

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

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Abstract:

Phytoplankton assemblages from tropical (Goa) and temperate (UK) locations were exposed to a 28 day dark period, followed by a period of re-exposure to light. During this time phytoplankton survival and changes in nutrient concentrations were mapped. The tropical plankton water samples showed high nutrient levels after the dark period which were utilised by cells during the re-exposure period. UK experiments looked at the effect of three different water types on population recovery after the 28 day dark period, and differences due to seasonal effects.

The population growth observed during the re-exposure period in the tropical population was comparable to that of the temperate population. Water type affected recovery and of the three tested media fresh seawater promoted the highest levels of growth. Seasonality had a significant influence on species survival. Understanding the effects of all these factors can aid the development of effective risk assessments in ballast water management.

Keywords: Phytoplankton, dark incubation, ballast water, risk assessment

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. It has been estimated that at any one time up to 10,000 species are being transported in this way (Carlton, 1999). Release of these organisms at destination ports is of major concern due to the economic, environmental and health impacts they have been demonstrated to cause worldwide (Aldridge et al., 2004; Castilla and Neill, 2008; Eno et al., 1997; Ferreira et al., 2008; Galil, 2000, 2008; Hayden et al., 2008; Herborg et al., 2003; Hoppenrath et al., 2007; Kideys et al., 2005; Leppakoski et al., 2002; Lewis et al., 2003; Ojaveer et al., 2002; Olenin and Leppakoski, 1999; Reise et al., 2002; Sliwa et al., 2008).

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 (Carlton and Geller, 1993; David et al., 2007; Dickman and Zhang, 1999; Hallegraeff and Bolch, 1991; Hamer et al., 2000; Medcof, 1975; Williams et al., 1988; Zhang and Dickman, 1999). 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 (Gollasch et al., 2000a; Gollasch et al.,

2000b; Klein et al., 2010; Lavoie et al., 1999; Olenin et al., 2000). 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 (Conover and Siferd, 1993; Drake et al., 2007; Marcus, 1980; Rinalta et al., 2007), reducing cell metabolism (Jochem, 1999; Peters, 1996; 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.

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).

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 100 μ m 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 vessels. The total volume of seawater in vessels was 5L (Goa) and 3.5L (Newcastle). Three of these vessels were covered for the initial four weeks to prevent light reaching the water (‘Dark-regime’ treatment), the other three vessels served as ‘Controls’. After the Dark experimental period the coverings were removed from the Dark-regime treatment vessels to reintroduce the phytoplankton assemblages to light conditions, and maintained under a similar regime as the Control vessels 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 vessels 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 vessels and in Newcastle glass bottles (Fisherbrand cat: 02-911-918) were used. Tubes were inserted into each vessel to allow air exchange. In Goa the temperature of the experimental vessels was maintained at a mean of $31 \pm 0.13^\circ\text{C}$ with a mean daily variance of $3.8 \pm 0.13^\circ\text{C}$, by suspension in an outdoor pool. In Newcastle the experimental vessels 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 vessel 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 vessels were split between the three media. From each vessel 650ml was filtered by $10\mu\text{m}$ and the filtered organisms were rinsed into a new experimental vessel 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 vessels 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.

2.2 Parameters measured

Chlorophyll α :

Samples (100 ml) were filtered through pre-ashed $0.7\ \mu\text{m}$ pore GF/F Glass fiber micro filters using a vacuum pressure $<400\ \text{mm Hg}$. The filters were then extracted in 90% acetone in the dark at 4°C for

24 hours, centrifuged and then analysed with a Turner Designs Trilogy™ fluorometer (Parsons et al., 1984).

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.

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.

FlowCAM assessment was performed in the dark using TriggerMode with only Channel 2 switched on for particle detection at a threshold of 400. FlowCAM is equipped with a blue laser which excites the FDA stain and then detects cells emitting a fluorescent signal above the threshold. The files were manually assessed and cells per ml calculated by FlowCAM.

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.

3.2 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.

3. Results

3.1 Tropical population:

Total cell numbers of 1204 ± 422 cells 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 $3.2 \mu\text{g/l}$ on Day 0 to the minimum level of $0.15 \mu\text{g/l}$ on Day 14 (Figure 1). 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 (Figures 2, 3 and 4), while phosphate and ammonia levels did not change accordingly (Figures 5 and 6). 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 $3.01 \mu\text{g/l}$, almost equal to Day 0 value. During the same period Control chlorophyll α levels continued to decrease (Figure 1). Nitrate, nitrite and silicate levels all decreased throughout the re-exposure period (Figures 2-4). 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).

