

Removal of *Prymnesium parvum* through clay and chemical flocculation

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Introduction

Prymnesium parvum Carter is a toxic, bloom-forming microalga found in low salinity lakes to coastal oceanic waters. Through its production of toxins, known collectively as prymnesins, *P. parvum* has been recognized as a potent fish-killing organism, affecting both wild and reared finfish (Komarovskiy, 1951; Holmquist and Willem, 1993; Igarashi et al., 1996; Lindholm et al., 1999). In the United States, *P. parvum* has been found in several southern states including Texas, Alabama, Arkansas, Georgia, North Carolina and South Carolina. In Texas, fish kills associated with *P. parvum* have been clearly documented as early as 1985 in the Pecos River (James and de la Cruz, 1989), but may extend as far back as the 1960's. Blooms have killed an estimated \$17.5 million fish in Texas waters, with direct costs totaling \$7 million and severe impacts on local economies involved in tourism, recreational fishing, and hatcheries. In 2003 alone, over 6.3 million fish were killed. In addition to fish, James and de la Cruz (1989) also reported some adverse impacts on bivalve populations. Recent trends and observations suggest that *P. parvum* blooms are increasing in frequency in Texas, with more areas being affected, but the manner and cause of this expansion remains unknown. Therefore, it appears prudent to investigate practical strategies to manage these blooms, in addition to methods currently in use (see below), and methods that directly target and eliminate the organisms (i.e. bloom control).

Currently, chemical control appears to be the primary means of dealing with *P. parvum* blooms, and possibly their toxins as well. These include addition of ammonium sulfate (Shilo and Shilo, 1953), ammonia (Glass et al., 1991), copper sulfate (Reichenbach-Klinke, 1973), acetic acid and other weak electrolytes (Glass et al., 1991). Shilo and Aschner (1953) showed that toxicity can be decreased through aeration and treatments with potassium permanganate and sodium hypochlorite. While successful, it should be noted that control methods in aquaculture facilities may not be applicable to natural systems also affected by *P. parvum* and its toxins, due to the risk of high collateral impacts and regulatory restrictions. Therefore, alternative strategies are still needed that can offer relief in both the hatcheries and natural systems.

In recent years, clay flocculation has received significant attention as an effective, economical and environmentally acceptable method of controlling harmful algal blooms (HABs) in open marine waters (Shirota, 1989; Yu et al., 1994; Kim 1996; Na et al. 1996; Choi et al., 1999; Sengco et al., 2001; Sengco and Anderson, 2004). This approach involves the addition of clay slurries directly over a bloom, leading to the formation of clay-cell aggregates that fall rapidly from the water column, and the entrainment of cells during settling. Clay minerals were selected

for this purpose because they can bind cells strongly and act rapidly (i.e. settling within minutes to hours in high salinity). Clays are also readily available, abundant, inexpensive, and considered environmentally friendly. Removal of 95-99% of the cells has been accomplished with small amounts of clay. Sengco et al. (2001) and Yu et al. (2004) further demonstrated substantial enhancement of removal by pre-treatment with chemical flocculants such as polyaluminum chloride (PAC), which increases bridging between particle surfaces. Typically, the addition of PAC reduces clay loadings by a factor of 3-10, depending on the clay and the algal species.

Of particular interest to Texas are studies that investigated the potential of Florida phosphatic clays to control *Prymnesium parvum* in culture (Hagström and Graneli, 2005). Experiments conducted in Sweden showed that 100% of *P. parvum* can be removed within 72 hrs with 4 g/L of Florida phosphatic clay, a sample consisting of smectite (i.e. montmorillonite), carbonate fluorapatite, and various other minerals, combined with 5 ppm of polyaluminum chloride (PAC). An 84% removal efficiency (RE) was achieved in 6 hrs at lower dosages. Experiments with nutrient-limited cultures showed that removal efficiency increased as the cells became more nutrient stressed. Lastly, the study also demonstrated the ability of phosphatic clay to remove *P. parvum* toxins. While these studies illustrated the possibility of clay control in general, the loading rates here were much higher than those in other studies, and used high salinity water (i.e. 26). Likewise, the time wherein cell removal reached maximum levels was much longer here than in previous studies.

More recently, laboratory experiments were conducted in Sweden to examine the ability of locally available clays to remove *Prymnesium parvum* from suspension (Sengco et al., 2005). As with smectite-rich phosphatic clays, the most effective mineral was a local bentonite (77% removal efficiency, at 0.50 g/L), treated with polyaluminum chloride (PAC). Cell removal varied depending on the cell concentration, as well as the physiological status of the organisms (i.e. nutrient replete or stressed). Finally, clay treatment reduced toxin concentration in suspension, presumably by the removal of intact cells. However, we saw that the toxin concentration increased in the pellet after treatment for nitrogen-limited cells, suggesting that clay addition may stimulate toxin production in these stressed cells, or that the presence of clays may be activating the toxins. This phenomenon was also observed when N-limited cells were treated with phosphatic clays (Hagström and Graneli, 2005). Overall, these studies demonstrated that clay flocculation can be effective at removing *P. parvum* and its toxins, only under certain conditions (i.e. cell concentration, physiological status, clay mineralogy and clay concentration).

Rationale and Objectives

Blooms of *Prymnesium parvum* are a growing nuisance and economic concern in Texas inland waters and reservoirs, with impacts on fish, shellfish, and local economies. Therefore, studies were initiated to examine whether domestic clays (and other flocculants) can control these organisms effectively, and mitigate their impacts, with minimal effects on environmental quality and local ecosystems.

The use of clay minerals to flocculate and sediment HAB organisms from the water column has shown promise in laboratory and field trials for a number of species and settings. To our

knowledge, however, there have been no attempts thus far to investigate the ability of locally-available clays to remove domestic strains of *Prymnesium parvum* under environmental conditions in which they occur in the U.S. While there have been attempts to examine the effectiveness of clay flocculation against *Prymnesium parvum* in Sweden, these studies offer some guidance, but cannot be readily extrapolated to conditions in Texas. First, differences among marine, brackish and freshwater conditions are critical. The rate of clay-clay and clay-cell flocculation depends greatly on the ionic strength and pH of the medium, which affects, in turn, the amount of time that clay and algal particles will interact and remain suspended in the water column. Therefore, clays that are effective in marine systems will not necessarily be effective in freshwater. Likewise, the effectiveness of chemical flocculants, alone or in combination with clays, may also be affected in the same manner. Thus, new efforts would be needed to investigate clay flocculation under these different conditions.

The specific objectives of this research were: (1) To identify the most effective clays from local sources against *Prymnesium parvum* under different conditions, including variations in cell concentration, physiological status, and salinity. (2) To test whether chemical flocculants alone can remove cells, and/or improve cell removal in combination with local clays. (3) To measure the ability of selected clays to reduce toxicity. (4) To examine cell removal in mesocosms and monitor changes in water quality following treatment. This report summarizes the results of the study.

Task 1. Experiment 1 – Screening of local clays against *Prymnesium parvum*

Materials and methods

Cultures: Two cultures of *Prymnesium parvum* (CMS2010 and CMS204) were obtained from Dr. Carmelo Tomas at the University of North Carolina, Wilmington, and one from the UTEX collection (ZZ181). At Woods Hole, all of the cultures were maintained in modified f/2 medium (salinity = 4) under conditions described in Anderson et al. (1999). For these experiments, CMS2010 was used due to its high growth rate and toxicity (Dr. Tomas, pers. comm.). This clone was isolated in 2000 during a winter bloom (3.65×10^5 cells/mL) in Possum Kingdom Lake reservoir in the Brazos River Basin. It was taken at a salinity of 4 with ambient temperatures around 15°C. For the removal experiments, CMS2010 was grown in 1 L batch cultures, at 20°C, at a 14-h light:10-h dark cycle. Growth was monitored using *in vivo* fluorescence on a Turner Model 10-AU fluorometer (Turner Designs, Sunnyvale, California, USA).

Clays: Clay minerals were obtained from several Texas companies or mines (Table 1). The samples consisted of bentonite, montmorillonite, kaolinite and others containing a mixture of minerals. Phosphatic clay from Florida (Hagström and Granèli, 2005) was used for comparison. Several attempts were made to obtain Phoslock[®], but this mineral is not available in the United States, and the Australian company did not deliver the sample requested for the screening. The clays were weighed and dispersed in distilled/deionized water (MilliQ) overnight to allow the minerals to be completely wetted.

Table 1. Clay screening experiment. Clay producers and sources in Texas were identified and small amounts were obtained for testing. Phosphatic clay from Florida was used for comparison (Hagström and Granelli, 2005).

Sample Name and Mineralogy	Code	Company
Bentonite WH	SPC-B	Southern Clay Products, Gonzales, TX
Mineral Colloid BP (montmorillonite or bentonite)	SPC-MC	Southern Clay Products, Gonzales, TX
Puregel (bentonite)	MI-PG	Messina, Inc., Dallas, TX
Texas sodium bentonite	QCS-B	Quality Clay Samples
Kaolin EG-44	TKC-K	Thiele Kaolin Company, Wrens, GA (with TX offices)
Dinsoil (soil mixture with humate, montmorillonite)	AL-DS	dinoSoil, Inc., Alpine, TX
Phosphatic clay (mixture of finely-divided minerals)	IMC-P4	Mosaic Company, Bartow, FL

Removal experiment: In previous experiments, *in vivo* cell fluorescence was used to estimate cell abundance (Sengco et al., 2001). Given the size of the organisms and the difficulty in gauging small changes in fluorescence, as well as possible interference of clay turbidity in the measurements, a flow cytometer was used to count cells following the methods established in Sweden by Sengco et al. (2005). Here cell population could be differentiated from clays through both their size (i.e. side-scatter properties) and their fluorescence. In preparation, the counts (using standard beads and actual *P. parvum* cells) from the flow cytometer were cross-compared with microscopic counts using a hemocytometer. They were within 10%, which is a reasonable difference in comparing different counting methods ($r^2 = 0.83$ for the calibration curve).

Ten milliliters of *P. parvum* culture (at 100,000 cells/mL) were added to a series of test tubes. One mL of clay slurry was then added dropwise to the surface of the water column, forming a turbid layer at the surface. For each clay, the final concentrations were 0, 0.05, 0.10, 0.25, and 0.50 g/L (in 11 mL), each concentration in triplicate. MilliQ water was added to the controls. The flocculation took place under quiescent conditions at room temperature. After 3 hrs, the supernatant – defined as the overlying 10 mL of water column – was carefully removed, placed in a new tube, and mixed thoroughly for the final count. Cell removal efficiency (%RE) was calculated using the following equation (Sengco et al., 2001):

$$\% \text{ RE} = [1 - (\text{final fluorescence} \div \text{final fluorescence of control})] \times 100$$

The final fluorescence of the control (i.e. 2.5 h after the addition of DI water) was used to account for cell sinking.

Results and implications

Except for the mineral colloid (SPC-MC), none of the clays examined in this study removed *Prymnesium parvum* (CMS2010 at 100,000 cells/mL) from the water column to any significant extent (Figure 1). Cell removal with SPC-MC was only 14%, and was only observed at 0.05 g/L of clay. Untreated phosphatic clay (IMCP-4), which showed some removal ability in earlier studies, was ineffective here (Hagström and Granelli, 2005), along with the other bentonites. In fact, cell removal was *negative* for nearly all of the clays. Based on this method of calculating removal efficiency, negative values suggest that the presence of the clay *reduced or prevented*

the sinking of the cells relative to the clay-free controls. This phenomenon was observed previously by Sengco et al. (2005) with Swedish clays and *Prymnesium parvum*.

Over the 3-hr quiescent period following clay addition, small flocs were seen to form near the surface of the water column, but no large agglomerates similar to those seen in experiments with marine species such as *Karenia brevis* (Sengco et al., 2001), *Alexandrium tamarense* (Sengco et al., 2001) and *Aureococcus anophagefferens* (Yu et al., 2004) were observed. No flocs were seen at all in cultures treated with kaolin (TKC-K). The water column remained turbid with fine particles. These observations suggest that the lower salinity of the medium limited the rate of particle flocculation – either clay-clay or clay-cell – leading to the absence of larger, more rapidly sinking particles. Under these conditions, therefore, the organisms would not become incorporated into sinking flocs, leading to low removal efficiency, while the absence of larger flocs would mean no entrainment of cells beneath the surface, also leading to low removal efficiency. Instead, the slowly flocculating suspension formed what appeared to be a gel-like, slightly viscous matrix, near the surface that may have led to the retention of cells, and thus, negative removal efficiency.

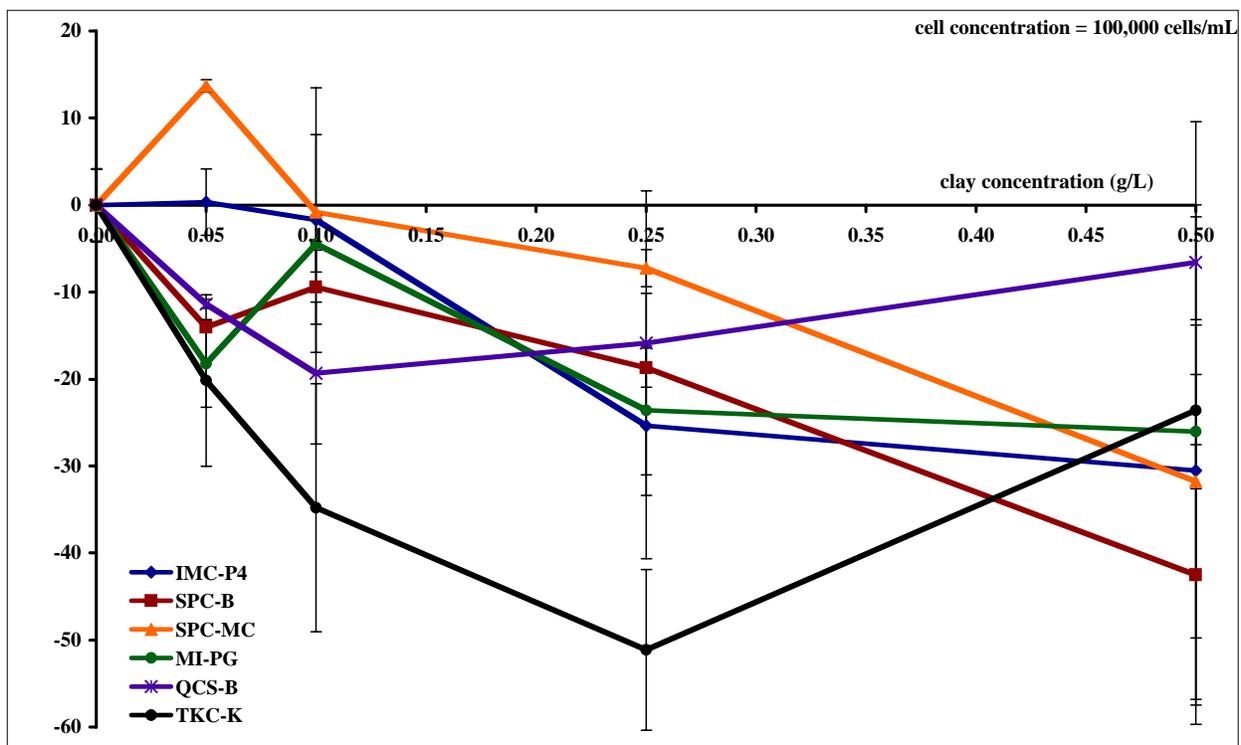


Figure 1. Removal ability of selected clays from Texas and Florida against *Prymnesium parvum* (CMS2010 at 100,000 cells/mL). Error bars represent standard deviations (n=3).

