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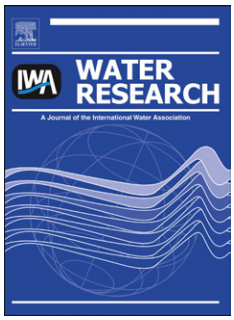

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Evaluation of a Bacterial Algal Control Agent in Tank-based Experiments

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Abstract

A bacterial-based bioremediation product, LakeRelief™ by Novozymes (Waterguru LakeRelief 2011), was tested in a series of experiments between October 2008 and March 2009 to evaluate its suitability as a short-term intervention technique to reduce algal blooms in the Swan-Canning River system. Results from fibreglass tank experiments (1100L) suggested that the product did not actively attack and lyse algal cells. The product decreased NH_4 and NO_x concentrations in treated tanks, both aerated and non-aerated. Product application decreased PO_4 concentrations in non-aerated tanks but not in aerated tanks. The product appeared to suppress algal growth in non-aerated tanks over short periods (several days). Algal growth regularly diminished after product application but reappeared shortly afterwards. Aeration had a negative effect on bacterial proliferation in the tanks, possibly through alteration of environmental conditions (e.g. water mixing). As a consequence of the environmental conditions in the tanks being counterproductive to the development of a representative microbial composition, several aspects regarding the product's effectiveness could not be assessed satisfactorily in the tank experiments. The importance of long-term nutrient immobilisation into a well developed food web and the subsequent nutrient removal through removal of the top order organisms is highlighted.

Keywords: Lake Relief, Bioremediation, Biological algal control, Algal lysing, Nutrient competition, Bloom prevention

1. Introduction

Algal blooms are part of the normal seasonal development in aquatic ecosystems, (Sigee 2005). In recent times, the use of fertilisers has dramatically accelerated eutrophication in aquatic systems (Carpenter 2005). In the Swan-Canning River system, Western Australia, long-term anthropogenic nutrient input has led to significant sediment enrichment, particularly in phosphorus (Swan River Trust 2005) The phosphorus can then be released from the river sediment under favourable P-release conditions (Xie, Xie, and Tang 2003) and becomes rapidly available for algal growth. Consequently, harmful algae bloom (HAB) outbreaks are symptomatic of the deteriorating health of the Swan-Canning River system.

A range of short-term intervention strategies have been trialled to reduce the frequency and severity of algal blooms but with limited success. Past methods of algal control included the use of algicides such as copper sulphate (Mason 1996). Several drawbacks from this method were its non-selective nature, the resulting destruction of other freshwater biota, and its prolonged presence in the water body. Other approaches that are more ecologically agreeable are artificial oxidation (Kim 2006; Swan River Trust 2000) or application of clay-based materials that bind phosphorus making it unavailable for algal growth (Pan et al. 2006; Swan River Trust 2001). While each of these methods is valuable, a mechanism to rapidly remove existing blooms is always attractive to managers dealing with immediate community concern.

An alternative approach to algal control is known as bioremediation or biological control. Here, specially selected bacterial strains are introduced, they quickly populate the treated

water body and take up excess phosphate and nitrogen, thereby out-competing algal populations (Kirchman 1994). Some bacteria in these products also excrete enzymes that break down algae cell walls, a process that is known as algal lysing (Lee et al. 2000). Bioremediation techniques are considered to have relatively low environmental impact and are said to be free of unintended side affects such as fish kills or degradation of plant life (Head 1998). Moreover, they attempt to stimulate or augment existing bacterial populations in a water body but not to replace them. It is further claimed, but not scientifically verified, that if these bio-control products are released into the environment, the introduced bacteria will have no selective advantage over indigenous microbes, unless the targeted pollutants are present (Waterguru Bertram Case Study 2011). However, attempts to clearly define the efficacy of these products are made more difficult by the confidentiality of the active constituents for various commercial-in-confidence reasons (Head 1998; Wang, Li, and Kang 2007).

Lake Relief™ by Novozymes is a biological algae control product that has been used during the last couple of years in field trials at Lake Bertram in Perth's southern suburbs and at the Joondalup golf course, Perth, Western Australia (Waterguru Case Studies 2011). Anecdotal findings from these trials suggest that the product has successfully reduced algal growth at these locations. Moreover, it is claimed by the product distributor that treatment with the product leads to a rapid disappearance of the surface scum in the treated water bodies, often within a few days (Lee, personal communication 01/10/2008) However, no independent scientific verification on the mechanism by which the product reduces algal growth has been conducted.

Only limited information is publically available regarding the active ingredients in LakeRelief. The product is described as a mixture of six different bacterial cultures with more than 10^{12} microorganisms contained per 1lb bag (Bjørn 2007). Due to these commercial-in-confidence restrictions, the present study took an ecological approach to determine the efficiency of LakeRelief. Owing to the potential ecological effects of product application into open water bodies, it was decided to conduct the study in mesocosms. This led to the design of several tank based experiments with the aim to verify if the product was able to reduce algal blooms and by which mechanism - whether by lysing algal cells, a requirement essential for the rapid disappearance of algal blooms within a few days as reported by the water manager, or via the reduction of nutrient concentrations, thus out-competing algae for vital nutrients. If LakeRelief was able to reduce or minimise the potential for algal blooms and effectively contribute to algal bloom decline without any adverse ecological impact, the product could play a significant role in improving the health of rivers and wetlands, in the Swan-Canning River system and elsewhere.

2. Methods

2.1. Investigating LakeRelief's algal lysing properties

The rapid disappearance of surface algal conglomerations within a few days, as described by the water manager, suggested that algal cells might be actively attacked by algicidal bacteria strains. These bacteria are capable of lysing algal cells on contact (Mayali and Doucette 2002). In order to verify the observations reported from the field and to assess LakeRelief's capability for algal lysing, sixteen fibreglass tanks (1000mm height; 1800mm internal diameter) located at Murdoch University were selected for this experiment. The tanks had been filled with rain water during winter and in spring a diverse algal community had

developed naturally with a species composition typical of wetlands and rivers in the area. Advantage was taken of this well-developed algal bloom of the type the product was designed to reduce. *Oocystis spp.* (green algae) was the dominant algal species existing in the tanks. The tanks also contained *Planktolyngbya subtilis* (blue-green algae), *Scenedesmus spp.* (green algae), *Synechocystis spp.* (blue green algae) and traces of numerous other algal species. Strongly elevated pH of 10.1 and dissolved oxygen (DO) concentrations above 140% saturation (11.5mg/L) in the tanks indicated substantial photosynthetic activity. At the start of the experiment as signified by the input of LakeRelief, orthophosphate (PO_4-) concentrations in the tanks were below 35µg/L and ammonium (NH_4-) concentrations were below 20µg/L. While both values were well within the range of healthy wetlands (ANZECC guidelines) and given that this experiment was solely aimed at testing the immediate effect of product bacteria on a well-established algal community, no additional nutrients were added to the tanks. Please note: ANZECC water quality guidelines were used throughout this study to reference conditions in the experimental tanks to those of natural systems in the region.

