyes and stains. In: Horobin R, Kiernan J (eds) Conns Biological stains: a handbook of dyes, stains and fluorochromes for use in biology and medicine. BIOS Scientific Publishers Oxford, UK

Keller RP, Drake JM, Drew MB, Lodge DM (2011) Linking environmental conditions and ship movements to estimate invasive species transport across the global shipping network. . Diversity and Distributions 17: 93-102

Kelly CB (1965) Disinfection of sea water by ultraviolet radiation. Salt water fisheries Division, Florida Board of Conservation., Tallahassee, Florida

Kelly CB (1971) Disinfection of seawater by ultraviolet radiation. American Journal of Public Health 51: 1670-1680

Kelly JM (1993) Ballast water and sediments as mechanisms for unwanted species introductions into Washington state. Journal of Shellfish Research 12: 405-410.

Kelly S, Sanderson S (1958) The effect of chlorine in water on enteric viruses. American Journal of Public Health 48: 1323-1334

Kent ML, Whyte JNC, LaTrace C (1995) Gill lesions and mortality in seawater pen-reared Atlantic salmon *Salmo salar* associated with a dense bloom of *Skeletonema costatum* and *Thalassiosira* species. Diseases of Aquatic Organisms 22: 77-81.

Keyser M, Muller IA, Cilliers FP, Nel W, Gouws PA (2008) Ultraviolet radiation as a non-thermal treatment for the inactivation of microorganisms in fruit juice. Innovative Food Science and Emerging Technologies. 9: 348-354

Kideys AE, Roohi A, Bagheri S, Finenko G, Kamburska L (2005) Impacts of invasive ctenophores on the fisheries of the Black Sea and Caspian Sea. Oceanography 18: 76-99

Klein G, MacIntosh K, Kaczmarska I, Ehrman J (2010) Diatom survivorship in ballast water during trans-Pacific crossings. Biological Invasions 12: 1031-1044.

Knudson GB (1985) Photoreactivation of UV irradiated *Legionella pnuemophila* and other *Legionella* species. Applied and Environmental Microbiology 49: 975-980

Komen J, Wolbers F, Franke H, Andersson H, Vermes I, van den Berg A (2008) Viability analysis and apoptosis induction of breast cancer cells in a microfluidic device: effect of cytostatic drugs. Biomedical Microdevices 10: 727-737

Korich DG, Mead JR, Madore MS, Sinclair NA, Sterling CR (1990) Effects of ozone, chlorine dioxide, chlorine and monochloramine on *Cryptosporidium parvum* oocyst viability. Applied and Environmental Microbiology 56: 1423-1428

Koutchma T, Keller S, Chirtel S, Parisi B (2004) Ultraviolet disinfection of juice products in laminar and turbulent flow reactors. Innovative Food Science and Emerging Technologies. 5: 179-189

Kowalski W (2009) Ultraviolet germicidal irradiation handbook: UVGI for air and surface disinfection. Springer Berlin

Kruithof JC, Kamp PC, Martjin BJ (2007) UV/H_2O_2 treatment: a practical solution for organic contaminant control and primary disinfection. Ozone: Science and Engineering 29: 273-280

La Carbona S, Viitasalo-Frosen S, Masson D, Sassi J, Pineau S, Lehtiniemi M, Corroler (2010) Efficacy and environmental acceptability of two ballast water treatment chemicals and an alkaline based-biocide. Science of the Total Environment 409: 247-255

Lahdes E (1995) Acute thermal tolerance of two Antarctic copepods, *Calnoides acutus* and *Calanus propinquus*. Journal of Thermal Biology 20: 75-78

Lavoie DM, Smith LD, Ruiz GM (1999) The potential for intracoastal transfer of non-indigenous species in the ballast water of ships. Estuarine Coastal and Shelf Science. 48: 551-564.

Leahy JG, Rubin AJ, Sproul OJ (1987) Inactivation of *Giardia muris* cysts by free chlorine. Applied and Environmental Microbiology 53: 1448-1453

Lebaron P, Catala P, Parthuisot N (1998) Effectiveness of SYTOX green stain for bacterial viability assessment Applied and Environmental microbiology 64: 2697-2700

Leigh T (2010) Advances in the application of ultraviolet light for swimming pool disinfection.

Leppakoski E, Gollasch S, Olenin S (2002) Alien species in European waters. In: Leppakoski E, Gollasch S, Olenin S (eds) Invasive aquatic species of Europe. Distribution, impacts and management. . Kluwer Academic Press, Dordrecht, pp 1-6.

Levitt J (1969) Introduction to plant physiology. C.V. Mosby Company, Saint Louis

Lewis PN, Hewitt CL, Riddle M, McMinn A (2003) Marine introductions in the Southern Ocean: an unrecognised hazard to biodiversity. Marine Pollution Bulletin 46: 213-223.