It is possible that unsettled clays added to the pool of suspended particles, bringing the number of particles higher than the controls. However, the flow cytometer clearly differentiated clay particles from *P. parvum* by their fluorescence (i.e. clay particles do not fluoresce). Therefore,

we are confident that we were comparing only the number of cells between the controls and treatments.

We also cannot rule out that the maximum amount of clay used in this experiment (0.50 g/L) may be insufficient to remove this number of organisms effectively. For example, Hagström and Granèli (2005) found higher *P. parvum* removal with Florida phosphatic clay at higher clay concentrations (up to 4 g/L), and longer incubation times (up to 24 hrs), than those used in this study. Likewise, Yu et al. (2004) found that prolonging flocculation and settling times (up to 7 days) produced high removals of the 2 µm cells of *Aureococcus anophagefferens*. Other authors have also demonstrated that removal of other algal species increases with clay loading (e.g. Shirota, 1989, Na et al., 1996; Sengco et al., 2001). Finally, Avnimelech et al. (1982) proposed the concept of an "ideal ratio" between cell numbers and clay loading for maximum overall removal of particles from suspension.

Certainly, particle concentration is an important factor in flocculation rates (Yu et al., 1994). From theory, the aggregation rate can be described by a second order equation (O'Melia and Tiller, 1993):

$$\frac{dn}{dt} = -k_a n^2$$

where n is the number concentration of particles in suspension at time t , and k_a is a rate constant that considers the physicochemical properties of the system. According to this equation, the change in particle concentration with time is related to the square of the concentration of particles in the system (in this case, clay particles). Therefore, increasing clay concentration can have a potentially strong effect in promoting the aggregation rate. However, our data suggested that increasing the number of clays does not increase the rate of flocculation and cell removal. Thus, it is likely that the system may be affected more by the physiochemical properties of the system (e.g. salinity, rate of interparticle collisions), instead of the number of particles in suspension.

Although additional clay amounts and longer incubations could have been tested, we chose not to do so in this study because we did not believe these to be reasonable conditions for treatments in natural waters. For instance, clay treatment at concentrations in excess of 0.50 g/L would be undesirable due to potential environmental issues and cost of treatment. Likewise, if flocculation required several hours to have a significant removal effect, this would be unrealistic as mitigation strategy since no water column would be static for such a length of time in nature.

Mixing the clay-cell suspension after clay addition yielded some improvement in cell removal, as has been shown with other small species like *Aureococcus anophagefferens* (Sengco et al., 2001; Yu et al. 2004). These authors proposed that the improvement in cell removal due to mixing was the result of higher collision rates between algal and clay particles, leading to higher flocculation rates, and the minimization of a process called hydrodynamic retardation: a phenomenon where interparticle contacts are decreased due to the inability of smaller particles (e.g. cells) to approach and overcome the hydrodynamic forces generated by the flowing layer of water displaced by a much larger particle (e.g. flocs) as it settles. By briefly mixing the suspension, the

clay particles would be dispersed throughout the medium, which minimizes the formation of large clay-clay aggregates near the surface, while larger aggregates already formed would be sheared apart and redistributed within the entire suspension. From a practical standpoint, however, mixing and over-turning the suspension may be feasible under laboratory conditions, where volumes are small, but it will be difficult to transfer this approach to larger scales or field conditions wherein vigorous mixing of the water column would be impossible. Certainly, the turbulent dispersal of the clay slurry on the surface may help in breaking apart aggregates, and in forcing collisions among the particles. Nevertheless, observations from this experiment suggest that the low salinity of the medium, and ultimately in TX reservoirs and lakes, may be the most important factor that limits flocculation rates in the system.

Task 1. Experiment 2 – Clay screening at higher cell concentrations

Materials and methods

Prymnesium parvum cultures and clay samples were similar to those used in Task 1, Experiment 1. The removal experiment was conducted in a similar manner as in the previous experiment, except the initial cell concentration was increased to 400,000 cells/mL.

Results and implications

As in the previous experiment, mineral colloid (SPC-MC) showed positive cell removal (11%) with 0.05 g/L (Figure 2). One bentonite sample, SPC-B, also displayed positive, but negligible removal efficiency (<1%) with 0.05 g/L. The remaining samples were ineffective against *P. parvum* at higher concentrations. Slow flocculation (i.e. little or no formation of flocs) was also observed during this experiment as in the previous experiment.

Increasing the number of cells did not yield higher cell removal despite expectations that higher particle numbers in the suspension (both clays and cells) would lead to higher rates of flocculation as predicted by O'Melia and Tiller (1993) (see previous experiment). In an earlier study, Soballe and Threlkeld (1988) found that there were fewer effects of species concentration in the removal efficiency compared to the change in mineral concentration and differences among the species themselves. Considering the results of this and the previous experiment, it appears that total particle concentration may not be the critical factor that determines the success of *Prymnesium parvum* removal in the system. Therefore, the focus should turn to other factors such as those that affect interparticle collision rates (e.g. particle size and water motion), and factors that influence the adhesiveness of particles (e.g. concentration of free cations (e.g. salinity), and molecular bridges in the medium).

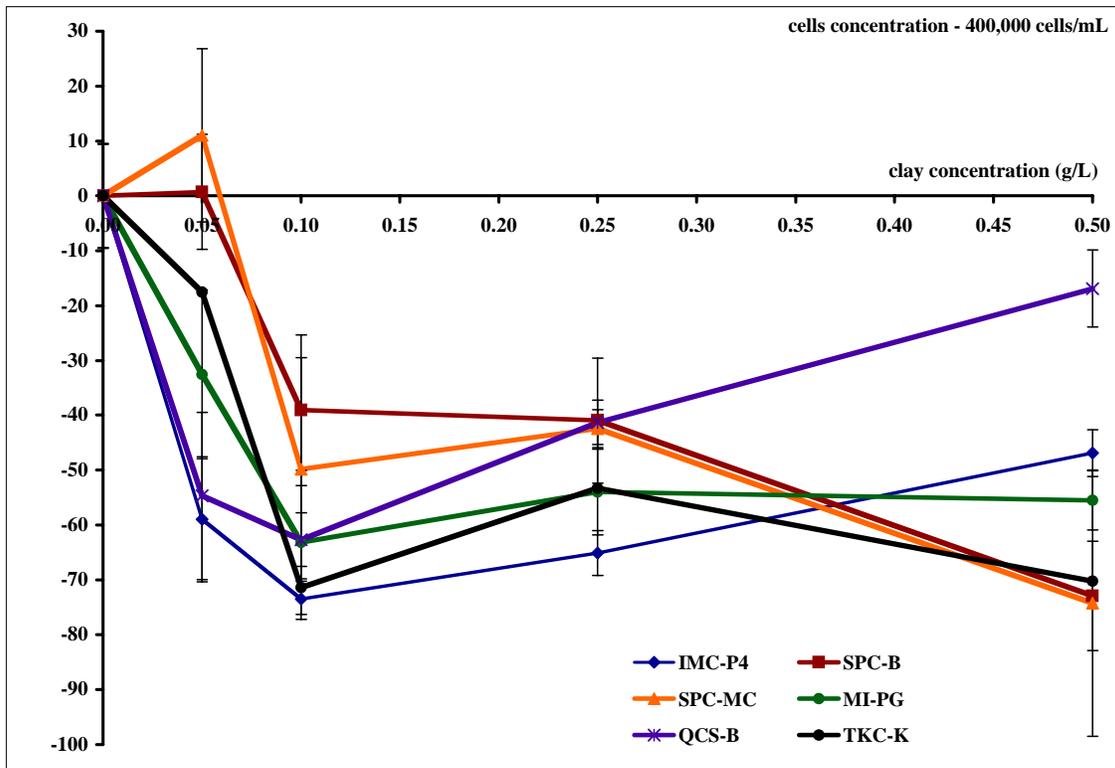


Figure 2. Removal ability of selected clays from Texas and Florida against *Prymnesium parvum* (CMS2010 at 400,000 cells/mL). Error bars represent standard deviations (n=3).

Task 1. Experiment 3 – Salinity gradient

Materials and methods

Cultures: *Prymnesium parvum* CMS2010 were grown in modified f/2 batch cultures over a range of salinities prepared by diluting 0.02 μm -filtered Vineyard seawater (salinity = 30) with MilliQ water: 1, 4, 10, 20, and 30. It should be noted that this isolate of *P. parvum* would not grow at 0 ppt. The cultures were grown in 1L batch cultures using f/2 medium, and maintained as described in Task 1, Experiment 1.

Removal experiment: Removal experiments were conducted in the same manner as in Task 1, Experiment 1. Cell concentration was 77,000 cells/mL, which corresponded to the maximum cell concentration achieved at salinity of 1 at late exponential growth. The same clay samples were used all at 0.10 g/L.

Results and implications

At salinities of 1, 4 and 10, none of the clays tested removed *Prymnesium parvum*. All of the clays displayed negative removal efficiencies. This was not unexpected because the two previous experiments showed that none of these clays can remove *P. parvum* with 0.10 g/L, at a salinity of 4. In this experiment, cell removal efficiency became more negative as salinity increased from 1 to 4, then became less negative as salinity increased to 10. Cell removal was

observed with phosphatic clay (IMC-P4) and one bentonite sample (QCS-B) when salinity increased to 20 and 30. With IMC-P4 phosphatic clays, large flocs were seen at the surface at higher salinities. Although the other three samples did not remove cells at these higher salinities, the removal values were less negative. These results suggest that increasing the salinity of the medium can improve the removal abilities for at least two of the clays tested, presumably by increasing their adhesiveness, and thus, increasing the rate of flocculation.

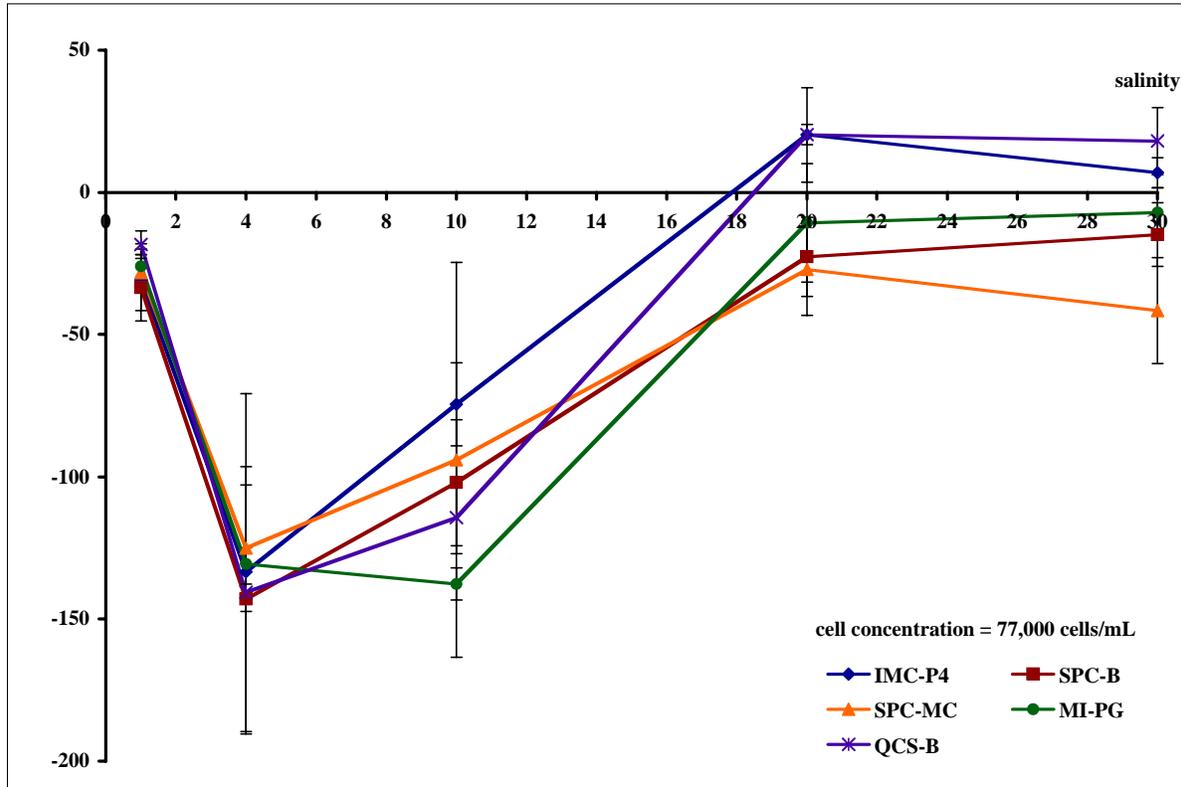


Figure 3. Removal ability of selected clays from Texas and Florida against *Prynnesium parvum* (CMS2010 at 77,000 cells/mL). Salinity range from 1 to 30. Clay concentration = 0.10 g/L. Error bars represent standard deviations (n=3).

Task 1. Experiment 4 – Cell physiology

Materials and methods

Cultures: *Prynnesium parvum* CMS2010 was grown in 1 L batch cultures. Filtered seawater diluted with MilliQ water to a salinity of 4 was enriched with standard f/2 medium except for the following modifications: (1) f/40 nitrogen for N-limited cultures, and (2) f/40 phosphorus P-limited cultures. The cultures were maintained at 20°C, at a 14-h light:10-h dark cycle. Growth was monitored using *in vivo* fluorescence on a Turner Model 10-AU fluorometer (Turner Designs, Sunnyvale, California, USA). The cultures were used in experiments after they reached late exponential stage. The cell concentration used in the removal experiment corresponded to the maximum number of cells achieved at each condition: 65,000 cells/mL for N-limited cultures, and 90,000 cells/mL for P-limited cultures.