Due to the rapid growth of oxygen consuming bacteria and the potential ecological consequences of deoxygenation, it was recommended to aerate treated water bodies when using the product in the field (Lee, personal communication 01/10/2008). The impacts of the product under aerated and non-aerated conditions were therefore investigated. The treatments of 'product' and 'aeration' resulted in a factorial experiment with four groups each with four repeats (1. product/aeration; 2. product/no aeration; 3. control/aeration; 4. control/no aeration). The recommended product dosage for field application was 2ppm (Lee, personal communication 01/10/2008). However, in order to observe the potential effect of a reduced dosage, it was decided to begin the experiment with a product application rate of 1ppm. Coinciding with the first product application, aeration was initiated in the corresponding

tanks (Precision SR 2500 aquarium air pumps). After three days and in the absence of an immediate response, the corresponding tanks were re-dosed at the higher dose of 2ppm. Over a period of one week, sets of water quality parameters (see Chapter 2.4) were analysed daily.

2.2. Investigating nutrient competition and algal growth suppression by LakeRelief

In order to evaluate the product's ability to out-compete algal organisms for the macronutrients nitrogen and phosphorus, a test was designed that simulated an unpolluted water body with little microbiological activity that suddenly experienced high nutrient pollution. The underlying theory was that product bacteria would rapidly multiply and incorporate the macronutrients phosphorus and nitrogen into bacterial biomass and thus, render them unavailable for algal growth (Doucette 1995; Doucette et al. 1998). Nutrient input as a trigger for microbial growth and product addition occurred simultaneously, thus allowing for an assessment of the product's ability to immediately out-compete and consequently, suppress or reduce algal bloom development.

For this experiment, the tanks were drained, cleaned, and refilled with 1100 litres of groundwater from the Murdoch veterinary farm at Murdoch University. The tanks were then left to settle for one week. Based on visual observation, no significant algal growth occurred during this period. In order to seed the tanks with river-specific algal cells, river sediment and water were collected from the Canning River (Western Australia), approximately 150 metres upstream from the Kent Street Weir. This location was chosen as it commonly has algal blooms in summer and autumn (Swan River Trust 2005). Approximately one kilogram of sediment and five litres of river water were then added to each tank.

After a one day settling period, all tanks were fertilized with 'Thrive soluble all purpose plant food' by Yates to a calculated total phosphorus (TP) concentration of 2mg/L and a calculated total nitrogen (TN) concentration of 10mg/L of tank water. These nutrient concentrations represented highly eutrophic conditions that would result in immediate and rapid algal growth. Aeration was initiated in the corresponding tanks and maintained over the duration of the experiment. The tanks selected for 'product' treatment were then supplied with 4ppm product. This higher dosage was chosen based on previous observations in the algal lysing experiment, where routine nutrient monitoring had not demonstrated a significant effect on the phosphorus and nitrogen levels in response to LakeRelief application. When the initial input did not enable an effect to be definitely observed, incremental doses were used throughout the experiment to attempt to produce a significant effect (as indicated by red arrows on Figures 4 to 9). Physiochemical parameters and chlorophyll *a* were measured twice weekly and nutrients were analysed once a week. On several occasions, samples for the microscopic identification of algal species were taken from the tanks and analysed, so as to monitor changes in algal composition over time. The experiment was conducted over a period of seven weeks.

2.3. Confirmation of product viability

Prior to each application into the corresponding tanks, the product was dissolved in tap water. For this purpose, the tap water had previously been aerated for twenty-four hours to facilitate the removal of chlorine, which could otherwise have impacted negatively on bacterial health. In order to verify the vitality of the product constituents, bacterial cultures were grown on Difco™ nutrient agar plates (Becton, Dickinson and Company). A small amount of the product (approximately 20µg) was dissolved in 5ml sterile isotonic NaCl solution. This

emulsion was inoculated in concentrated form onto one plate whereas a second plate received a 1:10 dilution.

2.4. Analysis

Nutrient analyses (TP, PO_4, TN, NH_4, NO_x) were carried out by the Marine and Freshwater Research Laboratory (MAFRL; NATA accredited No. 10603) at Murdoch University. Filterable reactive phosphorus was analysed by the ascorbic acid method (Johnson 1982); nitrate plus nitrite by copper-cadmium reduction (Johnson 1983) and ammonium by the alkaline phenate method (Switala 1993). Total nitrogen and total phosphorus were determined from autoclave digests with potassium persulphate (Valderrama 1981). Sediment samples were digested in concentrated sulphuric acid in the presence of a copper catalyst for total Kjeldahl nitrogen (TKN) and total Kjeldahl phosphorus (TKP). All analyses were carried out on a Lachat Quick-Chem 8000 Automated Flow Injection Analyser. The remaining parameters were determined either on-site (turbidity, pH, DO, temperature, conductivity), at the Loneragan laboratory facility at Murdoch University (COD), or at the MAFRL laboratory facility at Murdoch University (chlorophyll *a* by fluorescence method). The equipment used for these analyses is listed below (Table 1).

Table 1: Equipment list for physiochemical water analysis (used in all experiments in this study)

Statistical analysis was carried out via SPSS 16 for Windows (Statistical Product Service Solutions, Chicago, IL, USA, 2007). Because each microcosm was measured repeatedly, F-values and significance levels in the following results section were obtained from a statistical method known as ‘doubly multivariate repeated measures design’. Interaction terms were

198 initially included in the analysis but did not produce significant results and were consequently
199 removed from the model. Relationships between parameters were examined by computing
200 Spearmen rank-order correlation, a non-parametric test for data that is not normally
201 distributed (Dytham 2005).
202

203 3. Results

204 3.1. Investigating the vitality of LakeRelief bacteria

205 After an incubation period of twenty four hours at 30^0C, the cultivations prepared on Difco™
206 nutrient agar plates demonstrated strong bacterial growth. Both plates (concentrated and 1:10
207 dilution) were densely colonised, suggesting that a large number of vital bacteria were
208 contained in the product.
209

210 3.2. Investigating LakeRelief's algal lysing properties

211 3.2.1. pH

212 The naturally developed and well established algal blooms had created extremely alkaline pH
213 conditions in the tanks. Measurements revealed a pH of 10.1, well above the ANZECC
214 threshold of 8.0 that is indicative of the ecological health in local wetlands (ANZECC 2000).
215 Coincidentally, the random selection of tanks into the four test groups had led to a higher
216 algal incidence in the two product groups (both aerated and non-aerated). In response to these
217 unequal amounts of initial algal biomass, the product groups overall demonstrated higher pH
218 compared to control groups. A large amount of the variation between sampling dates resulted
219 from the prevailing climatic conditions (i.e. pH as a function of light intensity). Statistically,
220 neither product ($F_{1,12} = 3.621$, $P = 0.081$) nor aeration ($F_{1,12} = 0.000$, $P = 0.983$)
221 significantly affected pH. However, Figure 1 demonstrates that while pH in product/aeration

tanks behaved similarly to the pH in control groups, the pH in the product/no aeration group was slightly reduced in response to product application. This suggested that algal metabolism was somewhat diminished in this group.