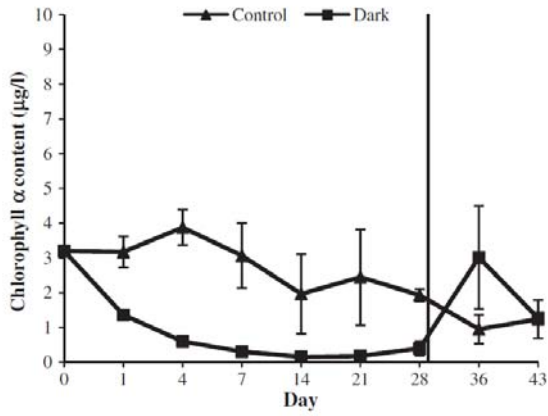


Fig 1

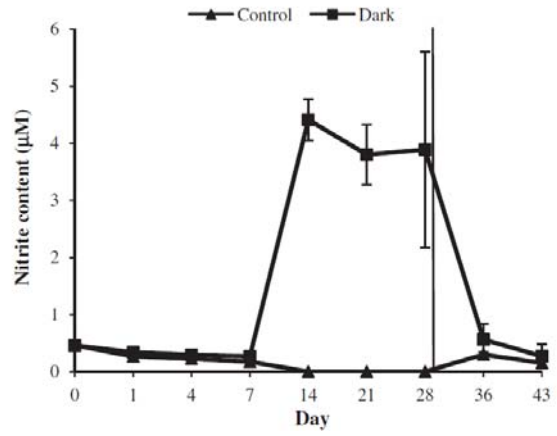


Fig 2

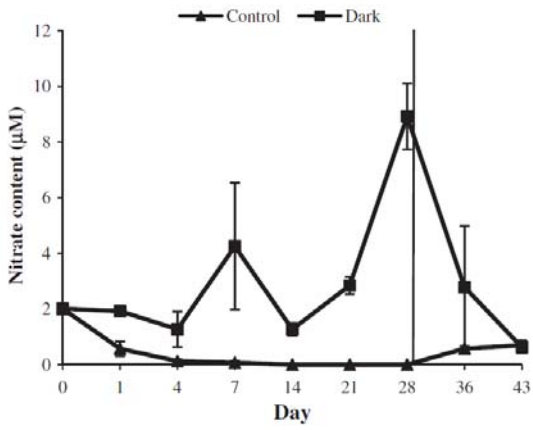


Fig 3

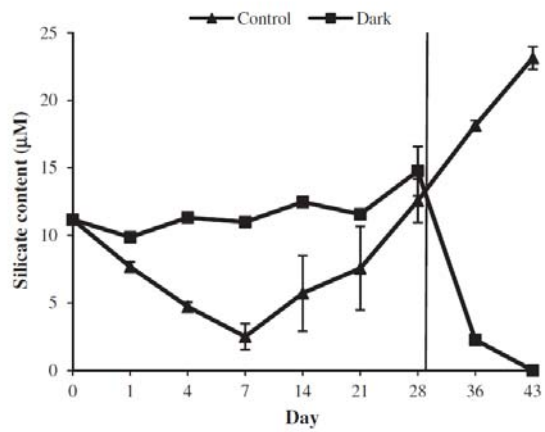


Fig 4

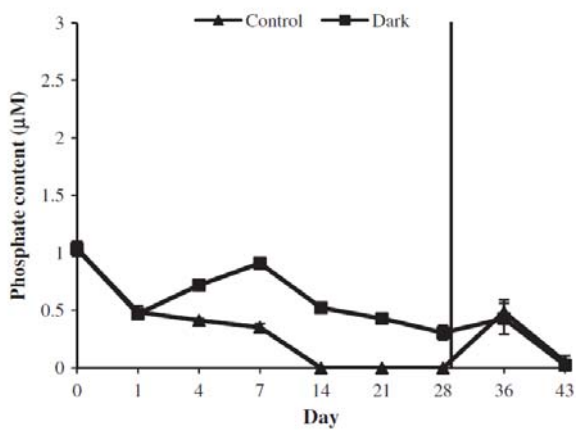


Fig 5

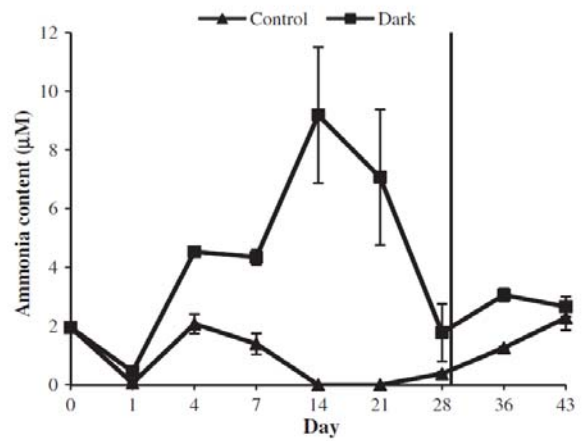


Fig 6

3.2 Temperate population: Autumn

In this experiment chlorophyll α levels were lower than the tropical population, and few live cells were present: 1.67 $\mu\text{g/l}$ 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 (Figures 7 and 8). 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 (Figures 9-11), as was observed in the tropical population.