Removal experiment: Removal experiments were conducted in the same manner as in Task 1, Experiment 1.

Results and implication

Overall, cell removal with clays occurred more readily with N-limited cultures than with P-limited cultures (Figure 4). Except for the kaolinite TKC-K at 0.50 g/L, all of the clay samples removed N-stressed *P. parvum*, with the highest value of 55% displayed by the mineral colloid (SPC-MC at 0.25 g/L) and puregel/montmorillonite (MI-PG at 0.50 g/L). In general, cell removal increased with increasing clay concentration. Four clays showed a decrease in cell removal when clay concentration increased from 0.25 g/L to 0.50 g/L (Figure 4A). The kaolinite TKC-K was the least effective among the group.

For P-stressed cells, removal with clays was ineffective to low (Figure 4B). Some appreciable removal for most of the clays was found at 0.25 g/L, with the highest value of 19% using the bentonite (SPC-B). For most of the clays, cell removal declined as clay concentration increased from 0.25 g/L to 0.50 g/L. The kaolinite TKC-K was completely ineffective across the entire concentration range.

Compared with the earlier experiments above, the highest cell removal was observed with N-limited cells compared to P-limited and nutrient-replete cells. Using arguments from flocculation theory, one explanation may be that the different nutrient conditions affected the surface charge properties of the cells, such that N-stressed cells were more adhesive towards the clays relative to cells grown in the two other conditions. It is known that microalgal cells can alter the types of proteins and other biochemical molecules on their surface to allow them to adapt to changing nutrient conditions. It is possible that changes in the type of molecules on the cells, or the quantity of certain types of molecules, can influence the adhesive properties of the cell. From a biological standpoint, nutrient stress may affect the cell's ability to avoid or resist flocculation with clay particles. Healthy cells with sufficient nutrients may have enough energy to avoid clay particles or aggregates, or to escape the floc during descent or the clay-cell pellet after deposition. On the other hand, nutrient-limited cells, may not have adequate energy to avoid or escape flocculation and settling.

From previous studies, Sengco et al. (2005) showed that nutrient-replete *P. parvum* were removed more readily than those in N- and P-limited conditions when treated with ≤ 0.25 g/L Swedish bentonite and 5 ppm polyaluminum chloride (PAC), a chemical flocculant. However, there was no significant difference in cell removal among the three nutrient conditions when clay concentration reached 0.50 g/L. Similarly, Hagström and Granéli (2005) found similar results using Florida phosphatic clay against *P. parvum*. Overall, these results clearly showed that the physiological status of the cell can influence the effectiveness of *P. parvum* with clays, even if the mechanism by which nutrient conditions affect the flocculation process remains unknown. If these observations can be extended to real-world, field conditions, the success or failure of clay control may be determined by the status of the bloom, among other factors. Blooms nearing the termination phase, presumably under nutrient stress, may be more readily controlled with clay, especially if the bloom is under nitrogen limitation. The consistency of this observation across

three independent studies indicates that this property of the bloom may be a factor to consider in deciding to use clay control.

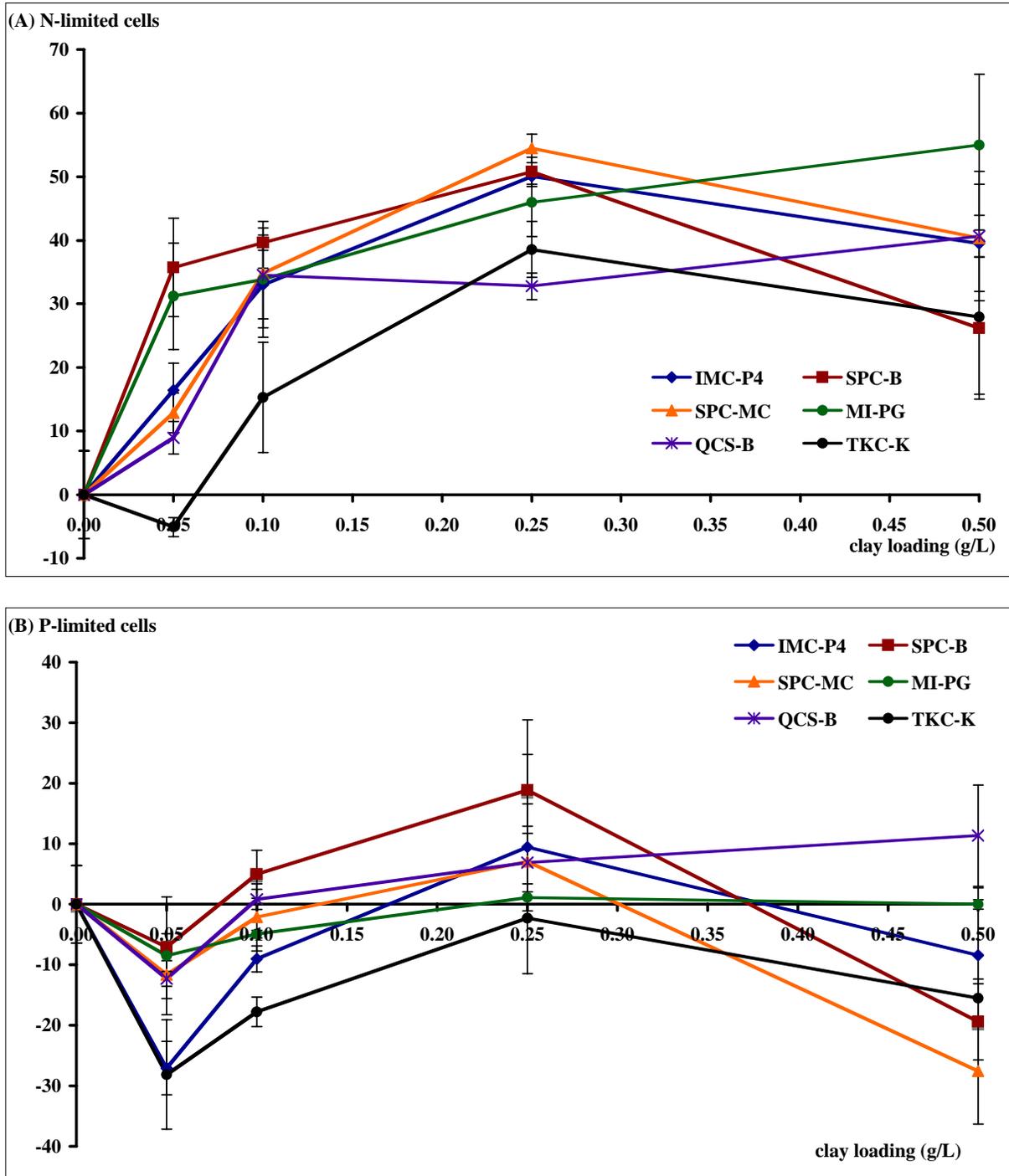


Figure 4. Removal ability of selected clays from Texas and Florida against *Prymnesium parvum*. (A) Nitrogen-limited. (B) Phosphorus-limited. Error bars represent standard deviations (n=3).

Task 2. Experiment 1 – Chemical flocculants against *Prymnesium parvum*

Materials and methods

Cultures: *P. parvum* cultures were grown similar to those used in Task 1, Experiment 1.

Flocculants: Chemical flocculants were obtained from CIBA Specialty Chemicals Corporation (Suffolk, VA) and Cytec Industries (Charlotte, NC) (Table 2). Out of the 20 samples provided, the choice was made based on toxicity information in the Material Safety Data Sheets (MSDS). Samples toxic to fish and other aquatic organisms were avoided. Generally, the flocculants could be used to a maximum of 20 ppm. The final list consisted of polyaluminum chloride, blends with cationic polymers, and anionic polymers. The chemicals are diluted in MilliQ to a working concentration of 1000 ppm. This was diluted to the range of concentrations needed for the experiments below.

Table 2. Chemical flocculants used in the study. Samples were obtained from Cytec Industries and CIBA Specialty Chemicals Corp. Samples were chosen based on information in their Material Safety Data Sheet (MSDS) regarding impacts on fish and aquatic organisms.

Product name	Chemical Family	Concentration
CYTEC INDUSTRIES		
Superfloc 607	cationic polymer and inorganic coagulant blend	40% (w/w) PAC
Superfloc 608	cationic polymer and inorganic coagulant blend	30% (w/w) PAC
Superfloc 9001	polyaluminum chloride	45% (w/w) PAC
CIBA SPECIALTY CHEMICALS CORPORATION		
Magnafloc E30	polyacrylamide	distillates, petroleum 500 ppm polyacrylamide

Removal experiment: Removal experiments were conducted in the same manner as in Task 1, Experiment 1. Ten milliliters of *P. parvum* culture (at 100,000 cells/mL) were added to a series of test tubes. One mL of flocculant suspension was then added dropwise to the surface of the water column. For each flocculant, the final concentrations were 0, 1, 5, 10 and 20 ppm (in 11 mL), each concentration in triplicate. MilliQ water was added to the controls. The flocculation took place under quiescent conditions under room temperature. After 3 hrs, the supernatant was carefully removed, placed in a new tube, and mixed thoroughly for the final count. Cell removal efficiency was calculated following Sengco et al. (2001).

Results and implications

Despite the number of sample choice provided by the manufacturers, only four had little or no known harmful effects on fish and other aquatic organisms. This is to be expected, as most of these chemicals were not designed for used in natural systems or open waters. Superfloc 606 and Superfloc 608 displayed low to moderate cell removal (Figure 5). For Superfloc 606,

removal efficiency peaked at 19% with 5 ppm, then it decreased as concentration increased to 10 and 20 ppm. Superfloc 608 had a peak removal efficiency of 39% with 5 ppm then decreased as the concentration of flocculant increased. Both of these flocculants contain small amounts of a cationic polymer mixed with polyaluminum chloride (PAC). PAC by itself (Superfloc 9001) was ineffective below 10 ppm, but displayed removal ability at 20 ppm (RE = 26%). In the all three flocculants, a cloudy layer formed near the surface of the water column after the flocculants were added. This may have been precipitates formed as the Al reacted with the medium. Lastly, the anionic polymer, Magnafloc E30, was ineffective across the entire concentration gradient. No flocs or aggregates were seen in the test tubes during the experiments.

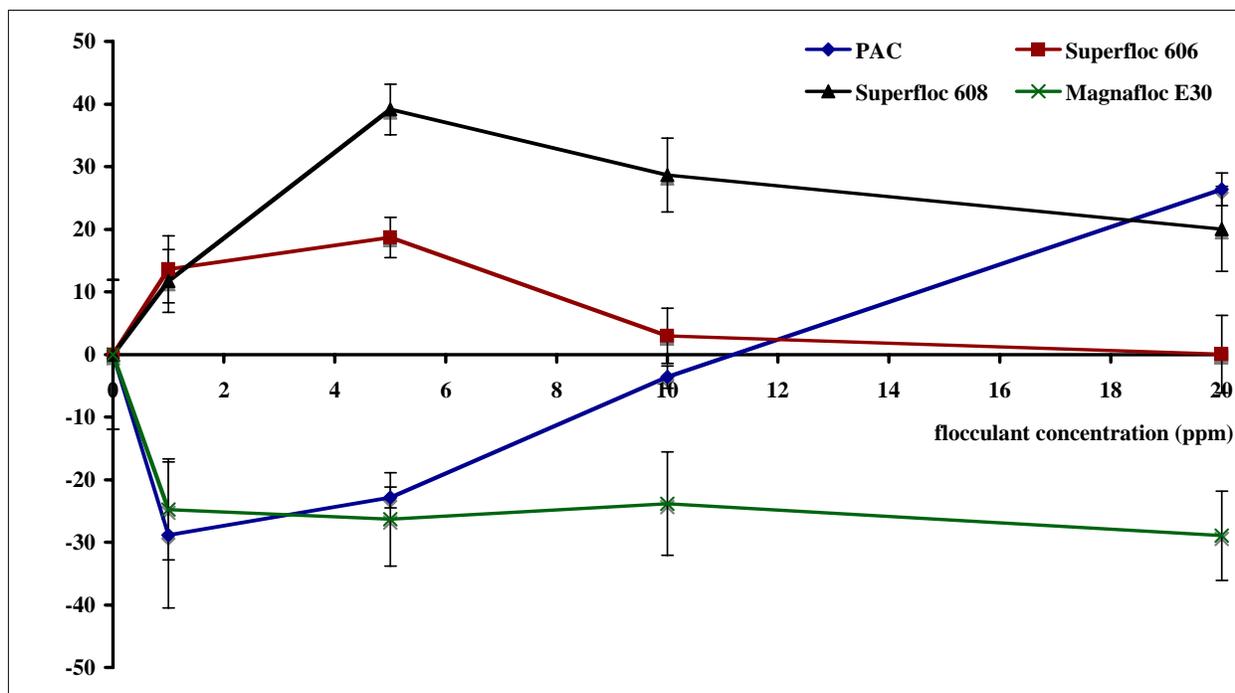


Figure 5. Removal of *Pymnesium parvum* using various chemical flocculants. Samples include polyaluminum chloride (PAC), and a combination of PAC and cationic flocculants (Superfloc 606 and 608), and an anionic flocculant (Magnafloc E30).

Superfloc 606 and Superfloc 608 appear to be very promising in that they can remove *Pymnesium parvum* cells in the absence of clay minerals (Figure 5). However, both are new to the market, and there is no information thus far on their potential impacts on fish and aquatic life. The presence of cationic polymer in the blend may explain its effectiveness as the positive charge of the molecule can bind to the negatively charged surfaces of the organisms (and the clays, as well). However, several cationic polymers have been shown to have some deleterious effects on fish, especially on the gills. While we study these two flocculants further, it will be important to focus on their potential impact on fish later. By contrast, the negative anionic charge of Magnafloc E30 did not appear to be appropriate to use in this system where the particles have a dominant negative charge.

Task 2. Experiment 2 – Flocculants over salinity range

Materials and methods

Cultures: *Prymnesium parvum* CMS2010 were grown in modified f/2 batch cultures over a range of salinities prepared by diluting filtered Vineyard seawater (salinity = 30) with MilliQ water: 1, 4, 10, 20, and 30. The cultures were grown and maintained as described in Task 1, Experiment 1.