Figure 1: Testing algal lysing properties - pH value of the water column in product and control tanks (P/A = microcosms with product and aeration, P/na = microcosms with product and no aeration, c/A = microcosms with no product (control) and aeration, c/na = microcosms with no product (control) and no aeration). Red arrows indicate product application.

3.2.2. Dissolved Oxygen (DO)

DO was supersaturated in all tanks and exceeded the ANZECC threshold of 120% saturation (10mg/L**)**, reflecting strong algal photosynthetic activity (Figure 2). The different DO concentrations between the groups signify the initial unequal presence of algal biomass and the variation between the sampling dates represents climatic influences. The induction of aeration immediately reduced DO in aerated tanks compared to non-aerated tanks. As DO concentrations in aerated tanks remained lower throughout the experiment, this was significant ($F_{1,12} = 8.369$, $P < 0.05$). In contrast, the second factor product did not produce a significant effect on DO concentrations ($F_{1,12} = 2.765$, $P = 0.122$).

Figure 2: Testing algal lysing properties - DO saturation of the water column in product and control tanks

3.2.3. Chlorophyll *a*

The unequal biomass distribution between the four test groups was once again demonstrated in the chlorophyll *a* concentrations at the start of the experiment (Figure 3). Regardless,

chlorophyll *a* concentrations in all groups exceeded trigger values of 3-5µg/L (ANZECC 2000). Due to large standard deviations, both factors product ($F_{1,12} = 3.252$, $P = 0.096$) and aeration did not significantly affect chlorophyll *a* concentrations ($F_{1,12} = 1.175$, $P = 0.300$). However, while chlorophyll *a* concentration increased in the product/aeration tanks at a rate comparable to control tanks, it reduced over time in the product/no aeration group, suggesting reduced algal metabolism.

Figure 3: Testing algal lysing properties - Chlorophyll *a* concentration of the water column in product and control tanks

3.3. Investigating nutrient competition by LakeRelief

3.3.1. Orthophosphate (PO_4)

PO_4 concentrations in the tanks were initially between 30 and 50 µg/L. Following nutrient input, they strongly increased over the next four days in product/aeration and in both control groups but remained much lower in product/no aeration (Figure 4). Nevertheless, the ANZECC threshold of 40µg/L was dramatically exceeded in all groups. Although the effect in the product/no aeration group was strongly masked by the dissimilar reaction in product/aeration tanks, the statistical analysis of product versus control groups produced an almost significant result. The measurable effect of factor product was just outside the 5% significance level ($F_{1,12} = 4.252$, $P = 0.062$). Based on the large variation within groups, aeration was not significant ($F_{1,12} = 1.601$, $P = 0.230$).

Figure 4: Testing nutrient competition - Orthophosphate concentration of the water column in product and control tanks.

3.3.2. Ammonium (NH_4)

While less than 35 µg/L NH_4 was initially found in the water column (twenty-four hours after massive nitrogen input as NH_4), all groups strongly exceeded the threshold value of 80µg/L (ANZECC 2000) shortly afterwards (Figure 5). The subsequent development of NH_4 concentrations was significantly determined by the factor product. In product tanks, NH_4 concentrations decreased faster (after Jan 26th) compared to control tanks ($F_{1,12} = 6.672$, $P < 0.05$). Deviating from the observations for orthophosphate, this response was demonstrated in both product groups. After Feb 10th, NH_4 was almost completely absent from all product tanks whereas around 200µg/L NH_4 were still available in control tanks. Aeration had no significant effect on NH_4 concentrations ($F_{1,12} = 0.106$, $P = 0.750$).

Figure 5: Testing nutrient competition - Ammonium concentration of the water column in product and control tanks.

3.3.3. Nitrite and Nitrate (NO_x)

The almost complete absence of NO_x (below 10µg/L) from all tanks twenty-four hours after fertiliser input was followed by a dramatic increase over the next four days (Figure 6). After NO_x had reached extreme concentrations (800% above the ANZECC threshold of 150µg/L), their subsequent reduction was significantly accelerated by the product ($F_{1,12} = 10.177$, $P < 0.01$). On Feb 3rd, NO_x concentrations in product tanks were below 100µg/L, whereas they were approximately five times higher in control tanks. After Feb 10th, NO_x was completely absent from the product tanks, compared to around 200µg/L in control tanks. The factor aeration was not significant ($F_{1,12} = 2.029$, $P = 0.180$).

Figure 6: Testing nutrient competition - Nitrite and Nitrate concentration of the water column in product and control tanks

3.4. Investigating LakeRelief's ability to prevent, reduce or eliminate algal blooms

3.4.1. pH

The immediate and extreme pH rise in response to algal seeding and nutrient addition reflects the dramatic increase in algal metabolism in all groups (Figure 7). Within four days, algal photosynthesis had resulted in extremely alkaline pH, dramatically exceeding ANZECC guidelines of 6.5-8.0 (ANZECC 2000). A slightly faster and initially higher pH rise occurred in non-aerated tanks. Furthermore, in aerated tanks pH fluctuation was less pronounced throughout the experiment, somewhat moderated by aeration. Consequently, aeration had a significant influence on pH ($F_{1,12} = 11.858$, $P < 0.01$). The pH in product/no aeration tanks did often reduce when product bacteria were added (particularly after the second, fourth and fifth dosing events), suggesting reduced algal photosynthesis. The same response could not be observed for the product/aeration group, where the pH often increased after product application (second, third and fourth dosing). The opposing reaction to product in this group lead to a large variation within the two product groups and consequently, resulted in a non-significant result for factor product overall ($F_{1,12} = 3.292$, $P = 0.095$).

Figure 7: Testing algal growth suppression - pH value of the water column in product and control tanks

3.4.2. Dissolved Oxygen (DO)

Similar to pH, the DO increase in all groups immediately after nutrient input corresponded to the dramatic increase in algal biomass and the intensified production of oxygen via photosynthesis in all groups (Figure 8). However, this rise was significantly stronger in non-aerated groups ($F_{1,12}$ = 12.752, P < 0.01), were DO rapidly exceeded the threshold value of 120% saturation (10mg/L; ANZECC 2000). Throughout the experiment, aerated tanks maintained 'healthier' DO concentrations than non-aerated tanks. In aerated tanks, DO never fell below 75% saturation (6.5mg/L). Equally, aeration prevented extreme DO-supersaturation. Whereas DO reached up to 200% saturation (>15mg/L) in non-aerated tanks, DO never rose above 160% saturation (12mg/L) in aerated tanks. Thus, aeration was effectively 'buffering' DO concentrations. Similar to previous findings for pH, DO concentrations in product/no aeration tanks appeared to reduce in correspondence to the input of product (second, fourth and fifth dosing). As this response to product application was not evident in the product/aeration group, the discrepancy within the two product groups lead to a non-significant result overall ($F_{1,12}$ = 1.697, P = 0.217).