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 (Figures 9-13).

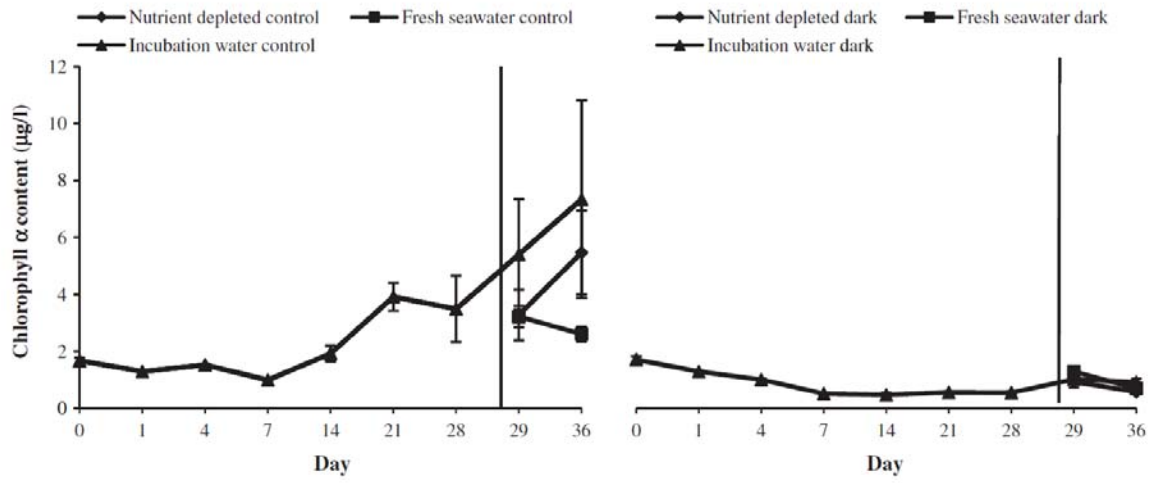


Fig 7

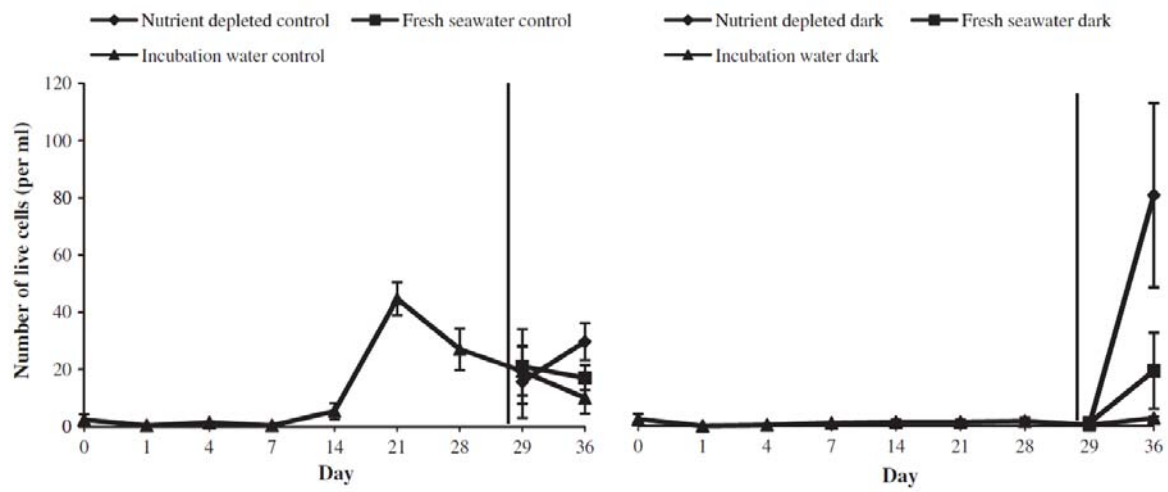


Fig 8

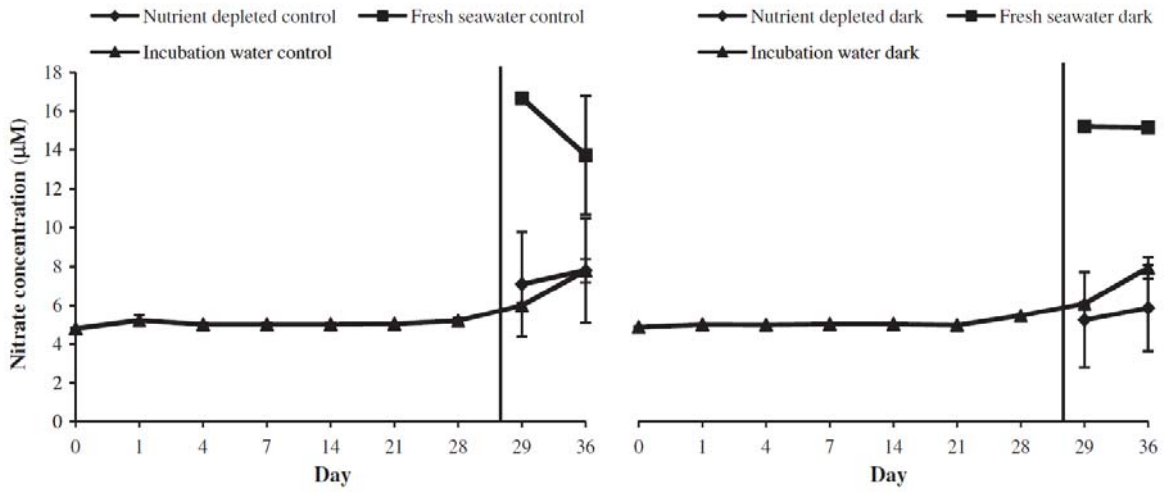


Fig 9

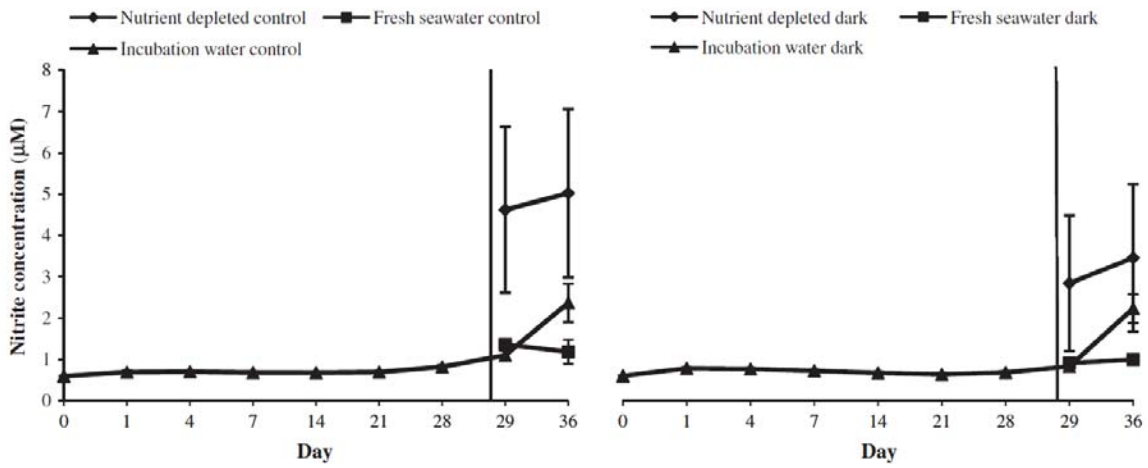


Fig 10

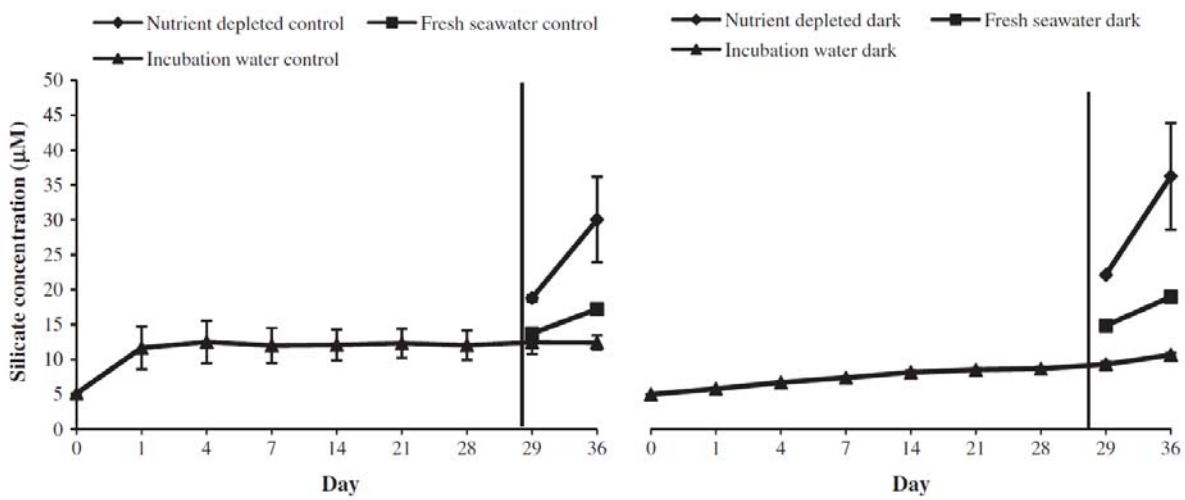


Fig 11