Removal experiment: Removal experiments were performed in the same manner as Task 1, Experiment 3. Cell concentration was 120,000 cells/mL. Flocculant concentration was 5 ppm.

Results and implications

Superfloc 608 retained its removal ability across the entire salinity gradient (Figure 6). Its average removal efficiency (RE) ranged from 24% at a salinity of 10, to 46% at a salinity of 30. Superfloc 606 showed a peak RE of 30% at the lowest salinity (1 ppt), but RE decreased linearly as salinity increased to 20. By contrast, the anionic flocculant, Magnafloc E30 was ineffective in the lower portion of the salinity range, but displayed cell removal as salinity increased to 10. Magnafloc E30 displayed a peak RE of 38% at a salinity of 30. PAC showed negligible cell removal between salinities of 1 and 4. Then, RE dropped to negative values between salinities of 4 and 20, but RE increased to positive values from 20 to 30. Based on these data, the salinity of the medium will clearly have an influence on the effectiveness of the flocculants alone, and probably in combination with the clays as well. This effect can be clearly seen in the performance of polyaluminum chloride, Superfloc 608 and Magnafloc E30 wherein cell removal occurred during certain salinity intervals. Among these samples, however, Superfloc 608 demonstrated great versatility in its ability to force flocculation of the organisms.

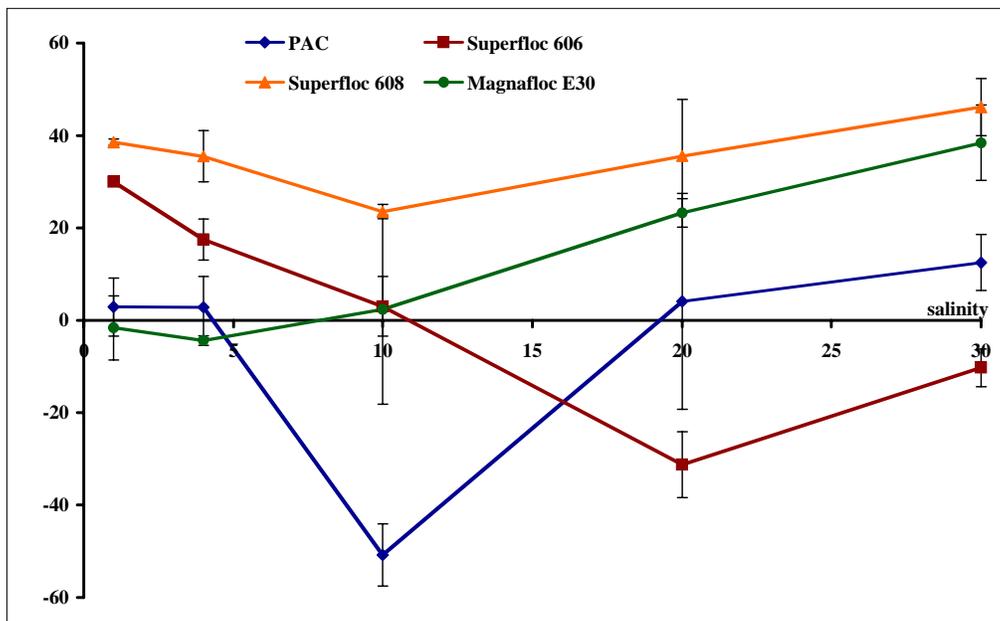


Figure 6. Removal of *Pymnesium parvum* using various chemical flocculants over salinity gradient. Samples include polyaluminum chloride (PAC), and a combination of PAC and cationic flocculants (Superfloc 606 and 608), and an anionic flocculant (Magnafloc E30).

Task 2. Experiment 3 - Clay and flocculant combination against *Prymnesium parvum*

Materials and Methods

Cultures: *Prymnesium parvum* CMS2010 was grown in 1 L batch cultures under nutrient-replete conditions (salinity = 4). The flasks were kept 20°C, in a 14-h light:10-h dark cycle. Growth was monitored using *in vivo* fluorescence on a Turner Model 10-AU fluorometer (Turner Designs, Sunnyvale, California, USA). Experiments were run with cultures at mid-exponential growth.

Clays and flocculants: The same series of clays and flocculants were used in this experiment. Magnafloc E30 was not used as it displayed poor removal ability in the salinity range of the cultures. Working suspensions of each clay and flocculant were prepared separately and were not combined until the removal experiment. This prevents the premature and irreversible flocculation of the clay minerals, leading to excessively large floc that sink rapidly without removing the organisms (Sengco et al., 2001).

Removal experiment: Ten milliliters of *P. parvum* culture (at 150,000 cells/mL) were added to a series of test tubes. 0.50 mL of flocculant was added to the surface of each tube to deliver a final concentration of 5 ppm (in 11 mL). After 20 min, 0.50 mL clay slurry was then added dropwise to the surface of the water column. For each clay, the final concentrations were 0, 0.05, 0.10, 0.25, and 0.50 g/L (in 11 mL), each concentration in triplicate. MilliQ water was added to the controls (no clay, no flocculant). Flocculation took place under quiescent conditions under room temperature. After 3 hrs, the supernatant – defined as the overlying 10 mL of water column – was carefully removed, placed in a new tube, and mixed thoroughly for the final count. Cell removal efficiency (%RE) was calculated using equation in Sengco et al. (2001).

Results and implications

Thus far in this study, the highest removal efficiencies were observed when clays and flocculants were combined, compared to clays alone (ineffective), and flocculants alone (low effectiveness). The best results were seen when the clays were combined with Superfloc 606 (Figure 7B). With as low as 0.05 g/L of each clay, removal efficiency (RE) ranged from 87% to 93%. RE remained high (between 85% and 95%) as clay concentration increased to 0.50 g/L, with phosphatic clay IMC-P4 showing the lowest RE across the concentration gradient relative to the other clays. Even the kaolinite TKC-K, which was ineffective in all previous experiments thus far, displayed some of the highest removal values when combined with this flocculant. It should also be noted that the variability among the replicates in this experiment was much smaller than in the previous experiment. These algal removal efficiencies have only been seen thus far in laboratory trials using *Karenia brevis* and phosphatic clays in seawater (Sengco et al., 2001).

Superfloc 608 combined with the various clays also led to higher removal values, although there were greater differences among the clays, especially as clay concentration increased (Figure 7C). At 0.05 g/L, RE ranged from 80% to 94% with TKC-K and IMC-P4 showing the lowest values among the clays. However, the removal ability of TKC-K improved considerably as clay concentration increased, while the removal ability of the other clays decreased.

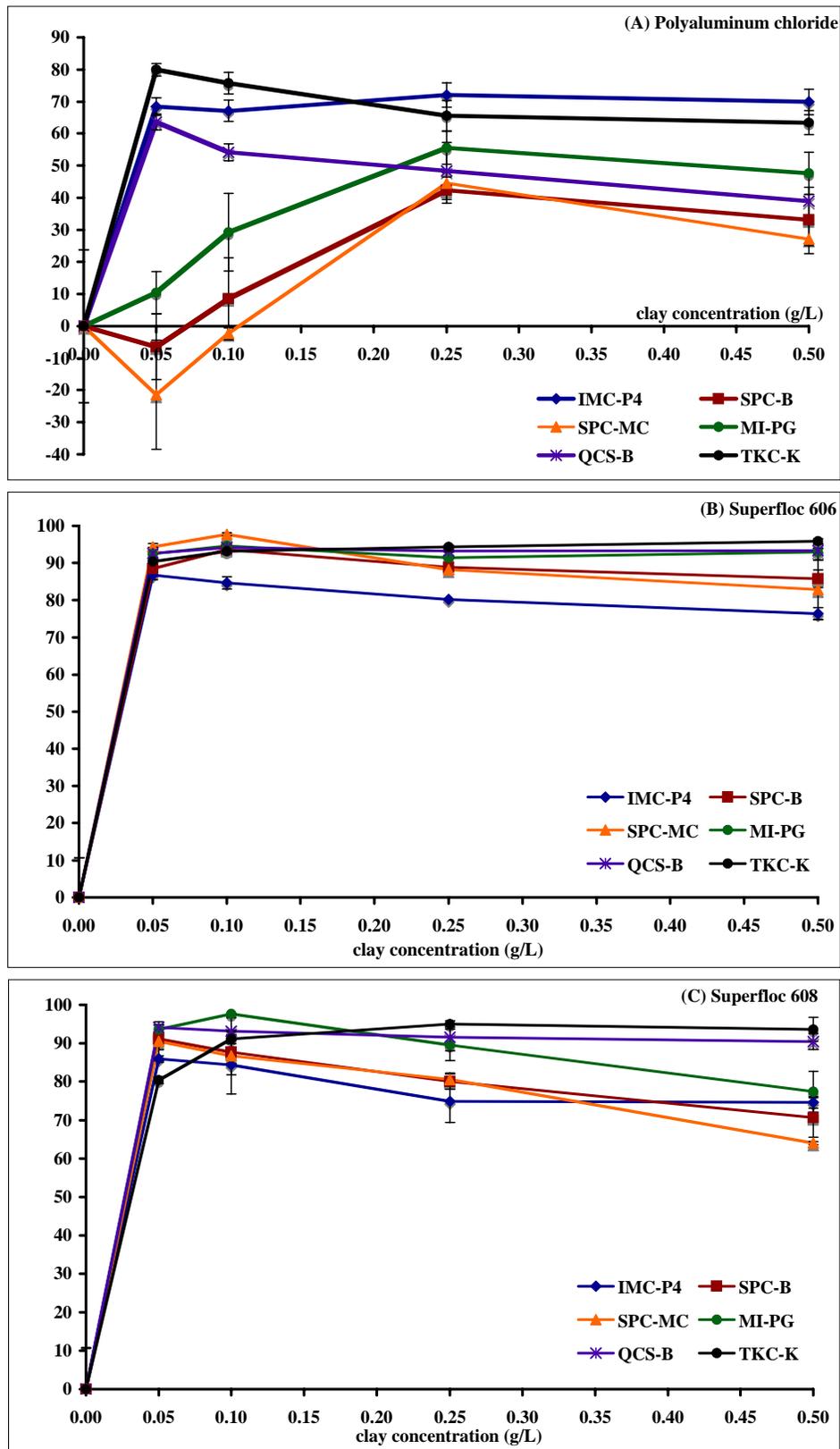


Figure 7. Removal of *Prynnesium parvum* using various clays treated with chemical flocculants (5 ppm). (A) Polyaluminum chloride. (B) Superfloc 606. (C) Superfloc 608.

PAC was especially useful when combined with kaolinite TKC-K, which displayed RE of 80% at 0.05 g/L, decreasing to 63% at 0.50 g/L. Phosphatic clay IMC-P4 also improved to a range of 67% to 72% across the concentration gradient. The bentonite QCS-B showed more moderate cell removal between 39% to 64%. Three clays were less improved by PAC addition at lower clay concentrations, but increased to moderate levels as clay concentration increased. Interestingly, SPC-B and SPC-MC, two clays that showed some promise earlier, performed poorly when combined with PAC.

Clearly, significant removal of *Prymnesium parvum* must rely on a combination of clays and flocculants. Based on these results, Superfloc 606 and 608 are the most promising. Taking all of the results thus far, we can hypothesize on the mechanism by which cell removal occurs in this system. The low salinity of the medium is insufficient to cause rapid flocculation as the particles - primarily the clays - are poorly adhesive. Increasing the number of particles in suspension will not cause significant improvement in the flocculation rates, if the particles do not adhere to one another upon collision. The addition of flocculants essentially replaces the lack of cations in the medium, and increases adhesiveness by binding to the surface of the particles, both clays and cells, changing their surface charges and promoting interparticle bridging. However, flocculants alone may not force the cells to sink rapidly even if flocculation is proceeding. This was seen when flocculants were used alone in Task 2, Experiment 1. The addition of clay particles not only increases the number of total particles in the system, but provides substantial ballast to the forming aggregates due to its density (generally 3 times more dense than water). The flocs can therefore sink more quickly when particles of clay are added.

Task 2. Experiment 4 – Clay and flocculants across salinity gradient.

Materials and methods

Cultures: *Prymnesium parvum* CMS2010 were grown and maintained as described in Task 1, Experiment 3 and in Task 2, Experiment 2. Cell concentration was 100,000 cells/mL.

Removal experiments: Removal experiments were performed in the same manner as in Task 2, Experiment 3. The best three clays were tested from Task 2, Experiment 3 (above), in combination with polyaluminum chloride (PAC) and Superfloc 606. PAC was still tested in this experiment in order to contrast a sample that contains no cationic flocculant (PAC), and one with cationic polymer. The final clay concentration was 0.05 g/L (in 11mL). The final flocculant concentration was 5 ppm (in 11 mL).

Results and implications

Removal efficiencies were moderately high in this experiment (Figure 8). The RE of clays combined with PAC were relatively similar to one another (Figure 8A). The average RE was 71% for most of the salinity gradient. RE then decreased to 50% at 30 ppm. Bentonite QCS-B was slightly less effective at salinity of 4 (RE = 61%), compared to the other two clays (RE = 71%).