Figure 8: Testing algal growth suppression – DO saturation of the water column in product and control tanks

3.4.3. Chlorophyll *a*

Shortly after nutrient input, chlorophyll *a* exceeded ANZECC guidelines (3–5µg/L) in all tanks (Figure 9). The subsequent increase of chlorophyll *a* reflects the build up of algal biomass. Chlorophyll *a* markedly dropped in both control groups around Feb 3rd. The product/no aeration curve confirmed the previously observed reduction in algal growth in response to the second, fourth and fifth product dosing events. As this response was again not demonstrated in the product/aeration group, the large variation between the product groups

resulted in overall insignificance for the factor product ($F_{1,12}$ = 1.996, P = 0.183). Similarly, aeration did not have a significant influence on chlorophyll *a* concentrations ($F_{1,12}$ = 0.951, P = 0.349).

Figure 9: Testing algal growth suppression – Chlorophyll *a* concentration of the water column in product and control tanks

4. Discussion

4.1. Investigating LakeRelief's algal lysing properties

The value of algicidal bacteria as a factor in the control of algal blooms and in limiting algal biomass has been highlighted by a number of studies (Lovejoy, Bowman, and Hallegraeff 1998; Mitsutani and Yamasaki 2001; Wu, Xi, and Zhao 2002). Studies in eutrophic stormwater detention ponds suggest that algicidal bacteria are a more effective factor in bloom termination than nutrient depletion (Bunker 2004; Lewitus et al. 2004; Lewitus et al. 2003). In regards to the mode of operation of the tested product, previous observations from field trials described the rapid disappearance of algal blooms (Waterguru Case Studies 2011). These anecdotal findings suggest that the product might contain algicidal bacterial strain(s), capable of breaking up algal conglomerations by actively lysing algal cells.

Algicidal activity is operationally defined as the induction of algal cell lysis within one to two days following bacterial introduction into algal cultures (Mayali and Doucette 2002). If LakeRelief was capable of lysing algal cells in the present experiment, this would have quickly led to dramatic changes in the tanks, signified by a strong and immediate reduction of the key indicators for algal photosynthetic activity, i.e. pH, DO and chlorophyll *a*. However,

such changes were not observed in the tanks (Figures 1, 2 and 3). This does not definitively preclude the presence of algal lysing strains in LakeRelief, it is possible that conditions in the tanks (high pH and DO) may have suppressed these strains.

4.2. Investigating nutrient competition by LakeRelief

A study on bacterial nutrient uptake in lake and sea water indicated that the amount of inorganic phosphate taken up by bacteria can exceed 50% of the entire phosphate pool (Kirchman 1994). This suggests that bacteria are strong competitors for phosphate under favourable circumstances. The situation is more complex for nitrogen, where bacteria are traditionally considered superior in competition for organic forms of nitrogen, while algae appear to be superior competitors for inorganic nitrogen supplied as NH_4 (Løvdal et al. 2008). Consequently, nutrient competition between the bacteria and algae varies depending upon which form of nitrogen, either organic or inorganic, is more readily available.

In the present experiment there was principal support for the product's ability to out-compete algae for nitrogen and phosphorus:

1. the product accelerated NH_4 and NO_x reduction in all treated tanks, both aerated and non-aerated; and
2. product application resulted in PO_4 reduction in product/no aeration tanks only. Aeration had a negative effect on bacterial proliferation, possibly through alteration of environmental conditions in the tanks.

However, visual observations and experimental data analysis suggested that nutrient cycling processes occurred at an accelerated rate in the confined tanks. As a result, the nutrient removal rate achieved by product bacteria could not be accurately quantified in this experiment.

The accelerated reduction of NH_4 and NO_x concentrations that was observed in both product treatments and the corresponding chlorophyll *a* measurements as an indicator for algal metabolism strongly suggested that product bacteria were responsible for the nitrogen uptake (Figures 5 and 6). Based on the corresponding reduction of NH_4 and NO_x after Jan 26th, the implication was that the product may have supplemented both nitrifying and denitrifying bacterial strains in the tanks (Blackburn et al. 1994).

Regarding phosphorus, the findings were more difficult to interpret. In product/no aeration tanks, algal biomass measured as chlorophyll *a* closely responded to the application of the product, diminishing strongly particularly after the high dosing rates on day 19 (Feb 3rd) and day 28 (Feb 12th) (Figure 4). Reduced algal activity in this group was demonstrated by several more parameters (turbidity, pH, DO, COD). Coinciding with these observations, PO_4 concentrations in the water column were markedly lower in the product/no aeration group compared to all other groups. The combination of standing algal biomass reduction and a reduction of PO_4 suggested that PO_4 had been taken up by product bacteria in this treatment type. The situation was different for the product/aeration group. Here, PO_4 concentrations were similar to those in control groups. Furthermore, the fluctuation in algal activity appeared to be unrelated to product application. This suggested that the effect of aeration had interfered and reduced bacterial ability to take up PO_4 and out-compete algae in this group.

4.3. Investigating LakeRelief's ability to prevent, reduce or eliminate algal blooms

Visual observations and analytical results (turbidity, chlorophyll *a*) provided good indication that algal growth was inhibited in product/no aeration tanks in response to LakeRelief input

(Figure 9). In one case, a DO reduction to 23% saturation (2mg/L) shortly after product application reflected the intensive bacterial metabolism that occurred in the tank. However, the algal population appeared to quickly recover within a few days. Once again, the product/aeration group did not demonstrate this short-term algal suppression in response to product application. This suggested that artificial aeration hindered the development of product bacteria in the confined mesocosms, possible related to the strong vertical mixing effect. While this was an unfortunate consequence of the experimental design, it must be stressed that due to the potential of aerobic bacteria to quickly metabolise the available oxygen, aeration is a specified requirement in bioremediation trials in natural water bodies.

An additional aspect that may have over time strongly influenced the product's performance in the aerated tanks was the algal species composition that evolved in response to continuous aeration. While two species of green algae Scenedesmus spp. and Selenastrum sp. 001 appeared to be favoured in non-aerated (= stagnant) tanks where they were detected in large numbers, cyanobacteria Synechocystis spp. was represented stronger in aerated tanks throughout the experiment. This might be related to the ability of bacteria to exhibit buoyancy migration (Sigee 2005), which would promote them over green algae as competitors for the available nutrients in the stagnant (= stratified) tanks.

4.4. Limitations in tank based experiments

Findings in the present study were in line with Duvall and Anderson (2001) who found that the conditions for observing the competition between algae and bacteria in the confined space of small scale experiments might not be optimal. Duvall, Anderson and Goldman (2001) reported little difference amongst microbial products and non-treated controls even in reactors of 61,000L capacity. This was despite the fact that they found a significant increase

444 of bacterial populations at least in the short term. The apparent interference of aeration on the
445 ability of bacteria to take up nutrients in the present study led to a more general consideration
446 of limitations in tank based experiments (see also Table 2).
447
448 An initial limitation to bacterial growth may have occurred in the nutrient competition
449 experiment, when algal seed material, nutrients and product bacteria were simultaneously
450 added to the tanks at the first product dosing event. Here bacterial growth was prevented due
451 to the absence of organic carbon as substrate (Bratbak 1987; Corina, Bussaard, and Riegman
452 1998). Thus, algal cells took up the available nutrients and rapidly increased their numbers to
453 the point where further population size increase was limited by other factors (e.g. reduced
454 light intensity, expressed as turbidity). Over time and in response to the build-up of algal
455 biomass, algal photosynthesis and algal excretion processes provided the organic substrate
456 necessary for bacterial growth (Brown and Zeiler 1993). This limitation would not have
457 occurred in a natural system where organic carbon is usually available as detritus etc.
458
459 Regarding physiochemical parameters, pH and DO strongly influenced the outcome of the
460 experiments. Recommendations for the optimum conditions that ensure good biological
461 activity of product bacteria are a pH within the range of 5.5-8.5 and DO concentrations of 6-
462 9mg/l (Lee, personal communication 01/02/2009). Unfortunately, the limited size of the tanks
463 promoted extreme pH and DO concentrations (Figures 1, 2, 7 and 8) and the recommended
464 thresholds were often dramatically exceeded in the tanks (pH up to 11; DO up to 200%
465 saturation or 15mg/L). It is likely that the high pH in the tanks led to a competitive advantage
466 of algae over bacteria and therefore restricted bacterial proliferation. Resulting from the finite
467 space of the tanks, several more factors likely affected the outcome of the tank experiments.
468 These factors are briefly summarised in Table 2.