Li D, Craik SA, Smith DW, Belosevic M (2009) The assessment of particle association and UV disinfection of wastewater using indigenous spore-forming bacteria. Water Research 43: 481-489

Li J, Song L (2007) Applicability of the MTT assay for measuring viability of cyanobacteria and algae, specifically for *Microcystis aeruginosa* (Chroococcales, Cyanobacteria). Phycologia 46: 593-599

Liltved H, Hektoen H, Efraimsen H (1995) Inactivation of bacterial and viral fish pathogens by ozonation or UV Irradiation in water of different salinity. Aquacultural Engineering 14: 107-122

Linden K, Shin G-A, Faubert G, Cairns W, Sobsey MD (2002) UV disinfection of *Giardia lamblia* cysts in water. Environmental Science and Technology 36: 2519-2522

Lindenauer KG, Darby JL (1994) Ultraviolet disinfection of wastewater: effect of dose on subsequent photoreactivation. Water Research 28: 805-817

Liu G (2005) An investigation of UV disinfection performance under the influence of turbidity and particulates for drinking water applications. Master of Applied Science. Civil Engineering, Waterloo, Ontario, Canada

Liu TK, Tsai TK (2011) Vessel traffic patterns in the Port of Kaohsiung and the management implications for preventing the introduction of non-indigenous aquatic species. Marine Pollution Bulletin 62: 602-608

Liu Z, Stout JE, Tedesco L, Boldin M, Hwang C, Yu VL (1995) Efficacy of ultraviolet light in preventing *Legionella* colonization of a hospital water distribution system. . Water Research 29: 2275-2280

Llabres M, Agusti S (2006) Picophytoplankton cell death induced by UV radiation: Evidence for oceanic Atlantic communities. Limnology and Oceanography 51: 21-29

Lloyds Register (2010) Ballast water treatment technology. Current status, London

Lloyds Register Fairplay (2009) Number of ships (by total and trade)

Loge FJ, Bourgeous K, Emerick RW, Darby JL (2001) Variations in wastewater quality parameters influencing UV disinfection performance: Relative impact on filtration. Journal of Environmental Engineering 127: 832-837

Loge FJ, Emerick RW, Thompson D, Nelson DC, Darby JL (1999) Factors influencing ultraviolet disinfection performance part 1: light penetration to wastewater particles. Water Environment Research 71: 377-381

Lorenzen CJ (1979) Ultraviolet radiation and phytoplankton photosynthesis. Limnology and Oceanography 24: 1117-1120

Lothrop TL, Sproul OJ (1969) High level inactivation of viruses in wastewater by chlorination. Journal of the Water Pollution Control Federation. 41: 567-575

Lovelock J (1972) Gaia as seen through the atmosphere. Atmospheric Environment 6: 579-580

Lovelock J (1979) Gaia: a new look at life on Earth. Oxford University Press, Oxford

Lovelock J (2000) Gaia: a new look at life on Earth. Oxford University Press, Oxford

Madge BA, Jensen J (2006) Ultraviolet disinfection of fecal coliform in municipal wastewater: effects of particle size. Water Environment Research 78: 294-304

Mamane H, Colorni A, Bar I, Ori I, Mozes N (2010) The use of an open channel, low pressure UV reactor for water treatment in low head recirculating aquaculture systems (LH-RAS). Aquacultural engineering 42: 103-111

Marcus NH (1980) Photoperiodic control of diapause in the marine calanoid copepod *Labidocera aestiva*. . Biological Bulletin 159: 311-318.

Martin C, Holtz K, Jensen J (2004) Evaluation of Ultraviolet (UV) Radiation Disinfection Technologies for Wastewater Treatment Plant Effluent

Masschelein WJ (2002) Ultraviolet light in water and wastewater sanitation. Lewis Publishers, Boca Raton, Florida

Matousek R, Hill D, Herwig R, Cordell J, Nielsen B, Ferm N, Lawrence D, Perrins J (2006) Electrolytic sodium hypochlorite system for treatment of ballast water. Journal of Ship Production 22: 160-171

Matulonis U, Rosenfeld CS, Shadduck RK (1993) Prevention of *Legionella* infections in a bone marrow transplant unit: multifaceted approach to decontamination of a water system. Infection Control and Hospital Epidemiology 14: 571-575

McCollin T, Quilez-Badia G, Josefsen KD, Gill ME, Mesbahi E, Frid CLJ (2007a) Ship board testing of a deoxygenation ballast water treatment. Marine Pollution Bulletin 54: 1170-1178

McCollin T, Shanks AM, Dunn J (2007b) The efficiency of regional ballast water exchange: Changes in phytoplankton abundance and diversity. Harmful Algae 6: 531-546

McCollin T, Shanks AM, Dunn J (2008) Changes in zooplankton abundance and diversity after ballast water exchange in regional seas. Marine Pollution Bulletin 56: 834-844

McCormick J (1989) The global environmental movement: reclaiming paradise. Belhaven Press, London

McCracken W (2001) Ballast water treatment with currently available biocides. In: Raaymakers S (ed) 1st International Ballast Water Treatment R&D Symposium, International Maritime Organisation, London, pp 81-87

McLeod K, Leslie H (2009) Ecosystem-based management for the oceans. Island Press, Washington

Medcof JC (1975) Living marine animals in a ship's ballast water. Proceedings of the National Shellfisheries Association 65: 54-55.