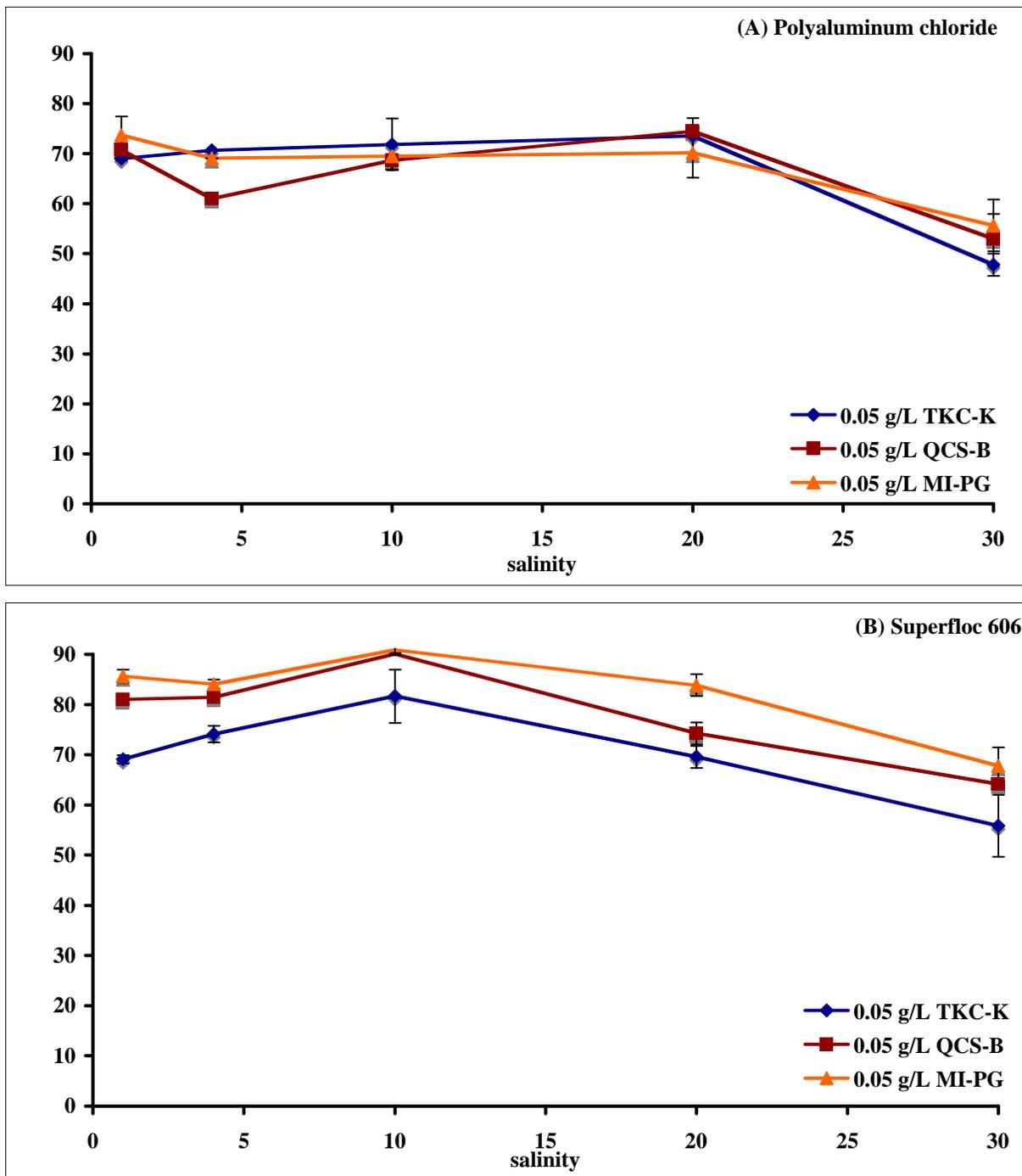


Figure 8. Removal of *Pymnesium parvum* using clays treated with (A) polyaluminum chloride and (B) Superfloc 606 across a salinity gradient. Clay concentration = 0.05 g/L. Flocculant concentration = 5 ppm. Cell concentration = 100,000 cells/mL.

Clays treated with Superfloc 606 displayed a wider range of removal ability, although two clays (MI-PG and QCS-B) had high removal efficiencies (>80%) between salinities of 1 and 10. As the salinity climbed over 10, their removal efficiencies dropped linearly. The kaolinite TKC-K treated with Superfloc 606 showed moderately high removal efficiencies (between 56% and

80%) across the salinity range, but it paralleled the removal pattern of the other two, PAC-treated clays.

These results emphasize the need to combine flocculants and clay to ensure moderate to high cell removal, even across the salinity gradient. PAC appeared to be less affected by increasing salinity, while Superfloc 606 was less effective at higher salinities. Nevertheless, both Superfloc 606 and Superfloc 608 seem to be the most appropriate flocculants for use in the relevant salinity range in Texas waters.

Task 2. Experiment 5 – Aquarium trials

Materials and methods

Cultures: To obtain larger volumes of culture for aquarium tests, 1 L batch cultures were transferred to 18 L glass carboys containing sterile-filtered, f/2-enriched medium (at salinity of 4). The carboys were gently bubbled and maintained at 20°C, at a 14-h light:10-h dark cycle.

Removal experiment. Studies were conducted in 10-gal glass aquaria (50 cm length by 26 cm width). The aquaria were filled to a depth of 10.7 cm (12 L). Initial cell concentration in each tank was 150,000 cells/mL. Three were used for the following treatments: Tank 1 = control (no clay, no flocculant), Tank 2 = TKC-K (0.05 g/L) + PAC (5 ppm), and Tank 3 = TKC-K (0.05 g/L + Superfloc 608 (5 ppm). TKC-K and Superfloc 608 was chosen based on the results of Task 2, Experiment 3. First, the cells were added into the aquaria (11.5 L diluted culture). Water samples were withdrawn using an automatic pipet device and counted. The samples were taken in 9 locations: at three equidistant points along the length of aquarium, and at three depths at each of these points (2.0, 5.5 and 9.5 cm above the bottom). The sample was collected using a premeasured pipet. The flocculant was dispersed over the surface using a hand-pressurized canister (0.25 L). After 20 min, the clay slurry was added (0.25 L). MilliQ water was added to the controls. The flocculation and settling of particles proceeded for 3 hrs. Samples were taken and counted.

Results and implications

Results are shown in Figure 9. In the control, no cell removal was observed within the aquarium. Three hours following their addition into the aquarium, the organisms had redistributed themselves and appeared to accumulate at the surface, towards the left side of the aquarium where the laboratory lights were stronger (Figure 9A). By contrast, *P. parvum* were removed close to the surface (at 9.5 cm above bottom, Figure 9B), using the combination of kaolinite TKC-K and polyaluminum chloride (PAC). The calculated removal efficiency (RE) at the surface was 75% after 3 hrs, based on the initial concentration in the aquarium. However, cell removal only took place at the surface in this treatment. At 5.5 cm and 2.0 cm above the bottom, RE's were -52% and -68%, respectively. These values suggest that cells were accumulating

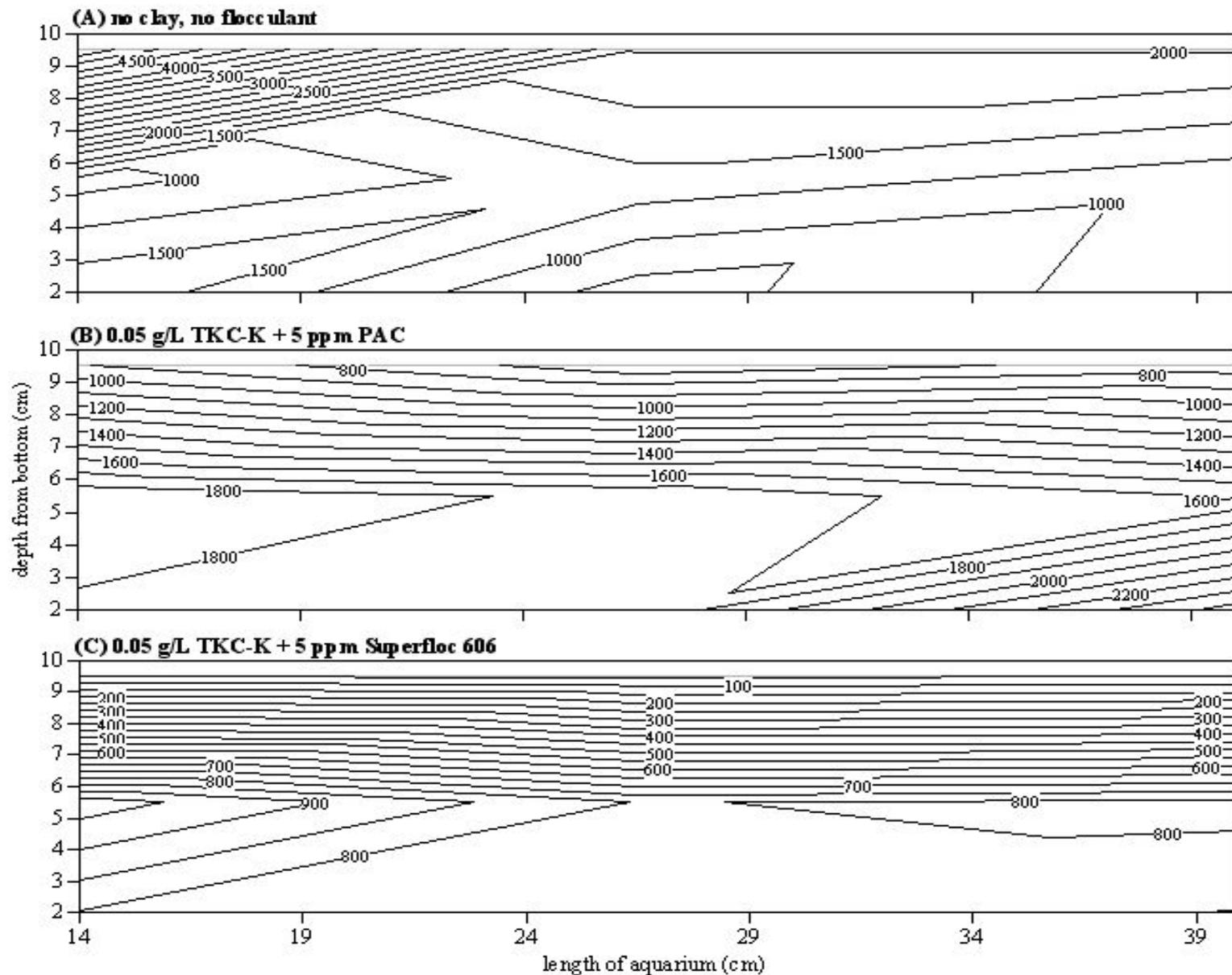


Figure 9. Removal of *Pymnesium parvum* using clay and flocculant combination in aquaria. Contour plots of cell counts taken after 3 h at three equidistant points along the center of the aquarium and at three discrete depths per point. (A) Control (no clay and flocculant) (B) 0.05 g/L kaolinite TKC-K with 5 ppm polyaluminum chloride PAC (C) 0.05 g/L kaolinite TKC-K with 5 ppm Superfloc 606.

beneath the surface. As we observed the flocculation and settling process, we can describe the sequence of events that led to cell removal. First, the flocculant is added to the surface. Several minutes afterwards, we saw the appearance of fine white precipitates along the surface layer, presumably from the reaction of aluminum from the flocculant and the small amount of chloride ions in the medium. This layer appeared to sink, and in the process, push the organisms down. The cells accumulated along the lower edge of the sinking layer. When the clay was added 20 min later, large flocs formed and surface turbid layer began to sink faster. However, the layer did not reach the bottom within the 3-hr period of the experiment. Hence, the accumulated cell along the interface was sampled at mid-depth and near the bottom.

The same general process was observed when Superfloc 606 was added to the surface, followed by the clay slurry. However, in this treatment, the flocculation and settling took place much faster, than in the PAC treatment. Again, a whitish, turbid layer appeared at the surface and sank, pushing down the cells. When the clay was added, a large number of flocs appeared. Settling was rapid and intense. As a result, cell removal efficiency near the surface (i.e. 9.5 cm above bottom) was calculated at 98% (Figure 9C). At 5.5 cm and 2.0 cm above bottom, cell removals were 24% and 35%, respectively, within 3 hrs. After 24 hrs, the water column was clear and no cells were found (Figure 10). The floc layer along the bottom has a brown-green discoloration, presumably from the incorporation of *Prymnesium parvum*. Therefore, the combination of kaolinite TKC-K and Superfloc 606 was the most effective at removing *P. parvum* in this experiment. This result has been consistent with previous experiments above. We recommend that further experiments use this combination.



Figure 10. Removal of *Prymnesium parvum* using combination of clays and chemical flocculants, 24 hrs after treatment and settling. Experiments began with 100,000 cells/mL of *P. parvum*. Two aquaria were treated with 0.05 g/L kaolinite TKC-K, and either 5 ppm polyaluminum chloride (PAC) or 5 ppm Superfloc 606.

Task 3. Experiment 1 – Removal of *Prymnesium parvum* cells and toxins

Materials and methods

Cultures: Three weeks prior to the experiment, *Prymnesium parvum* (CMS2010) were grown in 1L batch cultures at 20°C, salinity of 4, 14 h light:10 h dark cycle under three nutrient conditions: (1) nutrient replete f/2, (2) N-limited f/40-nitrogen, and (3) P-limited f/40-phosphorus. Nutrient limitation was used to induce higher toxin production. Cell counts three days prior to the study showed that the two nutrient cultures had reached stationary phase.

Removal experiment: The cultures were diluted to 150,000 cells/mL using 4 ppt water. Two beakers were each filled with 550 mL of culture: one beaker was used as the control (i.e. untreated), the other was to be treated with clay/flocculant. In parallel, six additional beakers were set-up following the same procedure, but here the cultures were sonified to break apart the cells and release toxins into the medium. The cultures were sonified for 3 min, and the status of the cells were checked under the microscope. The contents of each beaker was then filtered through a 0.20-micron filter to remove cell debris and any unbroken cells.

For the treatment, 5 ppm of Superfloc 606 was added dropwise to the surface of the beaker with a pipet. The flocculant was delivered in 25 mL of water. 25 mL of MilliQ was added to the controls. After 20 min, the TKC-C kaolinite slurry was added to the surface dropwise with a pipet to a final concentration of 0.05 g/L. Again, the clay was delivered in 25 mL. MilliQ water was added to the controls. The final volume of each beaker was 600 mL. To ensure full settling of the material, additional clay was added to the treatment to yield a final clay concentration of 0.25 g/L (in 620 mL). This supplement was added after 60 min. The suspension was allowed to flocculate and settle without disturbance for 3 hrs under room conditions. Afterwards, the overlying water was decanted carefully into a clean beaker. A small aliquot was taken for cell counts and the rest was poured into 500 mL bottles for fish bioassay, and 40 mL for toxin analysis. The samples were packed with ice packs and shipped overnight. Fish bioassay analysis was performed by Greg Southard (A.E. Woods Fish Hatchery, San Marcos, TX) following the Standard Bioassay of *Prymnesium parvum* Toxin, Version AEW-ITU 1.2. Toxin analysis was performed by Pamela Hamlett (Environmental Contaminants Lab, San Marcos, TX).

Results and implications

The results from this experiment are summarized in Table 3. Immediately following the experiment, the removal efficiency (RE) of intact cells (sequence 4 – 6) were 97%, 96% and 97%, respectively, relative to the controls. During overnight shipment, the cells from the control survived and grew based on the increased count performed the day after. Upon arrival at the San Marcos lab, the cells appeared viable and motile.

In Table 3, there appeared to be clear difference among the toxicity among the three cultures (Sequence 1-3). The P-limited culture was highly toxic, relative to the NP-replete culture. The N-limited culture was more ambiguous, ranging from 5 to 25 ITU's. Despite the high removal

efficiency during the experiment, there did not appear to be a noticeable difference in the toxicity of the sample following the treatment with both the P-limited and N-limited cultures. There may be some slight decreased in toxicity in the NP-replete sample.