Table 2: Limitations in tank experiments and their consequences

4.5. Long-term nutrient immobilisation

As previously discussed, visual observations and experimental data analysis led to the conclusion that nutrient cycling processes occurred at an accelerated rate in the confined space of the experiment tanks. A key requirement for long-term immobilisation of macronutrients nitrogen and phosphorus and thus, suppression of algal growth over any substantial time, is the establishment of a well developed tropic cascade. In order to be successfully employed as a long-term algal bloom management tool, optimum growth conditions for bacterial metabolism, a well developed trophic cascade and regular harvesting of the accumulated nutrients through removal of highest level organisms would be essential. This suggests that there may be an opportunity for biological control agents such as LakeRelief as a long-term algal bloom management tool in the aqua-cultural industry were nutrients are passed through a food web and are ultimately removed through harvesting of the top order organisms in the system e.g. prawns or fish.

In contrast to this, in natural wetland management efficient nutrient removal through harvesting of the top order organisms does not take place. As a result of the continuous nutrient recycling in such a system, bioremediation programs can only suppress algal bloom development by constantly dosing and maintaining unnaturally high levels of bio-control bacteria. This brings up another aspect regarding the impact of bio-control bacteria on food web structure. In a simplified model it could be hypothesised that when bacterial numbers are continuously kept at higher than natural levels due to constant external input, their predators (heterotrophic nanoflagellates and microzooplankton) would respond to the increased food

494 availability by increasing their own numbers over time. This would eventually lead to larger
495 numbers of organisms in the next higher trophic level (zooplankton grazers) and so forth.
496 While the build-up of particular food web species to unnaturally large numbers may occur in
497 natural systems in response to the temporary release of bottom-up or top-down pressure, both
498 pressures quickly increase in response to this build up. Thus, population density of the
499 previously uncontrolled species will decrease in response to the strengthened control
500 mechanisms. Therefore, it could be argued that the constant addition of bacteria as a food
501 source would lessen the regulatory strength of bottom-up pressure and ultimately lead to
502 ecosystem destabilisation and a much more rapid turnover of nutrients.
503

504 5. Conclusion

505 In this ecological study, a series of tank based experiments was conducted to investigate the
506 efficiency and the modus operandi of a bacterial based bioremediation product, LakeRelief^{TM}
507 by Novozymes. The ability to actively attack and lyse algal cells - essential for the rapid
508 disappearance of algal blooms within 1-2 days - was not observed. LakeRelief's capacity to
509 compete with algae for vital macronutrients was indicated. Under suitable conditions, the
510 product bacteria grew and incorporated available nutrients nitrogen and phosphorus into
511 bacterial biomass. However, due to the accelerated rate of nutrient recycling in the tanks,
512 resulting from the absence of a significant trophic cascade and the restricted space of the
513 mesocosms, it could not be statistically confirmed that the bacteria were successfully out-
514 competing algal organisms for vital macronutrients over any significant time period.
515 Essential conditions for bacterial growth were not satisfied in the confined space of the tanks
516 and thus, frustrated the process of algal growth suppression. Amongst these confining factors
517 were carbon limitation, extreme physiochemical conditions, water motion from aeration and
518 unfavourable light conditions in the tanks. While application of bio-control agents may

519 provide very temporary reduction in algal blooms in natural wetlands, the key requirements -
520 long-term nutrient immobilisation into an artificially amplified food web and the subsequent
521 removal of nutrients through removal of the top order organisms - are not satisfied. The
522 requirement to continually add product to retain an algal suppression effect could result in
523 rapid turnover of nutrients and ecosystem destabilisation.
524

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528 this research.
529

530 7. References

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The tested bacterial-based bioremediation product did not actively lyse algal cells

The product suppressed algal growth in tanks over short periods (several days)

Testing product performance in tank-based experiments was hindered by limitations

No long-term nutrient immobilisation, thus unsuitable for natural wetland treatment

Table 1: Equipment list for physiochemical water analysis (used in all experiments in this study)

Parameter	**Equipment**
Turbidity	Hach Portable Turbidimeter 2100P
pH	Hanna Microcomputer pH meter HI 8424
DO	Hanna Microprocessor Dissolved Oxygen meter HI 9145
Temperature	Hanna Microprocessor Dissolved Oxygen meter HI 9145
Conductivity	Hanna Conductivity meter HI 8733
COD	Hach Portable Data-logging Spectrometer DR 2010
Chlorophyll *a*	Spectrophotometer Novaspec II
Fluorescence	Turner Design Fluorometer Model 10-005

Table 2: Limitations in tank experiments and their consequences

Nutrient and carbon limitation	Bacterial growth was impeded by unfavourable nutrient and carbon supply ratios available at times of product dosing.
Physiochemical extremes	As strong algal photosynthesis occurred throughout the whole reactor, pH and DO raised to extreme levels, hostile for bacteria.
Uniform conditions	In the confined reactor space, pH and DO concentrations were homogenous. No buffer zones existed, where conditions are less extreme.
Water mixing from aeration	Water motion influenced algal species composition and promoted certain - mobile - species (cyanobacteria) over others (green algae).
Surface to tank volume ratio	Measurements of chlorophyll *a* and turbidity were strongly influenced by the rate of algal attachment onto reactor surfaces.
Light intensity	Light regime in the reactors affected algal responses and possibly, bacterial/algal interaction.
Absence of food web	While some nutrients appeared to be assimilated into bacterial biomass, fast re-release seemed to occur in the tanks, intimately linked to bacterial biomass die-off conditions. This was related to the absence of a tropic cascade and thus, no immobilisation and transport of nutrients into higher organisms occurred.