MEPC (1993) Guidelines for preventing the introduction of unwanted aquatic organisms and pathogens from ships' ballast water and sediment discharges. In: International Maritime Organisation (ed)

MEPC (1997) Guidelines for the control and management of ships' ballast water to minimize the transfer of harmful aquatic organisms and pathogens. In: IMO (ed), pp 17

MEPC (2005a) Guidelines for approval of ballast water management systems (G8). In: IMO (ed)

MEPC (2005b) Guidelines for ballast water exchange (G6). In: IMO (ed)

MEPC (2006a) Guidelines for sediment reception facilities (G1). In: IMO (ed), pp 4

MEPC (2006b) Guidelines on design and construction to facilitate sediment control on ships (G12). In: IMO (ed), pp 4

MEPC (2010) List of ballast water management systems which received Type Approval Certification by their respective Administrations (resolution MEPC 175 (58)).

Mimura H, Katakura R, Ishida H (2005) Changes of microbial populations in a ship's ballast water and sediments on a voyage from Japan to Qatar. Marine Pollution Bulletin 50: 751-757

Minchin D, Eno NC (2002) Exotics of coastal and inland waters of Ireland and Britain. In: Leppakoski E, Gollasch S, Olenin S (eds) Invasive aquatic species of Europe. Distribution, impacts and management. Kluwer Academic Publishers, Dordrecht, pp 267-275

Minchin D, Gollasch S (2003) Fouling and ships' hulls: how changing circumstances and spawning events may result in the spread of exotic species. Biofouling 19: 111-122

Minchin D, Gollasch S, Cohen AN, Hewitt C, Olenin S (2008) Characterising vectors of marine invasion. In: Heldmaier G, Jackson R (eds) Biological Invasions in marine ecosystems:ecological, management and geographic perspectives. . Springer, Dordrecht, pp 109-116

Minuer F, Belsher T, Johnson M, Maggs C, Verlaque M (2007) Experimental assessment of oyster transfers as a vector for macroalgal introductions. Biological Conservation 137: 232-247

Miron A, Garcia M, Gomez A, Camacho F, Grima E, Chisti Y (2003) Shear stress tolerance and biochemical characterization of *Phaeodactylum tricornutum* in quasi steady-state continuous culture in outdoor photobioreactors. Biochemical Engineering Journal 16: 287-297

Miyahara K, Nagai S, Itakura S, Yamamoto K, Fujisawa K, Iwamoto T, Yoshimatsu S, Matsuoka S, Yuasa A, Makino K, Hori Y, Nagata S, Nagasaki K, Yamaguchi M, Honjo T (1996) First record of a bloom of Thalassiosira diporocyclus in the eastern Seto Inland Sea. Fisheries Science 62: 878-882.

Mofidi AA, Meyer EA, Wallis PM, Chou CI, Meyer BP, Ramalingam S, Coffey BM (2002) The effect of UV light on the inactivation of *Giardia lamblia* and *Giardia muris* cysts as determined by animal infectivity assay (P-2951-01). Water Research 36: 2098-2108

Mohlenberg F (1987) A submersible net-pump for quantitative zooplankton sampling; comparison with conventional net sampling. Ophelia 27: 101-110

Morera C, Villanueva M (2009) Heat treatment and viability assessment by Evans blue in cultured *Symbiodinium kawagutii* cells. . World Journal of Microbiology and Technology 25: 1125-1128

Morita S, Namikoshi A, Hirata T, Oguma K, Katayama H, Ohgaki S, Motoyama N, Fujiwara M (2002) Efficacy of UV irradiation in Inactivating *Cryptosporidium parvum* Oocysts. Applied and Environmental Microbiology 68: 5387-5393

Morris JG, Sztein MB, Rice EW, Nataro JP, Losonsky GA, Panigrahi P, Tacket CO, Johnson JA (1996) *Vibrio cholera* O1 can assume a chlorine-resistant rugose survival form that is virulent for humans. The Journal of Infectious diseases. 174: 1364-1368

Mountfort D, Dodgshun T, Taylor M (2001) Ballast water treatment by heat - New Zealand laboratory and shipboard trials. In: Raaymakers S (ed) 1st International ballast water treatment R&D symposium, London, pp 45-50

Murphy AM, Cowles TJ (1997) Effects of darkness on multi-excitation in vivo fluorescence and survival in a marine diatom. Limnology and Oceanography. 42: 1444-1453

Murphy K, Boehme J, Coble P, Cullen J, Field P, Moore W, Perry E, Sherrell R, Ruiz G (2004) Verification of mid-ocean ballast water exchange using naturally occurring coastal tracers. Marine Pollution Bulletin 48: 711-730