Table 3. Results from fish bioassay following treatment of *Prymnesium parvum* cultures (whole cell and lysed) with 0.25 g/L kaolinite (TKC-K) and 5 ppm Superfloc606. Cell counts were done in replicate using a common hemacytometer for sequence 1 through 6, and an average density (cells/mL) were calculated. Arrows in the bioassay are used when there is incomplete mortality within a beaker after 2 hrs at 28°C (Greg Southard, pers. comm.). Since the bioassay is set up in a logarithmic scale (i.e. indicating 0, 1, 5 and equal to/greater than 25 ITU's), versus a linear scale, it would be difficult to determine an intermediate measurement would lie between two values when incomplete mortality occur (e.g. 2.5 between 1 and 5, or 15 between 5 and 25). Activity of cells was noted in comments section. Lysed cells (sequence 7-12) were not observed. Legend: BDL = below detection limit. N/A = not available. +NP = nutrient replete, -N = nitrogen-limited, -P = phosphorus-limited.

Sequence number	Cell Status	Nutrient Condition	Treatment	Density (cells/mL)	Ichthyotoxic Units (ITUs)	Comments (cell activity)	Toxin Conc (ppm)
1	Intact	+NP	control	242,000	5	Cells motile	25
2	Intact	-N	control	178,000	5 → 25	Slow; non-motile	31
3	Intact	-P	control	129,000	≥ 25	Cells motile	72
4	Intact	+NP	+clay/flocculent	0 / BDL	1 ← 5	N/A	48
5	Intact	-N	+clay/flocculent	0 / BDL	5 → 25	N/A	71
6	Intact	-P	+clay/flocculent	0 / BDL	≥ 25	N/A	lost
7	Lysed	+NP	control	N/A	1 ← → 5	N/A	63
8	Lysed	-N	control	N/A	5	N/A	86
9	Lysed	-P	control	N/A	≥ 25	N/A	84
10	Lysed	+NP	+clay/flocculent	N/A	1	N/A	35
11	Lysed	-N	+clay/flocculent	N/A	1 ← 5	N/A	66
12	Lysed	-P	+clay/flocculent	N/A	≥ 25	N/A	89

Based on these data, ichthyotoxins were present in the medium after the *P. parvum* were lysed and filtered (Table 3, Sequence 7-12). The highest amount of toxins were found in the P-limited cultures. Smaller amounts were found in NP-replete and N-limited samples. No change in toxicity was found in the P-limited cultures despite the addition of clay and flocculant. There may be some slight reduction in toxicity with the N-limited sample following treatment. The results for the NP-replete condition was ambiguous. Overall, there did not appear to be a clear effect of clay treatment on toxicity based on this bioassay method, with either intact cells or released toxins.

The results of the toxin analysis and quantification are presented in the last column of Table 3. Again, the toxin concentration in the two nutrient-limited cultures (with intact cells) was higher than in the nutrient-replete culture, with the P-limited culture having the highest amount. However, there appeared to be an increased in the toxin content of the cells following the treatment for the NP-replete and N-limited cultures. As the sample for sequence 6 was lost, we

cannot be determined whether the same occurred in the P-limited culture. This result has been seen in earlier studies (Sengco et al., 2005) in which the N-limited cells increased their toxicity (assessed with a horse-blood haemolysis test) following incubation with clay. The authors speculated that the stress of the treatment may have induced additional toxin production within the cells. However, it has also been suggested that the toxins may become activated by cations that are present on the clays. This is an area that requires further study to understand the mechanism by which toxicity increases in the presence of inorganic, clay particles.

When the toxins were released into the medium, the toxin concentration in the medium decreased following clay/flocculant treatment in the case of the NP-replete and N-limited samples. There was little change in the toxin content in the P-limited samples following treatment. Based on this result, it is possible that free toxins may be removed from the water column.

Task 4 – Mesocosm experiments at the Lewisville Aquatic Ecosystems Research Facility

Materials and methods

Cultures: Several strains of *Prymnesium parvum* were grown in various containers: 2 L batch cultures (Figure 11A), 20-L bottles plastic bottles, and 200-400 L in 1,600-L tanks (Figure 11B). Several strains were used in order to compare their growth rates and ability to survive under various environmental conditions. Prior to the experiment, all of the cultures were combined into one tank (Figure 11B), which was thoroughly mixed and aerated. The cell concentration was determined and the contents of the tank were divided equally into 6, 1600-L tanks using a submersible pump. The cultures were diluted using freshwater adjusted to a salinity of 4 using Instant Ocean. The cultures were diluted to a final cell concentration of 160,000 cells/mL in a final volume of 800 L, which corresponded to a water column height of 30 cm (1 ft) in these tanks. This was the maximum volume and cell density we can achieve under the current culturing conditions in order to meet the requirements of the experiment.

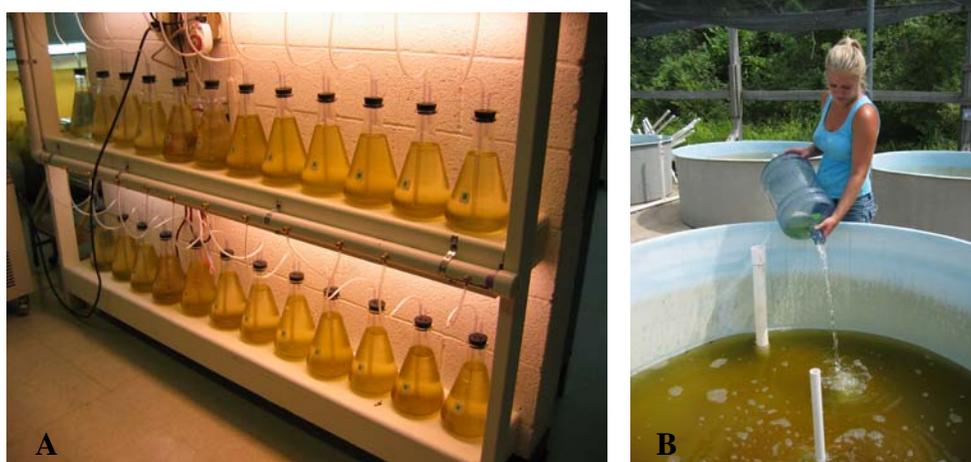


Figure 11. Culturing *Prymnesium parvum* for mesocosm experiments. (A) 2-L batch cultures. (B) 400-L outdoor tank cultures, into which all of the cultures were combined before distribution.

Removal experiment: Experiments were conducted in 1600 L mesocosm tanks (Figure 12A). We used 6 tanks: three controls and three clay treatments. The target clay concentration was 0.10 g/L. Kaolinite TCK-K was prepared by suspending the required amount in 20 L of water. The target flocculant concentration was 2.5 ppm of Superfloc 606. It should be noted that the flocculant concentration was reduced from 5 ppm to minimize possible reaction in the fish bioassay following the experiment. The flocculant was also dispersed in 20 L of water.



Figure 12. Mesocosm experiments. (A) Six tanks were filled with water and salinity adjusted to 4 with Instant Ocean. Water column height = 30 cm. (B) Cells were transferred using submersible pumps to initial cell concentration of 100,000 cells/mL.

After the tanks were filled with the cultures (Figure 12B), initial conditions within the tanks were determined. An integrated water sampler, consisting of a PVC pipe with a ball seal on the bottom, was lowered into the tank to collect water samples for cell counts, turbidity and nutrient analysis. The volume was collected in 1-L beakers and subdivided to for the various analyses. Initial water quality conditions were monitored using a Hydrolabs that were placed in four of the tanks (2 control and 2 treatment). Temperature, pH, and dissolved oxygen were measured.

Then, the flocculant was added to the surface using a submersible pump and garden hose nozzle. After 20 min, the clay slurry was also added in the same manner. For the controls, the same volumes of water was added instead. The suspensions were allowed to flocculate and settle for 6. Intermediate samples were taken at 2 and 4 hrs for cell count and turbidity. After 6 hrs, water samples were collected. Additional subsamples were taken for fish bioassay and toxin analysis, which were shipped overnight to San Marcos in ice (See Task 3). The cell counts were done using a haemocytometer and light microscope. Turbidity was measured using a benchtop turbidity meter. Nutrient analysis were performed at LAERF following standard procedures.

Results and implications

Water temperature gradually increased in all of the tanks during the course of the experiment (Figure 13A). The two control tanks (1 and 2) were similar to each other. Initially, Tank 4 was also similar in temperature to the two controls, but began to deviate around noon. Afterwards, the temperature in Tank 4 continued to increase, but at a slower rate. Tank 5, the other treatment

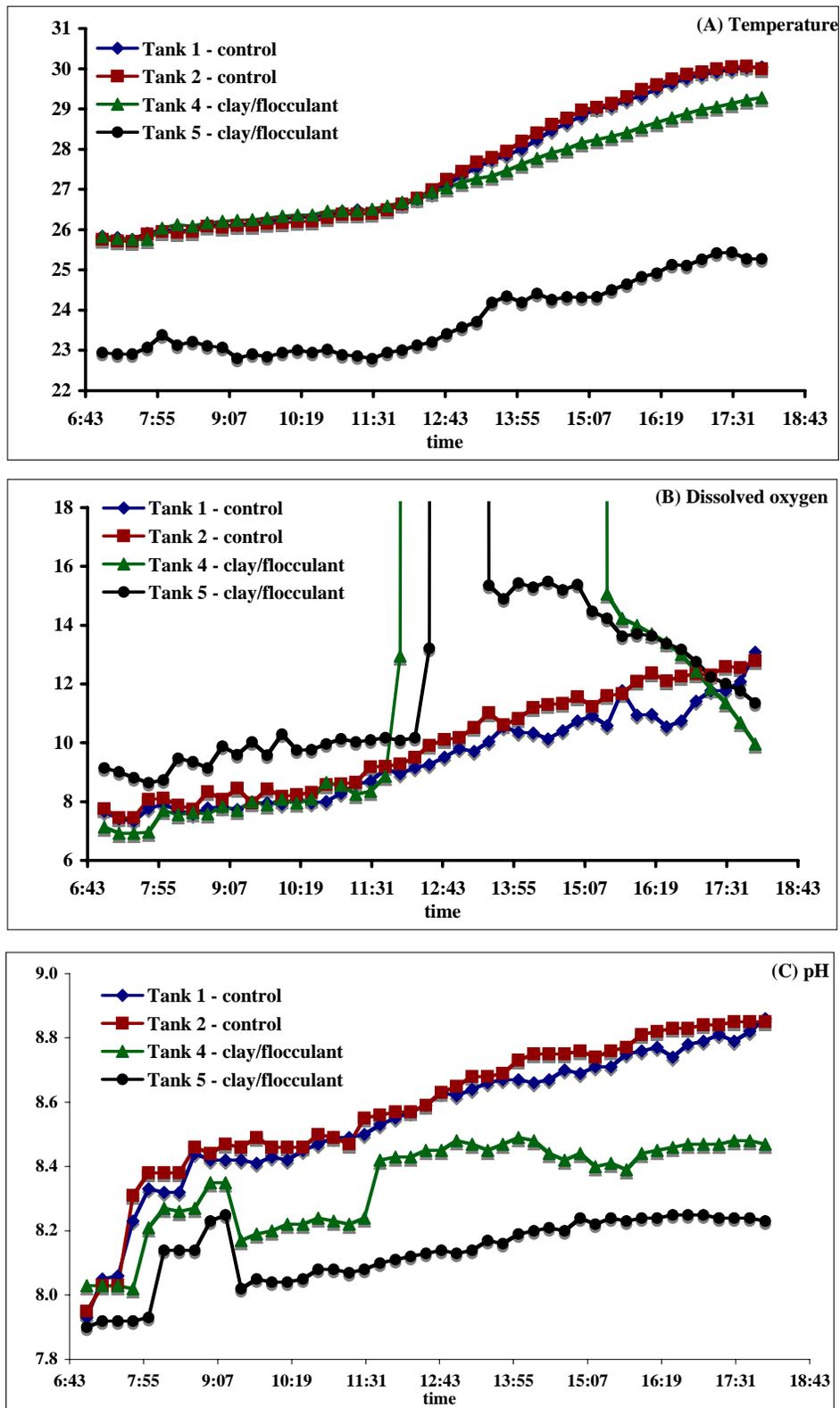


Figure 13. Mesocosm experiment. Water quality data from Hydrolabs placed in control (1 and 2) and treatment (4 and 5) tanks. (A) Temperature, (B) Dissolved oxygen, (C) pH.

tank being monitored, was different from the other three in that its initial temperature was 3 degrees lower. The temperature remained lower throughout the experiment. It is possible that the introduction of clay into the tank helped to reflect away solar irradiation, which kept the tank cooler. This phenomenon may also explain the deviation in temperature between Tank 4 and the two control tanks later in the experiment. However, we cannot explain why the temperature in Tank 5 was so different, unless the temperature sensor in the Hydrolab was malfunctioning. We have not observed such a significant difference in temperature between control and treatment tanks in earlier mesocosm experiments we have conducted.

Approximately one hour after clay addition, the dissolved oxygen concentration in Tank 4 went above scale, indicating that the water became supersaturated. It remained supersaturated for nearly 4 hrs. Likewise, Tank 5 went above the scale about 1 hr after Tank 4, but was above scale for about 1.5 hrs only. Both tanks returned to dissolved oxygen levels similar to the control tanks by the end of the 6-hr experiment. We have not observed this phenomenon before in our work with marine species treated with clay. In one unpublished study, we saw a slight increase in oxygen content near the surface, but we attributed this to the injection of bubbles during clay dispersal, as the force of the pump churned the surface. We had not seen over-saturation. At about the same time that the oxygen content was climbing in the tanks, we observed aggregates of cells and clays rising to the surface of the tanks (Figure 14). We hypothesize at this point that the increase in dissolved oxygen may have come from an increase in photosynthetic activity in *Prymnesium parvum*, and that the clay treatment may have facilitated this process. Unfortunately, we do not have enough information to say how this could take place. Certainly, the tanks and the water column are shallow enough to allow light to penetrate into the bottom of the tanks. Therefore, the organisms may have access to enough light for photosynthesis. However, we are not familiar with any biological explanation as to why the presence of clay would induce this organism, or any photosynthetic organism to suddenly increase their rate of carbon fixation. In one of the previous experiments in this report (Task 3) and in studies by Sengco et al. (2005), we observe that toxin content can increase with clay treatment. Therefore, it is possible that the addition of clay may have a stimulatory effect on *P. parvum*. More importantly, the flotation of the aggregates is an important consideration in the use of clays to treat this bloom species. If this phenomenon also occurs in nature, or can be replicated, then the effect of forcing the organisms to sink may be reversed if the aggregates can return to the surface. This result is surprising and should be studied further, from both biological and practical standpoints.