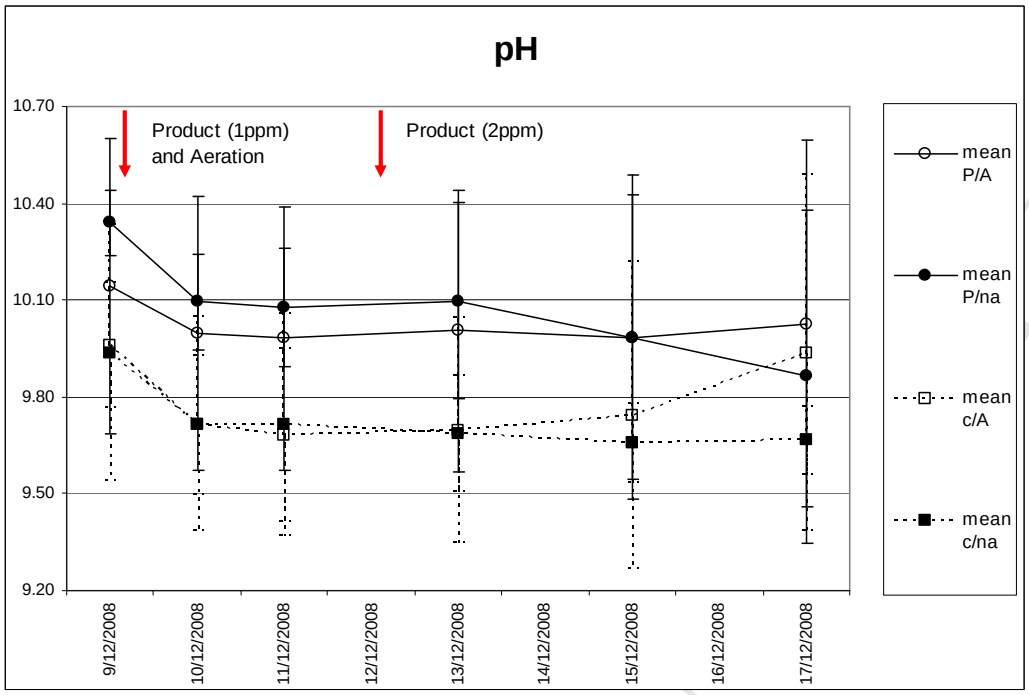

Figure 1: Testing algal lysing properties - pH value of the water column in product and control tanks (P/A = microcosms with product and aeration, P/na = microcosms with product and no aeration, c/A = microcosms with no product (control) and aeration, c/na = microcosms with no product (control) and no aeration). Red arrows indicate product application.

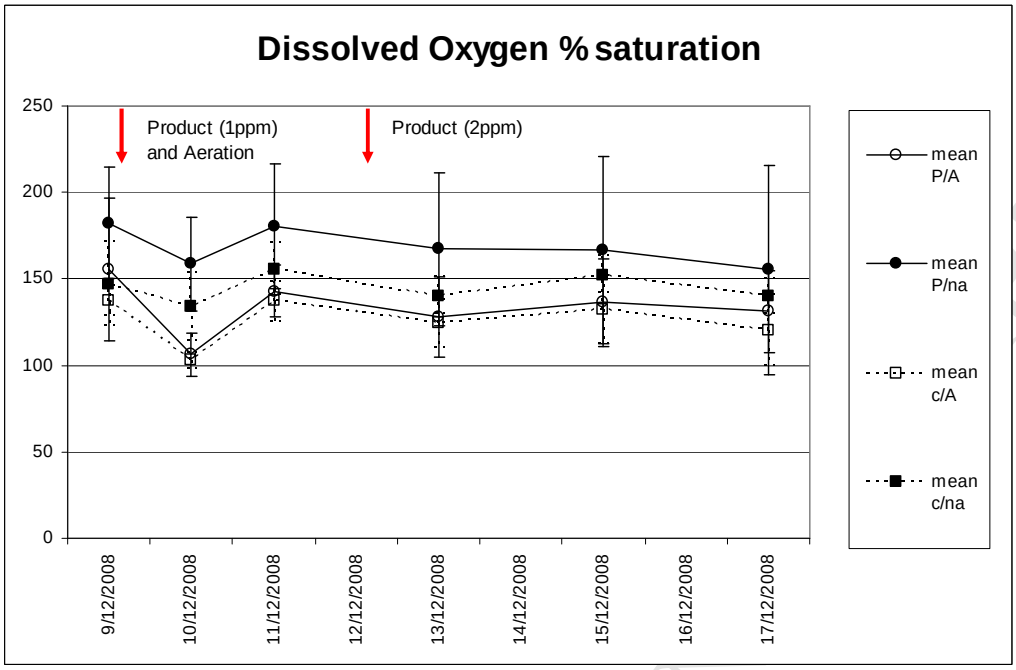

Figure 2: Testing algal lysing properties - DO saturation of the water column in product and control tanks

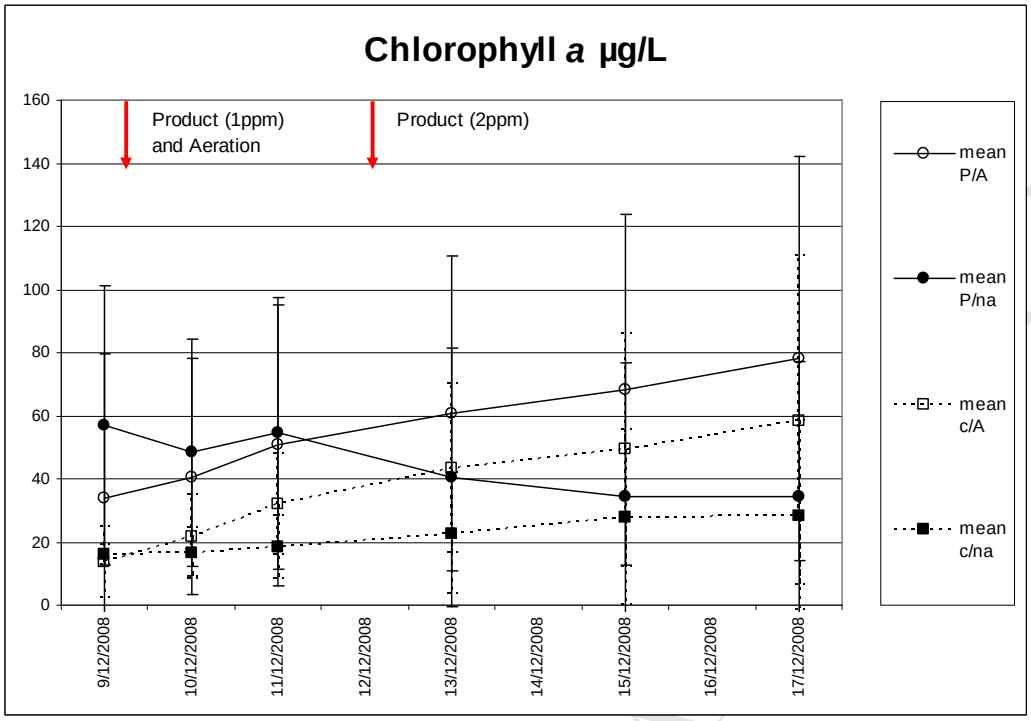

Figure 3: Testing algal lysing properties - Chlorophyll *a* concentration of the water column in product and control tanks