Nandakumar K, Obika H, Shinozaki T, Ooie T, Utsumi A, Yano T (2002) Impact of pulsed laser irradiations from Nd:YAG laser on the larvae of the fouling barnacle *Balanus amphitrite*. Biofouling 18: 257-262

Nandakumar K, Obika H, Shinozaki T, Ooie T, Utsumi A, Yano T (2003a) Laser impact on marine planktonic diatoms: an experimental study using a flow cytometry system. Biofouling 19: 133-138

Nandakumar K, Obika H, Shinozaki T, Ooie T, Utsumi A, Yano T (2003b) Lethal and sub-lethal impacts of pulsed laser irradiations on the larvae of the fouling barnacle *Balanus amphitrite*. Biofouling 19: 169-176

Nandakumar K, Obika H, Shinozaki T, Ooie T, Utsumi A, Yano T (2003c) Pulsed laser irradiation impact on two marine diatoms *Skeletonema costatum* and *Chaetoceros gracilis*. Water Research 37: 2311-2316

Nandakumar K, Obika H, Sreekumari K, Utsumi A, Ooie T, Yano T (2009) Laser damage to marine plankton and its application to checking biofouling and invasion by aquatic species: a laboratory study. Biofouling 25: 95-98

Nandakumar K, Obika H, Utsumi A, Ooie T, Yano T (2004) In vitro laser ablation of natural marine biofilms. Applied and Environmental Microbiology 70: 6905-6908

National Drinking Water Clearinghouse (1999) Ozone Tech Brief. A National Drinking Water Clearinghouse Fact Sheet, pp 1-4

National Research Council (1996) Stemming the tide: Controlling introductions of nonindigenous species by ships' ballast water treatment processes. National Academy Press, Washington, DC, USA

Nayar S, Goh BPL, Chou LM (2002) A portable, low-cost, multipurpose, surface-subsurface plankton sampler. Journal of Plankton Research 24: 1097-1105

Nemery B, Hoet PHM, Nowak D (2002) Indoor swimming pools, water chlorination and respiratory health. European Respiratory Journal 19: 790-793

Newcomer HS (1917) The abiotic action of ultra-violet light. Journal of Experimental Medicine 26: 841-849

Oemcke D, Van Leeuwen J (2004) Seawater ozonation of *Bacillus subtilis* spores: implications for the use of ozone in ballast water treatment. Ozone: Science and Engineering 26: 389-401

Oemcke D, van Leeuwen J (2005) Ozonation of the marine dinoflagellate alga *Amphidinium* sp. - implications for ballast water disinfection. Water Research 39: 5119-5125

Oguma K, Katayama H, Mitani H, Morita S, Hirata T, Ohgaki S (2001) Determination of pyrimidine dimers in *Escherichia coli* and *Cryptosporidium parvum* during UV light inactivation, photoreactivation and dark repair. . Applied and Environmental Microbiology 67: 4630-4637

Oguma K, Katayama H, Ohgaki S (2002) Photoreactivation of *Escherichia coli* after low- or medium-pressure UV disinfection determined by an Endonuclease Sensitive Site assay. Applied and Environmental Microbiology 68: 6029-6035

Ojaveer H, Leppakoski E, Olenin S, Ricciardi A (2002) Ecological impact of ponto-caspian invaders in the Baltic Sea, European inland waters and the Great Lakes: an inter-ecosystem comparison. In: Leppakoski E, Gollasch S, Olenin S (eds) Invasive aquatic species of Europe. Distribution, impacts and management. . Kluwer Academic Publishers, Dordrecht, pp 412-425.

Olenin S, Gollasch S, Jonusas S, Rimkute I (2000) En-route investigations of plankton in ballast water on a ship's voyage from the Baltic Sea to the open Atlantic coast of Europe. International Review of Hydrobiology 85: 577-596.

Oliver BG, Cosgrove EG (1975) The disinfection of sewage treatment plant effluents using ultraviolet light. The Canadian Journal of Chemical Engineering 53: 170-174

Onji M, Sawabe T, Ezura Y (2000) An evaluation of viable staining dyes suitable for marine phytoplankton. Bulletin of the Faculty of Fisheries Hokkaido University 51: 153-157

Ozturk B (2002) The Marmara Sea, a link between the Mediterranean and the Black Sea. In: Leppakoski E, Gollasch S, Olenin S (eds) Invasive aquatic species of Europe. Distribution, impacts and management. Kluwer Academic Publishers, Dordrecht, pp 337-340

Paerl HW, Bland PT, Bowles ND, Haibach ME (1985) Adaptation to high intensity, low wavelenght light among surface blooms of the cyanobacterium *Microcystis aeruginosa.* Applied and Environmental Microbiology 49: 1046-1052