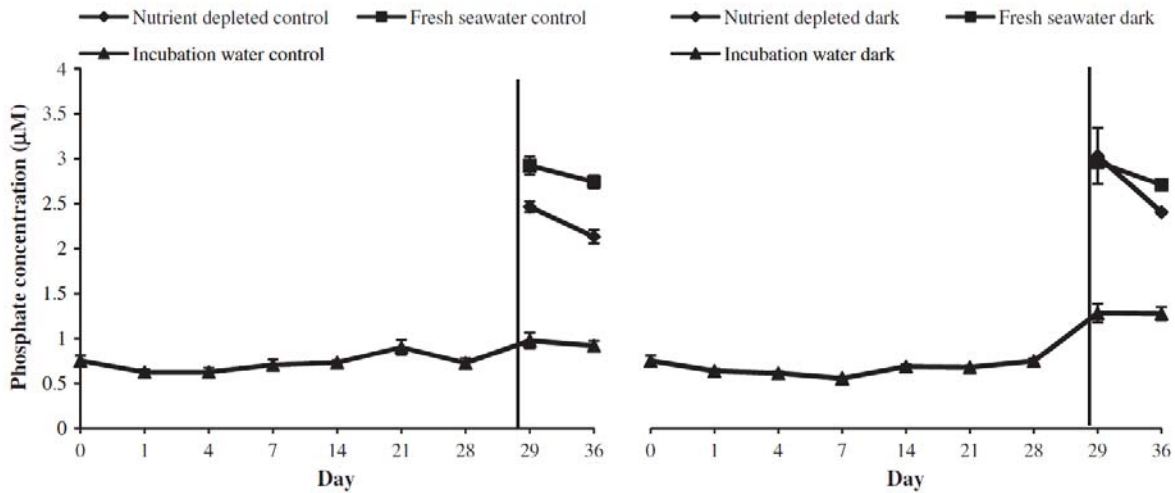


Fig 12

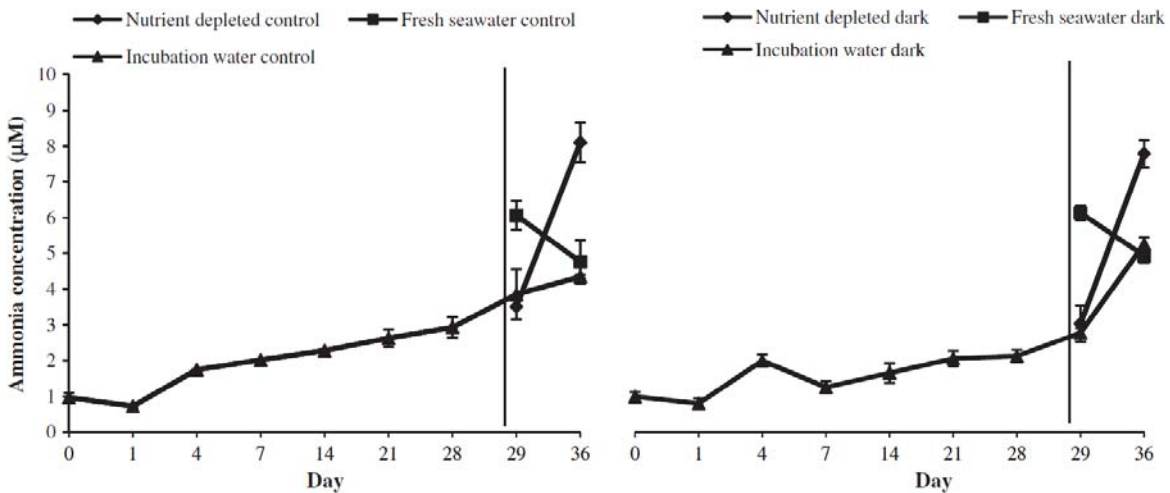


Fig 13

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 $3.8\mu\text{g/l}$ 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 (Figures 14 and 15). 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 (Figures 16-20). 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.

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 (Figures 14 and 15), and little change in nutrient levels was observed. The incubation water maintained the Dark-regime exposed population for the two week period, (Figures 14 and 15) and decreases in nitrate, nitrite, silicate and ammonia levels were observed.