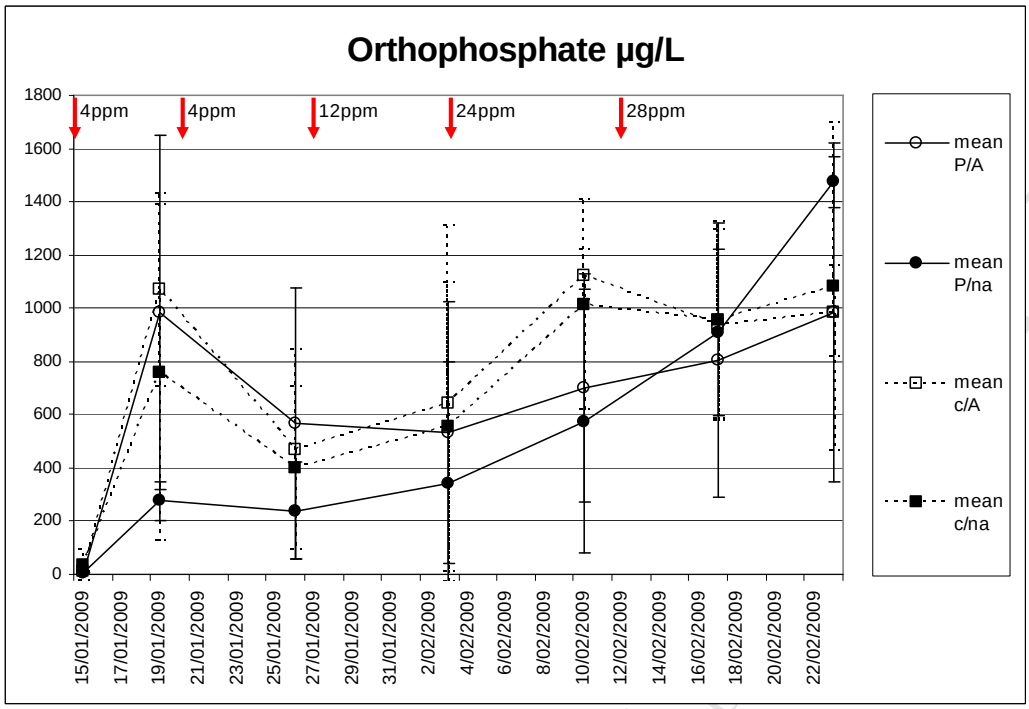

Figure 4: Testing nutrient competition - Orthophosphate concentration of the water column in product and control tanks.

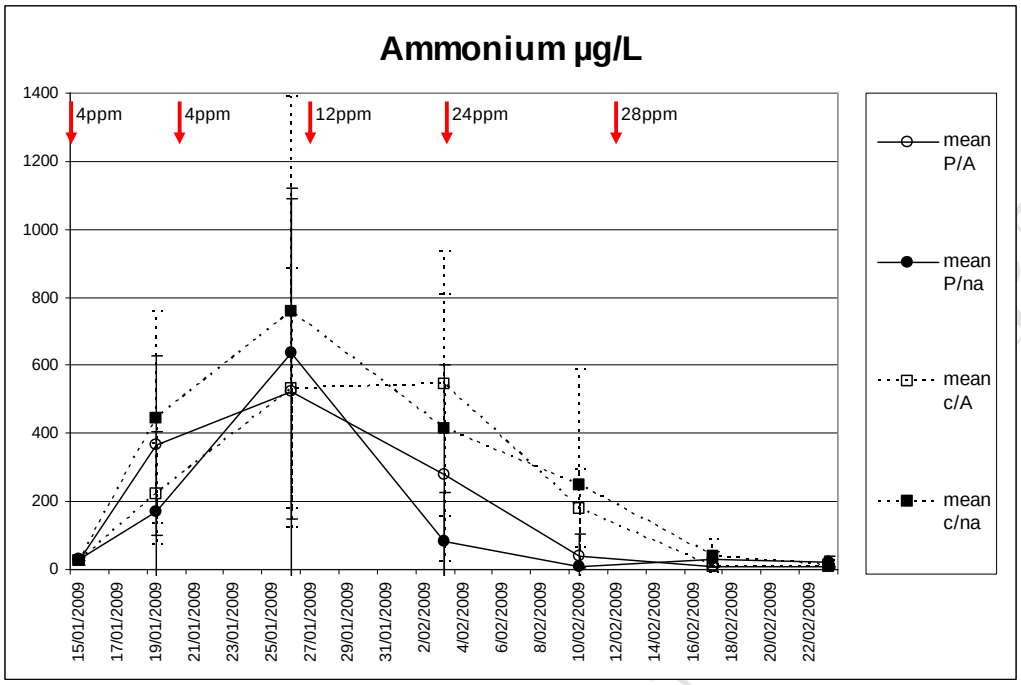

Figure 5: Testing nutrient competition - Ammonium concentration of the water column in product and control tanks.

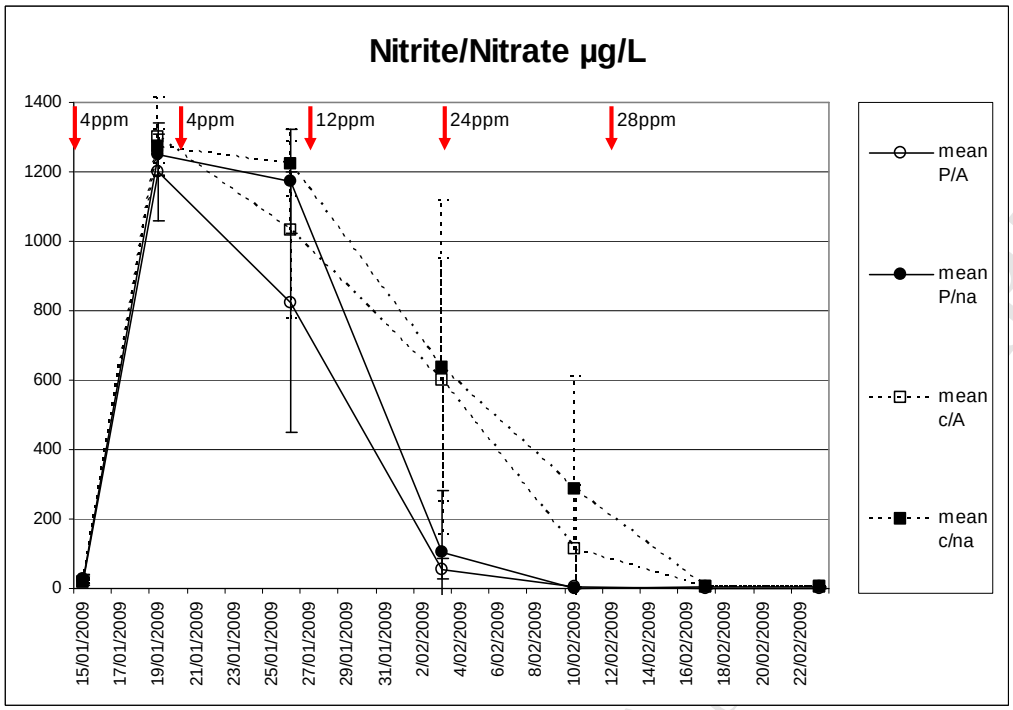

Figure 6: Testing nutrient competition - Nitrite and Nitrate concentration of the water column in product and control tanks