Parmegiani L, Accorsi A, Cognigni GE, Bernardi S, Troilo E, Filicori M (2010) Sterilization of liquid nitrogen with ultraviolet irradiation for safe vitrification of human oocytes or embryos. Fertility and Sterility 94: 1525-1528

Parsons M, Harkins R (2002) Full scale particle removal performance of three types of mechanical separation devices for the primary treatment of ballast water. Marine Technology 39: 211-222

Parsons TR, Maita Y, Lalli CM (1984) A manual of chemical and biological methods for seawater analysis. New York Pergamon Press., Oxford, UK

Perrins JC, Cordell JR, Ferm NC, Grocock JL, Herwig RP (2006) Mesocosm experiments for evaluating the biological efficacy of ozone treatment of marine ballast water. Marine Pollution Bulletin 52: 1756-1767

Pertola S, Faust MA, Kuosa H (2006) Survey on germination and species composition of dinoflagellates from ballast tanks and recent sediments in ports of the South Coast of Finland, North Eastern Baltic Sea. Marine Pollution Bulletin 52: 900-911

Peters E (1996) Prolonged darkness and diatom mortality: II. Marine temperate species. . Journal of Experimental Marine Biology and Ecology. 207: 43-58.

Peters E, Thomas D (1996) Prolonged darkness and diatom mortality: I. Marine Antarctic species. Journal of Experimental Marine Biology and Ecology. 207: 25-41.

Pienimaki M, Leppakoski E (2004) Invasion pressure on the Finnish Lake District: invasion corridors and barriers. Biological Invasions 6: 331-346

Pini R (1999) Ballast water purified with UV waves. Photonics Spectra March: 30-31

Popovich CA, Gayoso AM (1999) Effect of irradiance and temperature on the growth rate of *Thalassiosira curviseriata* Takano (Bacillariophyceae), a bloom diatom in Bahia Blanca estuary (Argentina). Journal of Plankton Research 21: 1101-1110

Pouneva I (1997) Evaluation of algal culture viability and physiological state by fluorescent microscopic methods. Bulgarian Journal of Plant Physiology 23: 67-76

Qualls RG, Flynn MP, Johnson JD (1983) The role of suspended particles in ultraviolet disinfection. Journal of Water Pollution Control Federation 55: 1280-1285

Quilez-Badia G, McCollin T, Josefsen KD, Vourdachas A, Gill ME, Mesbahi E, Frid CJ (2008) On board short-time high temperature heat treatment of ballast water: A field trial under operational conditions. Marine Pollution Bulletin 56: 127-135

Raaymakers S (2002) IMO ballast water update.

Rahkola M, Karjalainen J, Viljanen M (1994) Evaluation of a pumping system for sampling zooplankton. Journal of Plankton Research 16: 905-910

Rajagopal S, Nair KVK, van der Velde G, Jenner HA (1997) Response of mussel *Brachidontes striatulus* to chlorination: an experimental study. Aquatic Toxicology 39: 135-149

Rajagopal S, Van der Velde G, Jenner HA (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, Jenner HA (2003) How effective is intermittent chlorination to control adult mussel fouling in cooling water systems. Water Research 37: 329-338

Reavie ED, Cangelosi AA, Allinger LE (2010) Assessing ballast water treatments: Evaluation of viability methods for ambient freshwater microplankton assemblages. Journal of Great Lakes Research 36: 540-547

Reynolds AE, Mackiernan GB, van Valkenburg SD (1978) Vital and mortal staining of algae in the presence of chlorine-produced oxidants. Estuaries 1: 192-196

Rice EW, Clark RM, Johnson CH (1999) Chlorine inactivation of *Escherichia coli* O157:H7. Emerging Infectious Diseases 5: 461-463

Rice EW, Hoff JC (1981) Inactivation of *Giardia lamblia* cysts by ultraviolet irradiation. Applied and Environmental Microbiology 42: 546-547

Rice EW, Hoff JC, Schaefer FW (1982) Inactivation of *Giardia* cysts by chlorine. Applied and Environmental Microbiology 43: 250-251

Rigby G, Hallegraeff G (1994) The transfer and control of harmful marine organisms in shipping ballast water: behaviour of marine plankton and ballast water exchange trials on the MV Iron Whyalla. Journal of Marine Environmental Engineering 1: 91-110

Rigby G, Hallegraeff G, Taylor A (2004) Does heat offer a superior ballast water treatment option? In: Matheickal JT, Raaymakers S (eds) 2nd International Ballast Water Treatment R&D Symposium., IMO, London, pp 67-79

Rigby GR, Hallegraeff GM, Sutton C (1999) Novel ballast water heating technique offers cost effective treatment to reduce the risk of global transport of harmful marine organisms. Marine Ecology-Progress Series 191: 289-293

Riley S, Lemieux E, Robbins S (2005) Testing of filter technologies for ballast water treatment Oceans 2005. IEEE, 345 E 47th St, New York, NY 10017 USA, Washington DC, pp 2511-2518

Rilov G, Crooks J (2009) Marine Bioinvasions: Conservation hazards and vehicles for ecological understanding. In: Rilov G, Crooks J (eds) Biological invasions in marine ecosystems: ecological, management, and geographic perspectives (Ecological studies). Springer-Verlag., Berlin, pp 3-12

Rinalta J, Spilling K, Blomster J (2007) Temporary cyst enables long-term dark survival of *Scripsiella hangoei* (Dinophyceae). Marine Biology. 152: 57-62.