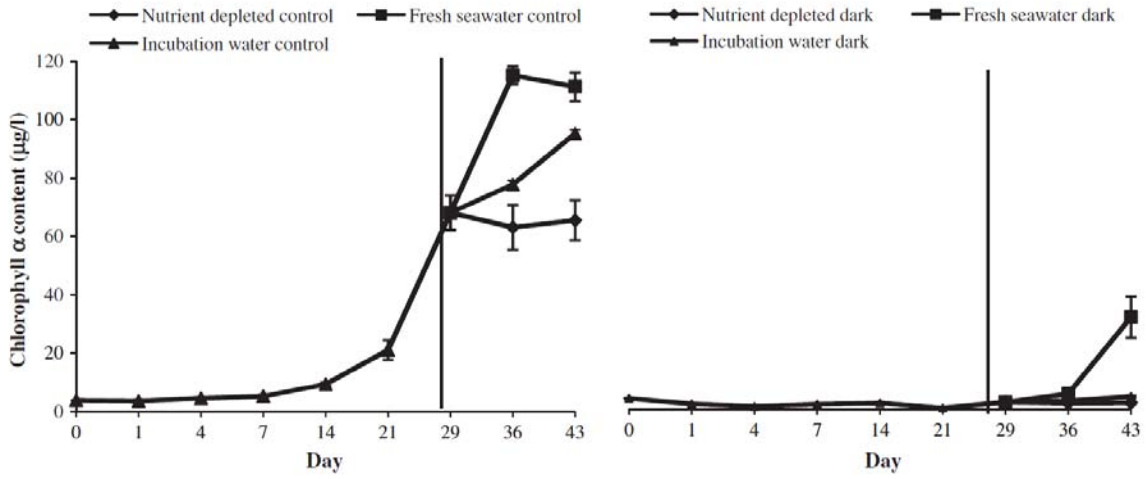


Fig 14

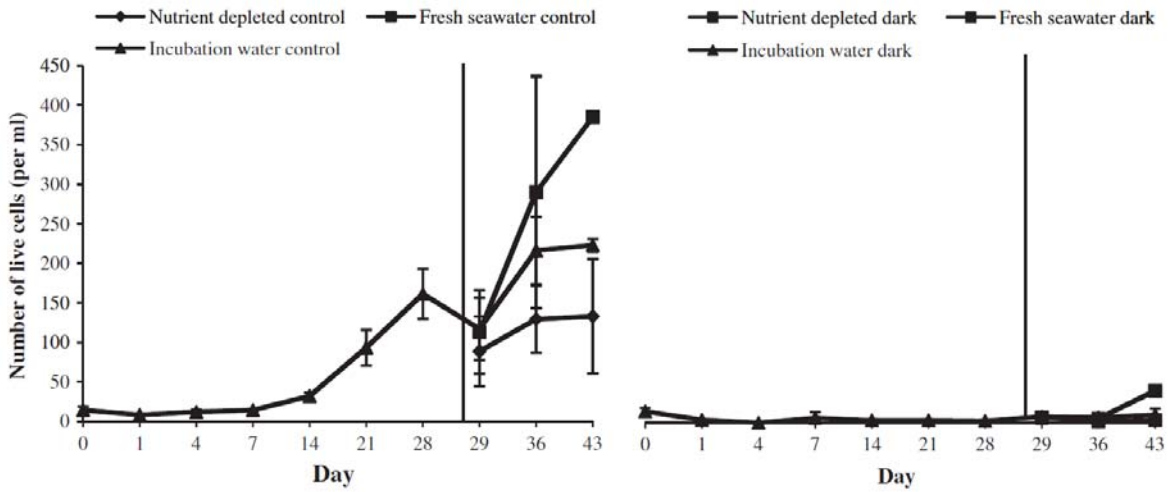


Fig 15

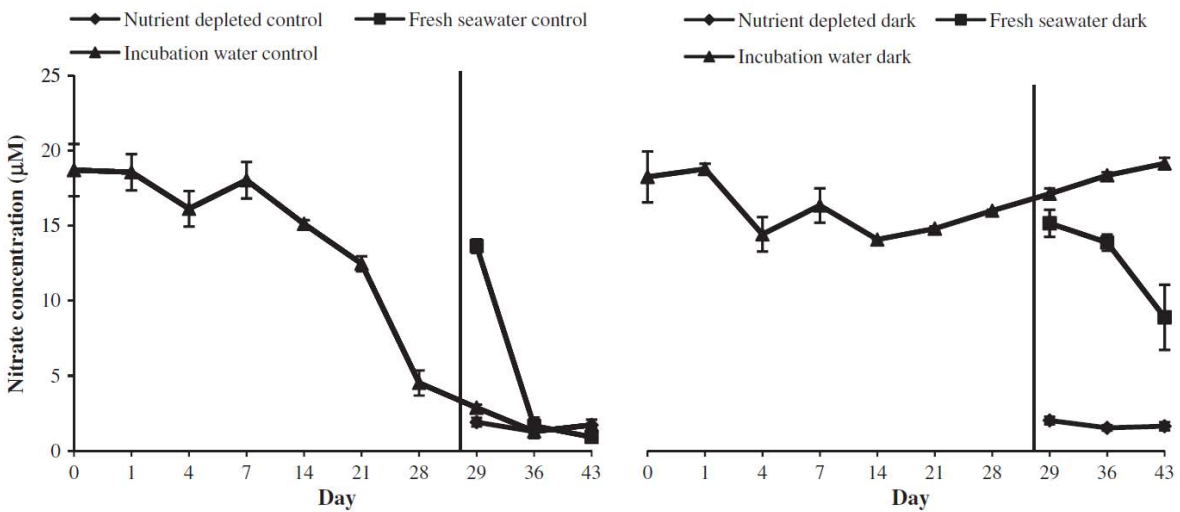


Fig 16