Figure 14. Clay/cell flocs floating at the surface of the mesocosm (2 hrs)

Finally, the pH in the two control tanks (1 and 2) showed similar trends to one another (Figure 13C). Although the changes in pH were small, the pH climbed slightly as water was added, and continued to increase gradually during the course of the study. Tank 4 showed a similar pH increase when the flocculant and clay were added. However, the water was slightly more acidic in this tank than in the two control tanks. The pH in Tank 5 was even less than that of Tank 4.

Despite the addition of flocculant Superfloc 606 and kaolinite clay, the pH in the tanks did not change dramatically.

Turbidity increased dramatically in the three treatment tanks (4, 5 and 6) following the addition of flocculant and clay (Figure 14A). The peak turbidity was approximately 90 NTU. Turbidity dropped quickly in Tank 4, but decreased more slowly in Tanks 5 and 6. After the 6-hr experiment, the turbidity in the tanks had decreased by about half to 40 NTU. After 24 hrs, the turbidity was similar to initial conditions as the clay settled. The floating aggregates remained at the surface, but most of the clays and flocs had settled to the bottom of the tank. There was no change in turbidity in the control tanks (1, 2, and 3).

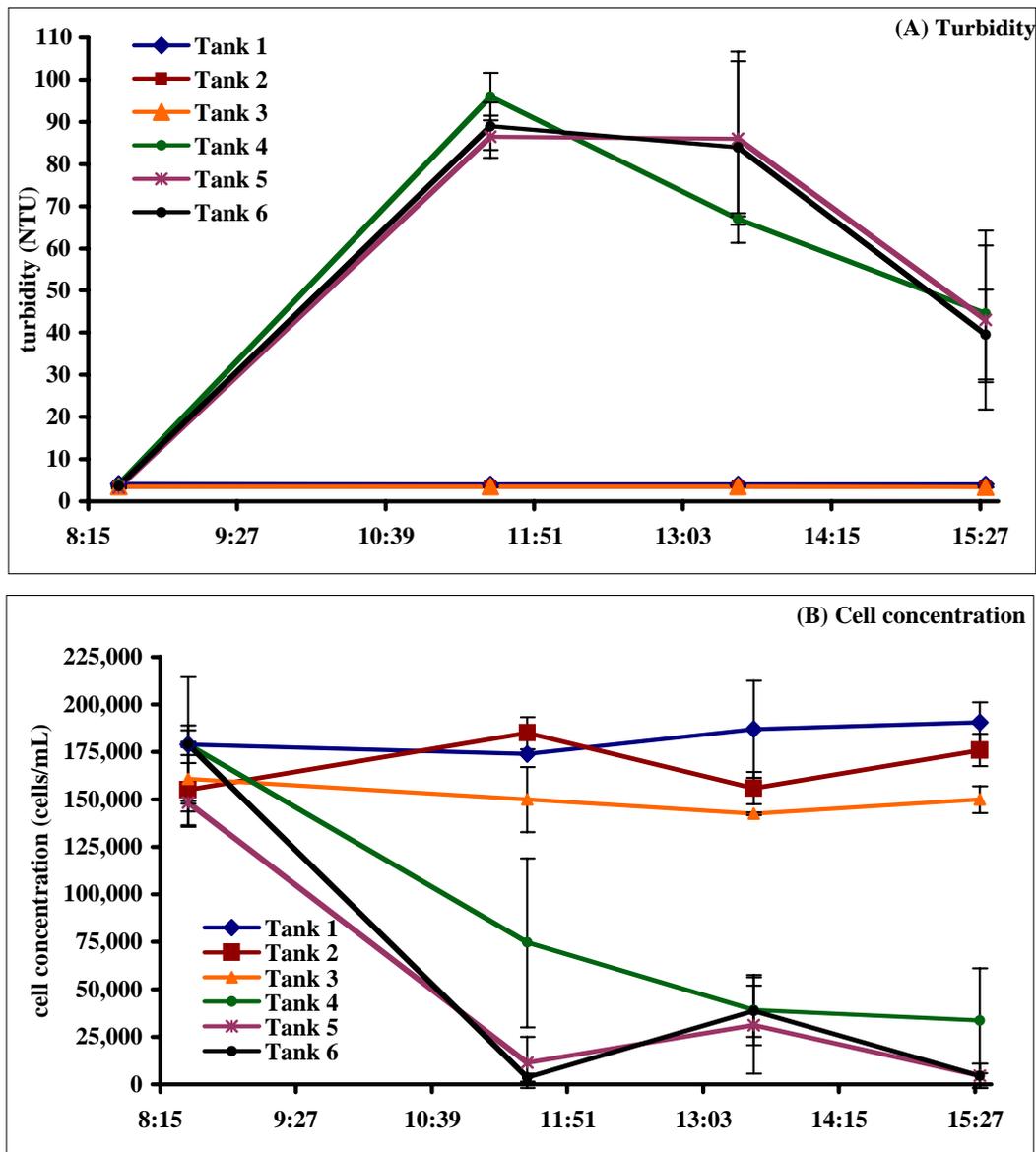


Figure 14. Removal of *Prymnesium parvum* using 0.10 g/L kaolinite clay (TKC-K) and 2.5 ppm Superfloc 606 in mesocosm tanks. (A) Turbidity. (B) Cell concentration. Initial cell concentration approximately 160,000 cells/mL.

As the flocculant and clays were added, and flocculation and settling took place, the cell concentration decreased in the treatment tanks (Figure 14B). Two hours after clay addition, the cell concentration dropped to below detection limit in Tanks 5 and 6 using the hemocytometer. In Tank 4, the cell concentration had dropped by 58%. At 4 and 6 hrs, the removal efficiency in Tank 4 was 78%. In Tank 5 and 6, more cells were detected 4 hrs after dispersal, which we believe is linked to the floatation phenomenon observed (see above). Therefore, the removal efficiency was calculated at 79% for Tank 5, and 78% for Tank 6. After 6 hrs, cell concentration in these two tanks dropped below the detection limit. The flocculation and settling process within the tanks were similar to that observed in the aquarium experiment. After the flocculant was added, a light, whitish layer appeared at the surface and small flocs began to form. When the clay slurry was added, flocculation rate increased quickly and flocs began to form. Flocculation also appeared to be patchy. Some areas became relatively free from particles, while others seemed to be high flocculation regions. We did not, however, observe floatation of aggregates in the aquaria as we did in these mesocosm tanks. This floatation can reverse some of the cell loss due to flocculation. However, the majority of the cells appeared to have remained in the floc layer along the bottom of the tanks. After 24 hours, the flocs had settled and a greenish-brown color was seen in the floc layer, suggesting the incorporation of *Prymnesium parvum* into the clay matrix.

The toxicity in all of the control tanks was consistently high (Table 4). After clay treatment, however, the toxicity dropped, except for one replicate from Tank 4 (i.e. sequence 7). This was also the tank in which cell removal efficiency took place, but not to the same extent as in Tanks 5 and 6. This tank also had more aggregates floating at the surface compared to the other two. Unlike the laboratory experiment from Task 3, there appears to be more consistency among the samples in this experiment with regards to the bioassay. Therefore, clay and flocculant treatment may reduce toxicity in the water column within a 6 hr treatment period, along with a reduction in cell numbers in the water column. However, there is again concern about the return of toxicity to the surface if floatation occurs to reverse the settling of particles. This should be addressed in further research. Samples for the toxin analysis were lost due to technical problems with the LC-MS and to the warming of the samples during shipment. Therefore, we have no data on the toxin concentrations from this experiment.

In this mesocosm study, we monitored changes in the concentration of inorganic nutrients, as a measure of water quality impacts. Phosphorus (SRP) decreased in all of the tanks after the 6 hr incubation, regardless of whether the tanks were treated or not treated with clay/flocculants (Figure 15A). By contrast, ammonia concentration increased in all of the tanks, whether treated or untreated with clay/flocculant (Figure 15B). There was no significant change in the nitrate concentration during the study. The presence of clay and flocculant did not have an effect on the concentration of these three nutrients, either significantly adding or removing them, based on these results.

Table 4. Removal of *Prymnesium parvum* using kaolinite clay (TKC-K) and Superfloc 606 in mesocosms. All samples had standard bioassay (Israeli protocol) conducted to detect lethal and sub-lethal amounts of ichthyotoxin present. Mortality recorded after 2 hrs at 28°C for all beakers (undiluted sample, undiluted sample + cofactor, 1/5 dilution + cofactor). Cell counts were done in replicate using a common hemacytometer for sequence 1 through 6, and an average density (cells/mL) were calculated. Arrows in the bioassay are used when there is incomplete mortality within a beaker after 2 hrs at 28°C (Greg Southard, pers. comm.). Since the bioassay is set up in a logarithmic scale (i.e. indicating 0, 1, 5 and equal to/greater than 25 ITU's), versus a linear scale, it would be difficult to determine an intermediate measurement would lie between two values when incomplete mortality occur (e.g. 2.5 between 1 and 5, or 15 between 5 and 25). Sequence 1 to 6 came from control tanks 1 to 3 (no clay, no flocculant), with two replicate measurements per tank. Sequence 7 to 12 came from the treatment tanks, with two replicate measurements per tank. Legend: BDL = below detection limit using haemocytometer.

Sequence #	Tank #	Replicate #	Treatment	Density (cells/mL)	Ichthyotoxic Units (ITUs)	Comments (cell activity)
1	1	1	Control	174,000	≥ 25	Motile
2	1	2	Control	186,000	≥ 25	Motile
3	2	1	Control	197,000	≥ 25	Motile
4	2	2	Control	200,000	≥ 25	Motile
5	3	1	Control	184,000	≥ 25	Motile
6	3	2	Control	186,000	≥ 25	Motile
7	4	1	+clay/flocculent	25,000	≥ 25	Motile and clay-cell clumps
8	4	2	+clay/flocculent	5,000	0 → 1	Most cells lysed, very few motile
9	5	1	+clay/flocculent	0 / detected	0 ← 1	Most cells lysed, very few motile
10	5	2	+clay/flocculent	0 / BDL	0 → 1	Cells lysed
11	6	1	+clay/flocculent	0 / detected	1	Non-motile
12	6	2	+clay/flocculent	0 / detected	1 ← 5	Most cells lysed, very few motile

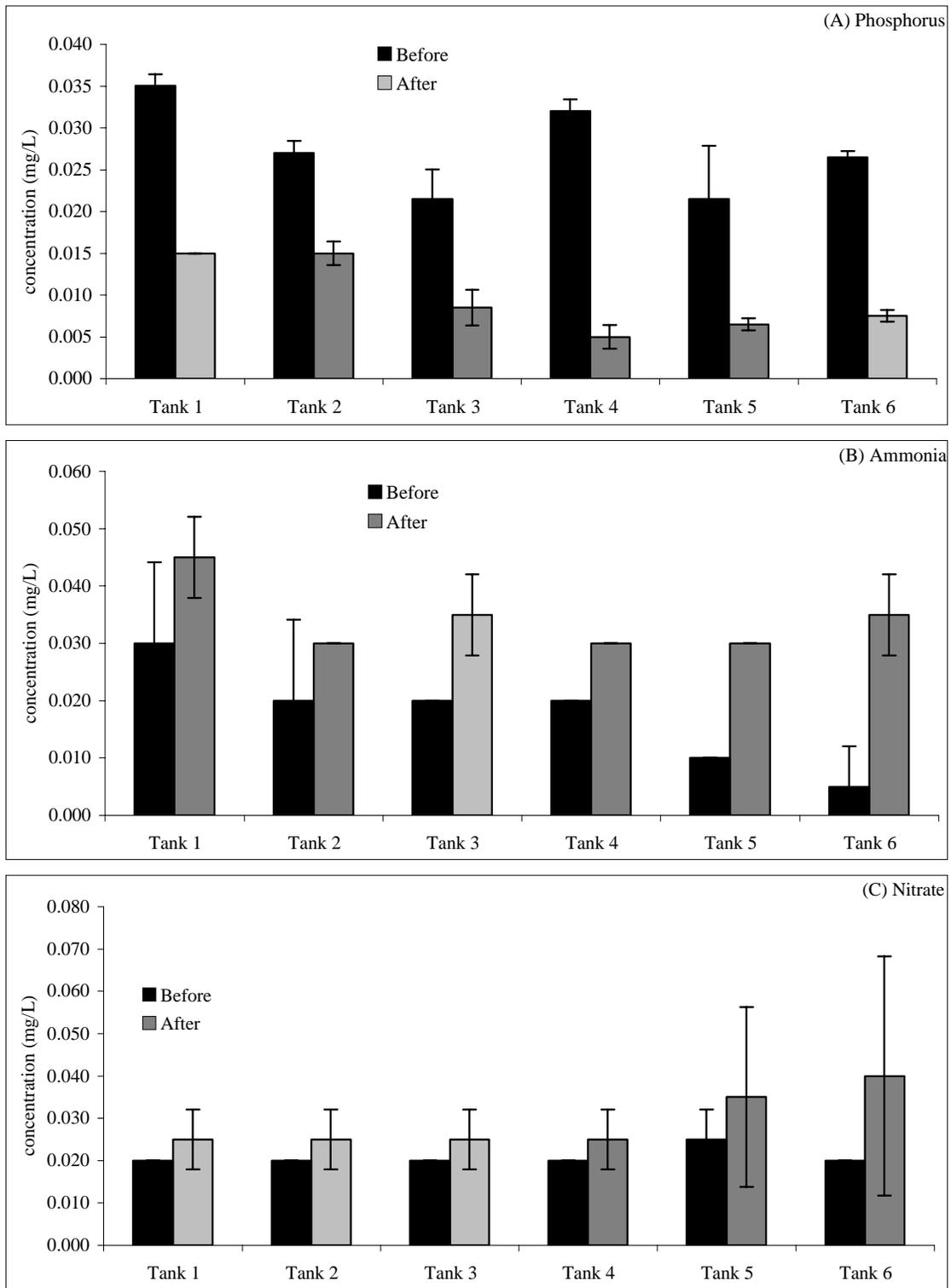


Figure 15. Mesocosm experiment. Inorganic nutrient concentration before and after clay/flocculant addition. Tank 1, 2 and 3 = control tanks. Tank 4, 5 and 6 = treatment tanks (0.10 g/L kaolinite TKC-K and 2.5 ppm Superfloc 606. Cell concentration approximately 160,000 cells/mL.