Ruiz G, Rawlings TK, Dobbs FC, Drake LA, Mullady TL, Huq A, Colwell RR (2000) Global spread of microorganisms by ships: Ballast water discharged from vessels harbours a cocktail of potential pathogens. Nature 408: 49-50

Ruiz GM, Carlton JT, Grosholz ED, Hines AH (1997) Global invasions of marine and estuarine habitats by non-indigenous species: Mechanisms, extent, and consequences. American Zoologist 37: 621-632

Sandlund OT, Schei PJ, Viken A (1999) The many aspects of the invasive alien species problem. In: Sandlund OT, Schei PJ, Viken A (eds) Invasive species and biodiversity management. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1-7

Sano L, Mapilli M, Krueger A, Garcia E, Gossiaux D, Phillips K, Landrum P (2004) Comparative efficacy of potential chemical disinfectants for treating unballasted vessels. Journal of Great Lakes Research 30: 201-216

Sarvel A, Kusel J, Araujo N, Coelho P, Katz N (2006) Comparison between morphological and staining characteristics of live and dead eggs of *Schistosoma mansoni*. Memorias do Instituto Oswaldo Cruz 101: 289-292

Sassi J, Viitasalo S, Rytkonen J, Leppakoski E (2005) Experiment with ultraviolet light, ultrasound and ozone technologies for onboard ballast water treatment

Seepersad B, Crippen RW (1978) Use of Aniline Blue for distinguishing between live and dead freshwater zooplankton. Journal of the Fish Research Board of Canada 35: 1363-1366

Selvin R, Reguera B, Bravo I, Yentsch C (1989) Use of Fluorescein diacetate (FDA) as a single-cell probe of metabolic activity in dinoflagellate cultures. . Biological Oceanography 6: 505-511

Seo KS, Lee Y (2008) A first assessment of invasive marine species on Chinese and Korean coasts. In: Caldwell M, Heldmaier G, Jackson R, Lange O, Mooney H, Schulze E, Sommer U (eds) Biological invasions in marine ecosystems: ecological, management, and geographic perspectives. Springer, Dordrecht, pp 577-586

Shi XL, Kong FX, Yu Y, Yang Z (2007) Survival of Microcystis aeruginosa and Scenedesmus obliquus under dark anaerobic conditions. Marine and Freshwater Research. 58: 634-639.

Shimizu Y, Gupta S, Masuda K, Maranda L, Walker CK, Wang R (1989) Dinoflagellate and other microalgal toxins: chemistry and biochemistry. Pure and Applied Chemistry 61: 513-516.

Shin G-A, Linden KG, Arrowood MJ, Sobsey MD (2001) Low pressure UV inactivation and DNA repair potential of *Cryptosporidium parvum* oocysts. Applied and Environmental Microbiology 67: 3029-3032

Sliwa C, Migus S, McEnnulty F, Hayes K (2008) Marine bioinvasions in Australia. In: Caldwell M, Heldmaier G, Jackson R, Lange O, Mooney H, Schulze E, Sommer U (eds) Biological invasions in marine ecosystems: ecological, management and geographic perspectives. Springer, Dordrecht, pp 425-438.

Slocombe S (1993) Implementing ecosystem based management. Bioscience 43: 612-622

Smith RC, Baker KS, Holm-Hansen O, Olson R (1980) Photoinhibition of photosynthesis in natural waters. Photochemistry and Photobiology 31: 585-592

Sousa O, Vieira R, Patel T, Hofer E, Mesquita V (2001) Effects of chlorine on cells of *Vibrio cholerae*. Food Microbiology 18: 355-359

Steeman Nielson E (1964) On a complication in marine productivity work due to the influence of ultraviolet light. Conseil International pour l'Exploration de la Mer 29: 130-135

Stovall WD, Fellow MD, Nichols MS (1923) Chlorination of swimming pool water. Americal Journal of Public Health 13: 478-480

Suez Canal Traffic Statistics (2010) Brief Monthly Statistics

Sugita H, Mitsuya T, Amanuma K, Hayashi K, Asai T, Maruyama C, Deguchi Y (1992) Ultraviolet susceptibility of three marine fish and pathogens. Bulletin of the college of the Agricltural and Vetinary Medicine 49: 117-121

Sutherland K (2008) Filters and filtration handbook. Butterworth - Heinmann, Oxford

Sutherland TF, Levings CD, Elliott C, Hesse WW (2001) Effect of a ballast water treatment system on survivorship of natural populations of marine plankton. Marine Ecology Progress Series 210: 139-148