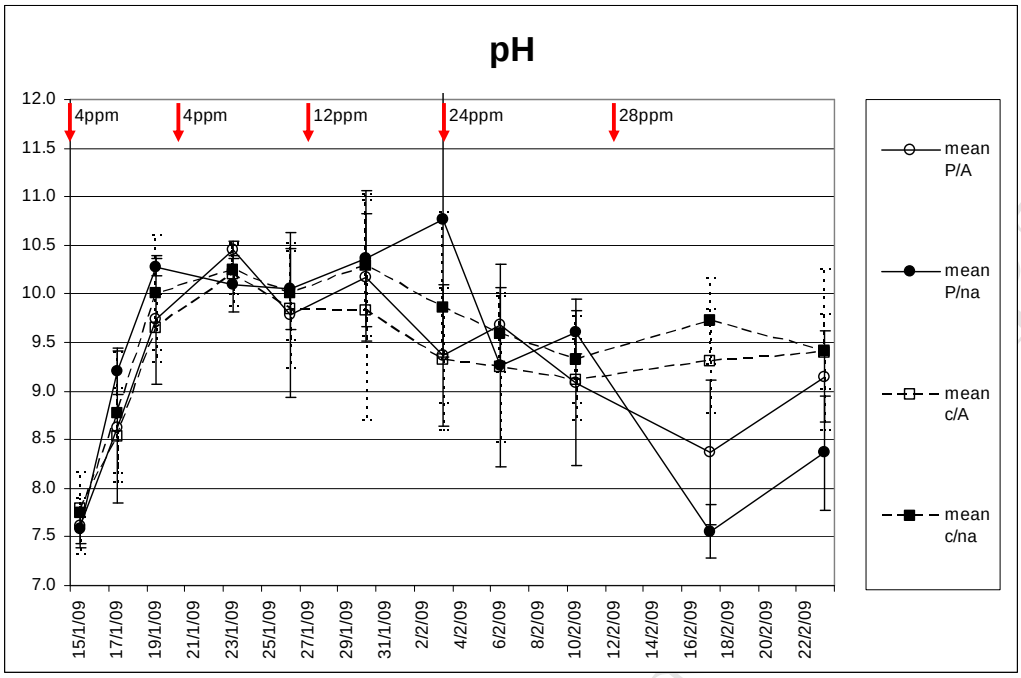

Figure 7: Testing algal growth suppression - pH value of the water column in product and control tanks

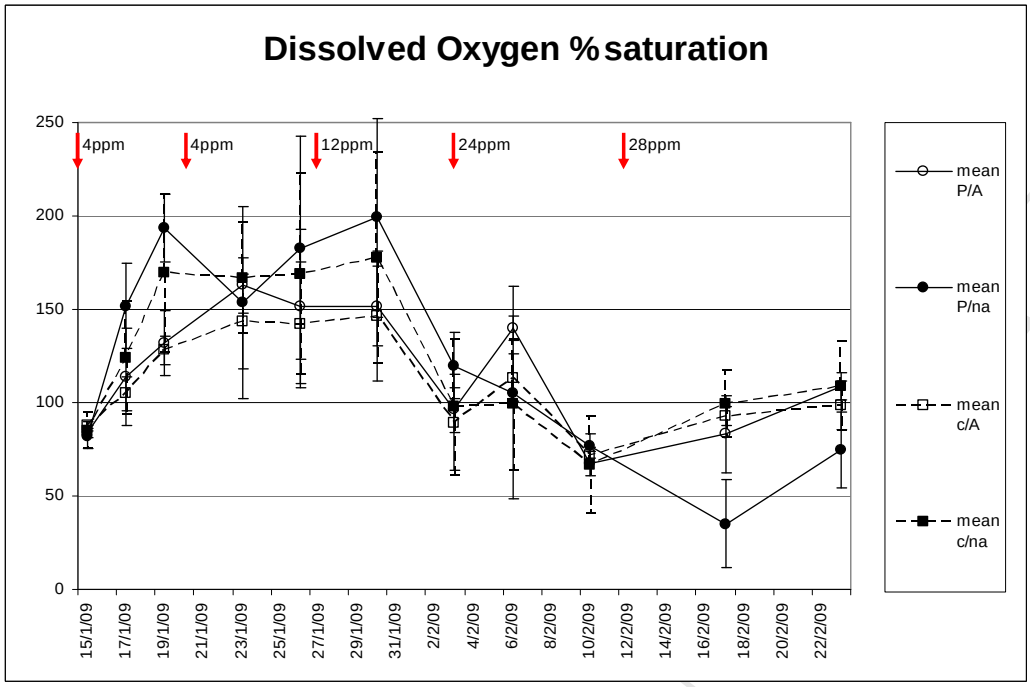

Figure 8: Testing algal growth suppression – DO saturation of the water column in product and control tanks

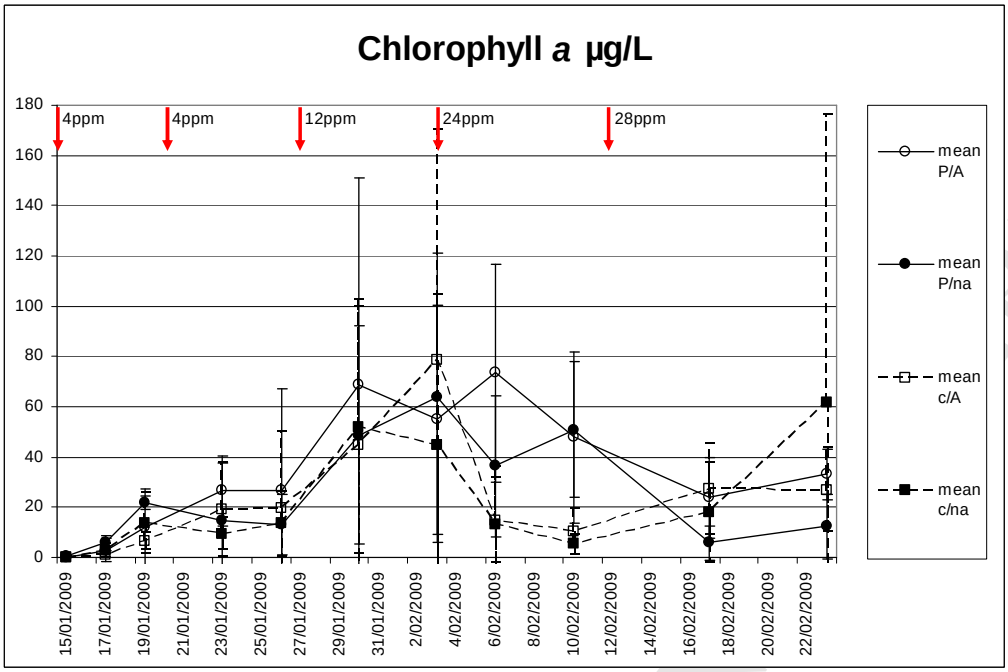

Figure 9: Testing algal growth suppression – Chlorophyll *a* concentration of the water column in product and control tanks