Summary and recommendations

Blooms of *Prymnesium parvum* are a growing nuisance and economic concern in Texas inland waters and reservoirs, with impacts on fish, shellfish, and local economies. Therefore, studies were initiated to examine whether domestic clays (and other flocculants) can control these organisms effectively, and mitigate their impacts, with minimal effects on environmental quality and local ecosystems.

In this study, we found that clays alone were completely ineffective at removing *Prymnesium parvum* within a range of cell concentrations in which fish kills have been reported (100,000 – 400,000 cells/mL). Bentonites and Florida phosphatic clays, both demonstrating moderate to high removal ability in earlier studies, were unable to flocculate the organisms within a study range of 0.05 g/L to 0.50 g/L. Kaolinite and other mixed minerals samples from Texas sources were equally ineffective. Some cell removal occurred when nitrogen-limited cells were treated with clays alone, but not with P-limited cells. Also, low cell removal was observed from two clays when the salinity of the medium increased above 20. Observations of the suspension indicate that the flocculation rate is slow at ambient salinity (= 4), relative to flocculation rates at higher salinities, indicating that particle adhesiveness – which is affected by the chemical properties of the medium – is an important limiting factor over particle concentration and size.

Cell removal efficiency was better when the cultures were treated with chemical flocculants compared to clays. Flocculants containing some cationic molecules was moderately effective in flocculating *Prymnesium parvum*, relative to polyaluminum chloride (PAC) and an anionic flocculant. The salinity of the medium affected the ability of the flocculant to remove *P. parvum*: in one case, cell removal increased as salinity increased, while in another case, the opposite was observed. The best results in the study were found when clays and flocculants were combined. The highest removal efficiencies were seen when Superfloc 606 was used with any of the clays, followed by Superfloc 608. Both flocculants were new blends of polyaluminum chloride and a cationic flocculant. We speculate that the flocculant acts to increase the adhesiveness of the clay and cell particles in the low salinity medium. This is accomplished when the positively charged species on the flocculants bind to the negatively charge surfaces of clays and cells, acting as molecular bridges between the particles. The presence of clays adds sufficient density to the flocs that leads to rapid settling. However, the salinity of the medium will again influence the process by affecting charges on the flocculants clays. Finally, the combination of kaolinite TKC-K and the flocculant Superfloc 606 was the most effective at removing cells in aquarium trials, compared to the same clay treated with PAC, or the controls. This combination was selected for use in toxin removal and mesocosm tests.

The combination of TKC-K and Superfloc 606 repeatedly displayed high cell removal, but the results of fish bioassay were ambiguous. We could not confidently determine whether the treatment reduced toxicity towards fish. Moreover, the toxicity and toxin content of the cell may increase in some cases due to clay treatment, particularly when the cells are N-limited (but not P-limited). When the clays were used to treat extracellular toxins alone, in the absence of cells, the data suggested that clays may be able to remove the toxins in some cases.

The mesocosm experiments demonstrated that the kaolinite TKC-K, combined with the flocculant, can effectively remove *Prymnesium parvum* from suspension ($RE \geq 78\%$) within a period of 6 hrs. Turbidity in the water column increased dramatically during treatment, but water clarity was restored to initial conditions within 24 hrs. Along with cell removal, toxicity in the water column also decreased. The inorganic nutrient pool was also not affected by the clay treatment. However, dissolved oxygen content increased dramatically about 1 to 1.5 hrs after clay addition, and the floatation of aggregates was seen at the surface. This process has not been observed before, and should be further investigated, as it may negate the removal process. Temperature and pH do not appear to be affected by the clay treatment, although there was some question as to the possibility that clays in suspension can prevent the heating of the water.

Overall, this study showed the potential usefulness of clays to remove *Prymnesium parvum* and its toxins from suspension, when combined with the proper flocculant, and consideration of the salinity. When the flocculant is chosen, any clay mineral that is finely divided can be used in small amounts. Clearly, it is the adhesiveness of the particles in suspension that strongly influence the flocculation and eventual settling. Therefore, future work should include the biological and environmental impacts of some of these new flocculant blends that contain cationic polymers. Furthermore, some studies should address questions regarding the effect of clay/flocculant treatment on the physiology of *Prymnesium parvum*, especially in relation to toxin production and photosynthesis/oxygen production. Both of these can determine whether clays can or should be used. In case of oxygen production, this may reverse the removal process by causing aggregates to float. It will be necessary to try and reproduce this phenomenon in the laboratory and understand the conditions under which this occurs. Finally, the removal process should be studied in much larger scales and under more realistic physical and hydrodynamic conditions, such as those that would be seen in hatcheries or in lakes. These experiments, by design, were done in relatively quiescent conditions in order to simplify the system and make it more tractable. However, we have learned that water motion can have significant effects on the rate of flocculation, the viability of the organism, and the fate of the settled flocs.

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References

- Anderson, D. M. 1997. Turning back the harmful red tide. *Nature*, 388:513-514.
- Anderson DM, Kulis DM, Keafer BA, Berdalet B. (1999) Detection of the toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae) with oligonucleotide and antibody probes: variability in labelling intensity with physiological condition. *J. Phycol.* 35: 870-883.
- Archambault, M.C., Bricelj, V.M., Grant, J. and Anderson, D.M. (2004) Effects of suspended and sedimented clays on juvenile hard clams, *Mercenaria mercenaria*, within the context of harmful algal bloom mitigation. *Mar. Biol.* 144: 553-565.
- Avnimelech, Y., Troeger, B.W., Reed, L.W. (1982) Mutual flocculation of algae and clay: evidence and implication. *Science.*, 216(4541): 63-65.
- Bae, H. M., Choi, H. G., Lee, W. C. & Yoon, S. J. (1998) Control of the red tide by yellow loess dispersion. *In: Kim, H. G., Lee, S. G. & Lee, C. K. (ed.), Proceedings of the Korea-China Joint Symposium on Harmful Algal Blooms*, 53-60.
- Boesch, D. F., Anderson, D. M., Horner, R. A., Shumway, S. E., Tester, P. A., & Whitedge, T. E. 1997. Harmful Algal Blooms in Coastal Waters: Options for Prevention, Control and Mitigation. NOAA Coastal Ocean Program Decision Analysis Series No. 10. NOAA Coastal Office, Silver Spring, MD. 46 pp + Appendix.
- Choi, H.G., Kim, P.J., Lee, W.C., Yun, S.J., Kim, H.G., Lee, H.J. (1998) Removal efficiency of *Cochlodinium polykrikoides* by yellow loess. *Journal of the Korean Fisheries Society* 31(1): 109-113.
- Choi, H.G., Lee, P.Y., Yun, S.J., Lee, W.C., Bae, H.M. (1999) Control of *Cochlodinium polykrikoides* blooms and adsorption of nutrients in the seawater by clay and yellow loess. *Bulletin of the National Fisheries Research and Development Institute of Korea*, 57: 105-110.
- Cranford, P.J. and Gordon Jr., D.C. 1992. The influence of dilute clay suspensions on sea scallop (*Placopecten magellanicus*) feeding activity and tissue growth. *Netherlands J. Sea Res.* 30: 107-120.
- Edvardsen, B. and Paasche, E. (1998) Bloom dynamics and physiology of *Prymnesium* and *Chrysochromulina*. *In: Anderson, D.M., Cembella, A.D. and Hallegraeff, G.M. (eds.) Physiological Ecology of Harmful Algal Blooms*, Springer-Verlag, Berlin, pp. 193-208.
- Fistarol, G.O., Legrand, C. and Granéli, E. (2003) Allelopathic effect of *Prymnesium parvum* on a natural plankton community. *Mar. Ecol. Prog. Ser.*, 255:115-125.
- Glass, J., Linman, G., and Ralph, J. (1991) The association of *Prymnesium parvum* with fish

kills in Texas. Texas Parks and Wildlife Report.

- Granéli, E. and Johansson, N. (2001). Nitrogen or phosphorus deficiency increases allelopathy in *Prymnesium parvum*. In: Harmful Algal Blooms 2000, Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J., (eds.). Intergov. Oceanographic Commission of UNESCO, pp. 328-331.
- Granéli, E. and Johansson N. (2003a) Effects of the toxic haptophyte *Prymnesium parvum* on the survival and feeding of a ciliate: the influence of different nutrient conditions. Mar. Ecol. Prog. Ser., 254: 49-56
- Granéli, E. and Johansson N. (2003b) Increase in the production of allelopathic substances by *Prymnesium parvum* cells grown under N- or P- deficient conditions. Harmful Algae, 2: 135.
- Hagström, J. A. and Granéli, E. (2005). Removal of *Prymnesium parvum* (Haptophyceae) cells under different nutrient conditions by clay, *Harmful Algae*, 4(2): 249-260.
- Holmquist, E. and Willém, T. (1993) Fish mortality caused by *Prymnesium parvum*. *Vatten*, 49:110-115.
- Igarashi, T., Satake, M., Yasumoto, T. (1996) Prymnesin-2: A potent ichthyotoxic and haemolytic glycoside isolated from the red tide algae *Prymnesium parvum*. *J. Am. Chem. Soc.*, 118: 479-480.
- James, T. and de la Cruz, A. (1989) *Prymnesium parvum* Carter (Chrysophyceae) as a suspect of mass mortalities of fish and shellfish communities in western Texas. *Texas J. Sci.*, 41(4) 429-430.
- Kim, H. K., Kim, K. I., Kim, D. S., Park, K. S., Hong, T. H. & Jeong, H. J. (2000) Developing a method of controlling the outbreak and maintenance of red tides using NaOCl produced by electrolysis of natural seawater. Abstract. 9th International Conference on Harmful Algal Blooms, Hobart, Tasmania, Australia, 28.
- Komarovsky, B. (1951) Some characteristic water-blooms in Lake Tiberias and fish ponds in the Jordan Valley. *Ver. Int. Ver. Theor. Angew. Limnol.* 11: 219-223.
- Lewis, M.A., Dantin, D.D., Walker, C.C., Kurtz, J.C., Greene, R.M. (2003) Toxicity of clay flocculation of the toxic dinoflagellate, *Karenia brevis* to estuarine invertebrates and fish. *Harmful Algae* 2(4): 235-246.
- Lindholm, T., Öhman, P., Kurki-Helasma, K., Kincaid, B. and Meriluoto, J. (1999) Toxic algae and fish mortality in a brackish water lake in Åland, SW Finland. *Hydrobiol.*, 397:109-120.
- Marvin, K. T. & Proctor, R. R. (1967) Laboratory evaluation of red-tide control agents. *Fish.*

Bull., 66:163--164.

- Moestrup, Ø. (1994) Economic aspects: 'blooms', nuisance species and toxins. In: Green, J.C., and Leadbeater, B.S.C. (eds.), *The Haptophyte Algae*, Clarendon Press, Oxford, pp.265-285.
- Na, G., Choi, W. & Chu, Y. 1996. A study on red tide control with Loess suspension. *J. Aquacult.*, 9: 239-245. (In Korean with English abstract).
- O'Melia, C.R., Tiller, C.L. (1993) Physicochemical aggregation and deposition in aquatic environments. In: *Environmental Particles*, Vol. 2, Buffle, J., Van Leeuwen, H.P. (eds.), Lewis Publishers Inc., Boca Raton, FL, pp. 353-386.
- Pierce, R. H., Henry, M. S., Higham, C. J., Blum, P., Sengco, M. R. & Anderson, D. M. (2004) Removal of harmful algal cells (*Karenia brevis*) and toxins from seawater culture by clay flocculation. *Harmful Algae*, 3(2): 141-148.
- Reichenbach-Klinke, H. (1973) *Fish Pathology*. T.F.H. Publications, Neptune City, NJ. p. 512.
- Sengco, M.R. and Anderson, D.M. (2004) Controlling harmful algal blooms through clay flocculation. *J. Eukaryot. Microbiol.*, 51 (2): 169-172.
- Sengco, M. R., Li, A., Tugend, K., Kulis, D. & Anderson, D. M. (2001) Removal of red- and brown-tide cells using clay flocculation. I. Laboratory culture experiments with *Gymnodinium breve* and *Aureococcus anophagefferens*. *Mar. Ecol. Prog. Ser.*, 210:41-53.
- Sengco, M.R., Hagström, J.A., Granéli, E. and Anderson, D.M. (2005) Removal of *Prymnesium parvum* (Haptophyceae) and its toxins using clay minerals. *Harmful Algae*, 4: 261-274.
- Shilo, M. and Aschner, M. (1953) Factors governing the toxicity of cultures containing phytoflagellate *Prymnesium parvum* Carter. *J. Gen. Microbiol.*, 8:333-343.
- Shilo, M. and Shilo, M. (1953) Conditions which determine the efficiency of ammonium sulphate in the control of *Prymnesium parvum* in fish breeding ponds. *Appl. Microbiol.*, 1: 330-333.
- Shirota, A. (1989) Red tide problem and countermeasures (2). *Int. J. Aquat. Fish. Technol.*, 1:195-223.
- Soballe, D.M., and Threlkeld, S.T. (1988) Algal-clay flocculation in turbid waters: variations due to algal and mineral differences. *Verh. Internat. Verein. Limnol.* 23: 750-754.
- Sridhar, P., Namasivayam, C. & Prabhakaran, G. (1988) Algae flocculation in reservoir water. *Biotechnol. Bioengineer.*, 32:345-347.

- Tenney, M. W., Echelberger, W. F., Schuessler, R. G. & Pavoni, J. L. (1969) Algal flocculation with synthetic organic polyelectrolytes. *Appl. Microbiol.*, 18: 965-971.
- Tillmann, U. (2003) Kill and eat your predator: a winning strategy of the planktonic flagellate *Prymnesium parvum*. *Aquat. Microb. Ecol.*, 32:73-84.
- Valkanov, A. (1964) Untersuchungen über *Prymnesium parvum* Carter und seine toxische Einwirkung auf die Wasserorganismen. *Kiel Meeresforsch.*, 20: 65-81.
- Yu, Z., Zou, J. & Ma, X. (1994) Applications of clays to removal of red tide organisms I. Coagulation of red tide organisms with clays. *Chin. J. Oceanol. Limnol.*, 12:193-200.
- Yu, Z. Sengco, M.R. and Anderson, D.M. (2004) Flocculation and removal of the brown-tide organism, *Aureococcus anophagefferens* (Chrysophyceae), using clays. *Journal of Applied Phycol.*, 16: 101-110.



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