Sutherland TF, Levings CD, Petersen S, Hesse WW (2003) Mortality of zooplankton and invertebrate larvae exposed to cyclonic pre-treatment and ultraviolet radiation. Marine Technology Society Journal 37: 3-12

Sylvester F, MacIsaac H (2010) Is vessel hull fouling an invasion threat to the Great Lakes? Diversity and Distributions 16: 132-143

Taghi-Kilani R, Gyurek L, Millard P, Finch G, Belosevic M (1996) Nucleic acid stains as indicators of *Giardia muris* viability following cyst inactivation. International Journal for Parasitology 26: 637-646

Takano H (1956) Harmful blooming of minute cells of *Thalassiosira decipiens* in coastal water in Tokyo Bay. Journal of the Oceanography Society Japan. 12: 63-67.

Tamburri MN, Little BJ, Ruiz GM, Lee JS, McNulty PD (2003) Evaluations of Venturi Oxygen Stripping as a ballast water treatment to prevent aquatic invasions and ship corrosion. In: Matheickal JT, Raaymakers S (eds) 2nd International Ballast Water Treatment R&D Symposium, IMO London, pp 34-47

Tamburri MN, Smith GE, Mullady TL (2006) Quantitative shipboard evaluations of Venturi Oxygen Stripping as a ballast water treatment. 3rd International Conference on Ballast Water Management, Singapore, pp 1-13

Tamburri MN, Wasson K, Matsuda M (2002) Ballast water deoxygenation can prevent aquatic introductions while reducing ship corrosion. Biological Conservation 103: 331-341

Tang Z, Butkus M, Xie Y (2006) Crumb rubber filtration: A potential technology for ballast water treatment. Marine Environmental Research 61: 410-423

Tang Z, Butkus M, Xie Y (2009) Enhanced performance of crumb rubber filtration for ballast water treatment. . Chemosphere. 74: 1396-1399

Tao Y, Zhang X, Au D, Moao X, Yuan K (2010) The effects of sub-lethal UV-C irradiation on growth and cell integrity of cyanobacteria and green algae. Chemosphere 78: 541-547

Taylor J, West D (1980) The use of Evans blue stain to test the survival of plant cells after exposure to high salt and high osmotic pressure. Journal of Experimental Biology 31: 571-576

Templeton MR, Andrews RC, Hofman R (2005) Inactivation of particle associated viral surrogates by ultra violet light. Water Research 39: 3487-3500

Thompson IS, Seed R, Richardson CA, Hui L, Walker G (1997) Effects of low level chlorination on the recruitment, behaviour and shell growth of *Mytilus edulis* Linnaeus in power station cooling water. Scientia Marina 61: 77-85

Thornton GA, Chapman JE (2004) The use of heat for ballast water disinfection - the AquaTherm method. In: Matheickal JT, Raaymakers S (eds) 2nd International Ballast Water treatment R&D Symposium IMO, London, pp 88-102

Tran MTT, Farid M (2004) Ultraviolet treatment of orange juice. Innovative Food Science and Emerging Technologies. 5: 495-502

Transport Statistics Great Britain (2009) TSGB/Maritime Statistics: Shipping - data tables

Tsolaki E, Diamadopoulos E (2009) Technologies for ballast water treatment: a review. Journal of Chemical Technology and Biotechnology

Tuchman N, Schollett M, Rier S, Geddes P (2006) Different heterotrophic utilization of organic compounds by diatoms and bacteria under light and dark conditions. Hydrobiologia. 561: 167-177.

UK Met Office (2009) Warming. Climate change - the facts. Met office

United States Environment Protection Agency (2006) Ultraviolet disinfection guidance manual for the final long term 2 enhanced surface water treatment rule

Veldhuis M (1997) Cellular DNA content of marine phytoplankton using two new fluorochromes:taxonomic and ecological implications. Journal of Phycology 33: 527-541

Veldhuis M, Fuhr F, Boon J, Ten Hallers-Tjabbers C (2006) Treatment of ballast water; How to test a system with a modular concept? Environmental technology 27: 909-921

Veldhuis MJW, Gijsbert WK, Timmermans KR (2001) Cell death in phytoplankton: correlation between changes in membrane permeability, photosynthetic activity, pigmentation and growth. European Journal of Phycology 36: 167-177

Vianna da Silva J, da Costa Fernandes F (2003) Use of chlorine for ballast water treatment. In: Matheickal JT, Raaymakers S (eds) 2nd International Ballast Water Treatment R&D Symposium, International Maritime Organisation, London, pp 158-163

von Recklinghausen M (1914) The ultra-violet rays and their application for the sterilization of water. Journal of the Franklin Institute 1057-1062: 681-704

Waite TD, Kazumi J, Lane PVZ, farmer LL, Smith SG, Smith SL, Hitchcock G, Capo TR (2003) Removal of natural populations of marine plankton by a large scale ballast water treatment system. Marine Ecology Progress Series 258: 51-63