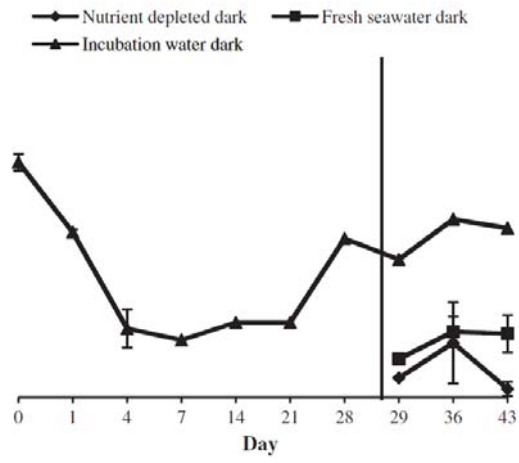
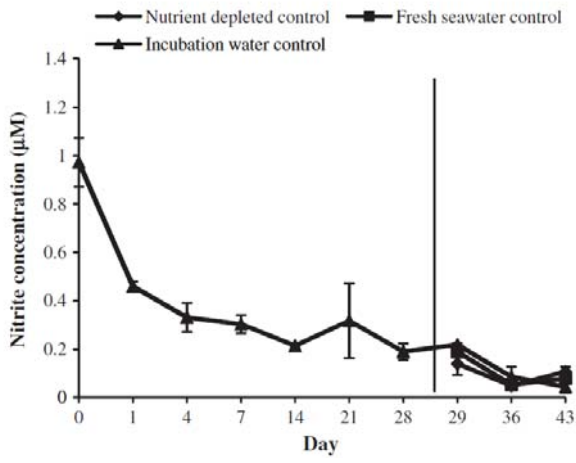


Fig 17

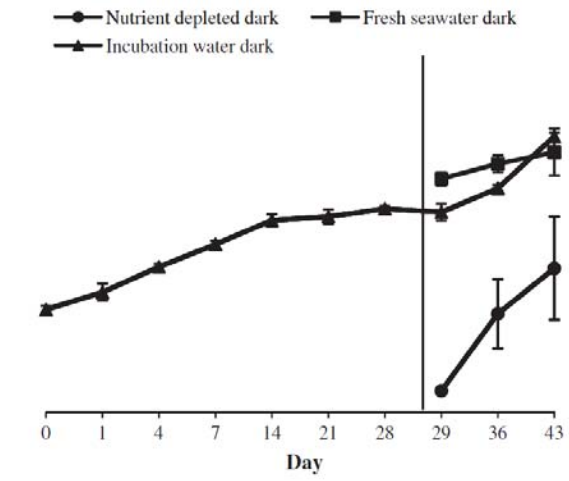
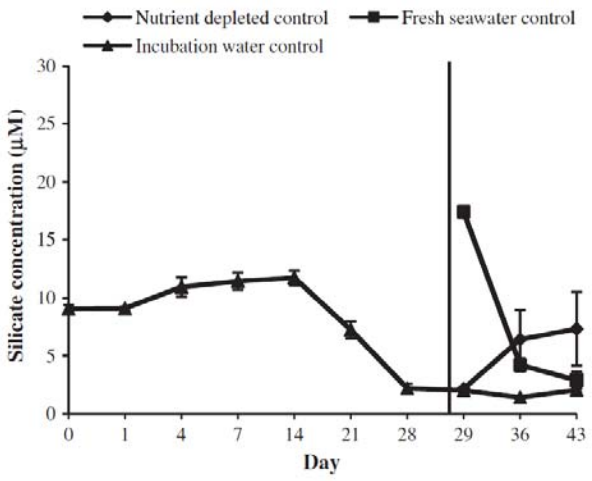