Ward HM (1892) Experiments on the action of light on *Bacillus anthracis*. Proceedings of the Royal Society of London 52: 393-403

Water Pollution Control Federation. Technical Practice Committee. (1976) Chlorination of wastewater, prepared under direction of the Technical Practice Committee by the Subcommittee on Chlorination of Wastewater., Washington

Weidenkopf SJ (1958) Inactivation of Type I Poliomyelitis virus with chlorine. Virology 5: 56-67

White G (1999) Handbook of chlorination and alternative disinfectants. Wiley-Interscience

Williams RJ, Griffiths FB, Vanderwal EJ, Kelly J (1988) Cargo Vessel Ballast Water as a Vector for the Transport of Non-Indigenous Marine Species. Estuarine Coastal and Shelf Science 26: 409-420.

Wolfe RL (1990) Ultraviolet disinfection of potable water: current technology and research needs. Environmental Science and Technology 24: 768-773

Wonham MJ, Walton WC, Ruiz GM, Frese AM, Galil BS (2001) Going to the source: role of the invasion pathway in determining potential invaders. Marine Ecology-Progress Series 215: 1-12

World Health Organisation (2009) Guidelines for safe recreational waters.

World Health Organisation (2010) Guidelines for drinking water quality., pp 62

Worrest RC (1983) Impact of solar ultraviolet-B radiation (290-320 nm) upon marine microalgae. Physiologia Plantarum 58: 428-434

Worrest RC, Thomson BE, Van Dyke H (1981) Impact of UV-B radiation upon estuarine microcosms. Photochemistry and Photobiology 33: 861-867

Wright D, Dawson R (2001) SeaKleen - a potential natural biocide for ballast water treatment. In: Raaymakers S (ed) 1st International Ballast Water Treatment R&D Symposium, International Maritime Organisation, London, pp 73-75

Wright DA, Dawson R, Mackey TP, Cutler HG, Cutler SJ (2004) Some shipboard trials of ballast water treatment systems in the United States. In: Matheickal JT, Raaymakers S (eds) 2nd International Ballast Water Treatment R&D Symposium., London., pp 243-257.

Wright DA, Dawson R, Orano-Dawson CE (2007a) Shipboard trials of Menadione as a ballast water treatment. Marine Technology 44: 68-76

Wright DA, Dawson R, Orano-Dawson CE, Moesel SM (2007b) A test of the efficacy of a ballast water treatment system aboard the vessel *Coral Princess*. Marine Technology 44: 57-67

Wright H, Cairns W Ultraviolet light. Trojan Technologies Inc

Wright JLC, Boyd RK, Defreitas ASW, Falk M, Foxall RA, Jamieson WD, Laycock MV, McCulloch AW, McInnes AG, Odense P, Pathak VP, Quilliam MA, Ragan MA, Sim PG, Thibault P, Walter JA, Gilgan M, Richard DJA, Dewar D (1989) Identification of domoic acid, a neuroexcitory amino acid, in toxic mussels from eastern Prince Edward Island. Canadian Journal of Chemistry-Revue Canadienne De Chimie 67: 481-490.

Wright JR, Sumner SS, Hackney CR, Pierson MD, Zoecklein BW (2000) Efficacy of ultraviolet light for reducing *Escherichia coli* O157:H7 in unpasteurized apple cider. Journal of Food Protection 63: 563-567

Wu Z, Song L, Li R (2008) Different tolerances and responses to low temperature and darkness between waterbloom forming cyanobacterium *Microcystis* sp. and a green alga *Scenedesmus* sp.. Hydrobiologia. 596: 47-55.

Wulff A, Roleda MY, Zacher K, Wiencke C (2008) Exposure to sudden light burst after prolonged darkness - a case study on benthic diatoms in Antarctica. Diatom Research 23: 519-532

www.lenntech.com/water-disinfection/disinfectants-chlorine.htm

www.lvpg-international.com

Zenetos A, Meric E, Verlaque M, Galli P, Bourdouresque C, Giangrande A, Cinar M, Bilecenoglu M (2008) Additions to the annotated list of marine alien biota in the Mediterranean with emphasis on Foraminifera and Parsites. Mediterranean Marine Science 9: 119-165

Zhang FZ, Dickman M (1999) Mid-ocean exchange of container vessel ballast water. 1: Seasonal factors affecting the transport of harmful diatoms and dinoflagellates. Marine Ecology-Progress Series 176: 243-251.

Zhang S, Chen X, Yang W, Gong Q, Wang J, Xiao J, Zhang H, Wang Q (2004) Effects of the chlorination treatment for ballast water 2nd International Ballast Water Treatment R&D Symposium, pp 148-157

Zimmer JL, Slawson RM (2002) Potential repair of *Escherichia coli* DNA following exposure to UV radiation from both medium- and low-pressure UV sources used in drinking water treatment. Applied and Environmental Microbiology 68: 3293-3299