Fig 18

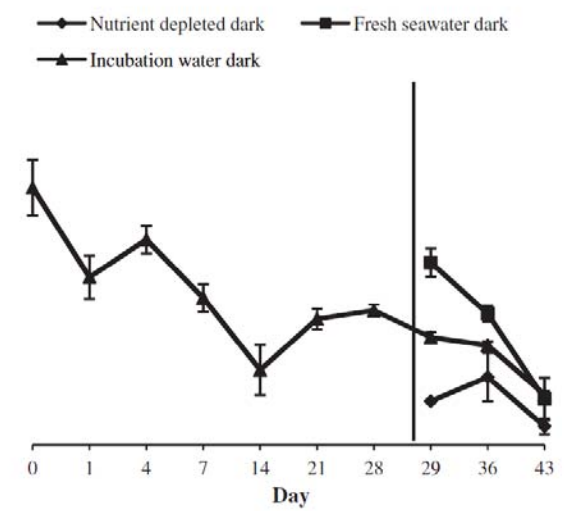
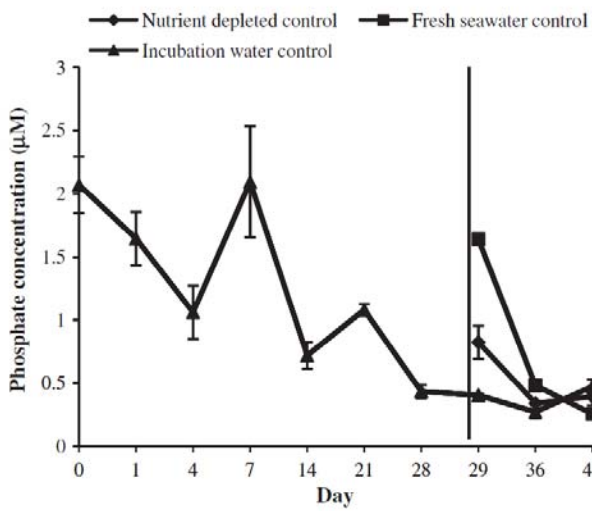


Fig 19

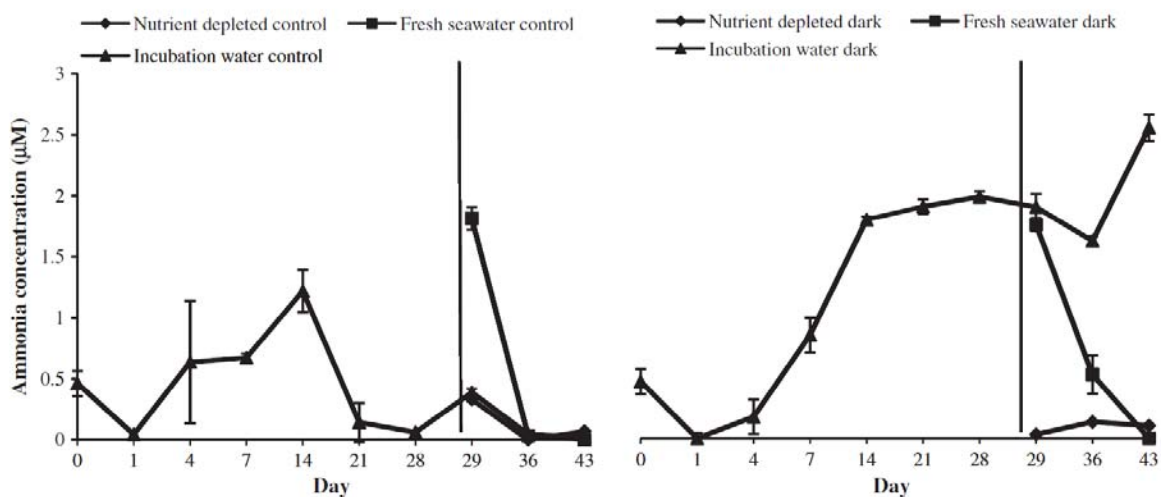


Fig 20

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; Hess-Erga et al., 2010; Wulff et al., 2008). 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 prevented, 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 (Kent et al., 1995; Miyahara et al., 1996; Takano, 1956). 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 (Barry et al., 2008; Behrens et al., 2005; Gollasch and Leppakoski, 1999, 2007; Hayes and Sliwa, 2003; Hayes, 1998; Hayes and Hewitt, 1998, 2000). 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.

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Figure legends

Figures 1 - 6. Chlorophyll α , nitrate, nitrite, silicate, phosphate and ammonia levels on all sampling days of the tropical population experiment performed in Goa. All values are the mean of three replicates \pm SE. The line indicates the end of the dark experimental period.

Figures 7 - 13. The temperate population experiment performed in autumn in Newcastle. Figures show nitrate, nitrite, ammonia, phosphate, silicate, chlorophyll α levels and live cell numbers obtained on all sampling days. Values from Control and Dark treated samples are separated, Control on the left graph and Dark treated on the right. The line indicates end of dark experimental period. All values are the mean of three replicates \pm SE.

Figures 14 - 20. The temperate population experiment performed in spring in Newcastle. Figures show chlorophyll α levels, live cell numbers, nitrate, nitrite, silicate, phosphate and ammonia levels obtained on all sampling days. Values from Control and Dark treated samples are separated, Control on the left graph and Dark treated on the right. The line indicates end of dark experimental period. All values are the mean of three replicates \pm SE.