

Article

## Mitigating Fish-Killing *Prymnesium parvum* Algal Blooms in Aquaculture Ponds with Clay: The Importance of pH and Clay Type

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**Abstract:** Clay minerals have previously been used to mitigate algal blooms because of their ability to flocculate algal cells or remove nutrients, but also offer considerable potential to remove ichthyotoxins. When a barramundi farm in tropical Australia suffered substantial fish mortalities due to a bloom of the ichthyotoxic haptophyte *Prymnesium parvum*, the farm manager decided to manipulate pond water N:P ratios through removal of phosphorus by the addition of lanthanum-modified bentonite clay (Phoslock™) to successfully mitigate ichthyotoxic effects. We conducted *Prymnesium* culture experiments

under a range of N:P ratios, screening 14 different clays (two zeolites, four kaolins, six bentonites and two types of Korean loess) at pH 7 and 9 for cell flocculation and removal of ichthyotoxicity assessed with the RTgill-W1 cell line assay. Application of Phoslock™ to cultures grown at different N:P effectively removed 60%–100% of water-soluble toxicity of live *Prymnesium* (dependent on nutritional status). While most clays efficiently flocculated *Prymnesium* cells ( $\geq 80\%$  removal), cell removal proved a poor predictor of ichthyotoxin adsorption. Extensive clay screening revealed that at elevated pH, as commonly associated with dense algal blooms, most clays either exacerbated ichthyotoxicity or exhibited significantly reduced toxin adsorption. Interpretation of changes in clay zeta potential at pH 7 and 9 provided valuable insight into clay/ichthyotoxin interactions, yet further research is required to completely understand the adsorption mechanisms. Bentonite-type clays proved best suited for ichthyotoxin removal purposes (100% removal at ecologically relevant pH 9) and offer great potential for on-farm emergency response.

**Keywords:** *Prymnesium parvum*; mitigation; clay; pH; ichthyotoxicity; gill cell line assay; RTgill-W1; zeta potential

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## 1. Introduction

The globally-distributed haptophyte *Prymnesium parvum* has been identified as the causative organism behind several mass fish mortality events [1–5]. Blooms are characterized by their seasonal recurrence, rapid proliferation, wide salinity and temperature tolerance, as well as the production of highly potent ichthyotoxins, the chemical nature of which remains incompletely known [5–7]. Recurring *Prymnesium parvum* blooms have become a routine problem for the Israeli *Tilapia* [8,9], the Texas striped bass [4,5] and, more recently, (June/July, 2009) the barramundi (*Lates calcarifer*) pond-based aquaculture industry in tropical Australia [2].

*Prymnesium parvum* blooms vary considerably in toxicity, from being weakly toxic during exponential growth in nutrient-replete conditions (nitrogen:phosphorus 16:1), to generating fast, irreversible gill cell damage under nitrogen or phosphorus limitation [10–12]. Due to the eutrophic nature of high throughput aquaculture ponds, these systems are deemed to be ecologically unstable and prone to disturbance. However, as semi-closed systems, they lend themselves to careful manipulation of nutrient ratios through either ammonium sulfate addition [13,14] or phosphorus removal by Phoslock™ clay [2,15].

The large economic and ecological damages incurred from *P. parvum* blooms across the globe provide a strong incentive to formulate effective mitigation strategies. Previous investigations have focused on the use of clay minerals as mitigation agents of *Prymnesium parvum* because of their ability to flocculate algal cells and, thereby, remove intracellular ichthyotoxins [12,16]. Later work, focusing on the clay-mediated removal of *Karenia brevis* dinoflagellate cells and *Microcystis aeruginosa* cyanobacterial toxins, reported evidence of direct adsorption of water-soluble, extracellular ichthyotoxins to bentonite-type clays [17,18]. This latter property of clay deserves special attention,

since algal cell lysis in response to collisions with clay particles has previously been reported [19–22]; a process that could greatly amplify ichthyotoxic effects through the release of intracellular ichthyotoxins [23,24]. The potential of clays to adsorb *P. parvum* ichthyotoxins and the factors governing adsorption remain largely unknown. From studies modelling the adsorption of pesticide and pharmaceutical compounds to clay minerals in terrestrial systems, it appears likely that the increased seawater pH encountered during *P. parvum* blooms could constitute a significant variable influencing ichthyotoxin adsorption [25–27].

Recently (2009), after a *Prymnesium* bloom at an Australian barramundi farm resulted in complete pond mortality (AUS \$95,000 of fish lost), the manager of the farm engaged in a preventative pond nutrient and pH manipulation experiment. An experimental pond was treated with Phoslock™ clay (30 kg/kg phosphate) and pH adjusted to <7.7 by stimulating microbial growth through the addition of molasses [2]. Phoslock™, a bentonite clay, has been specially modified to irreversibly bind water-dissolved phosphorus to lanthanum cations incorporated into its clay matrix, a strongly pH-dependent process [15]. After application, phytoplankton biomass became more stable, with reduced diurnal oxygen fluctuations, and *Prymnesium* did not reoccur in the treated pond. By contrast, an untreated control pond developed a further *Prymnesium* bloom in June, 2011, and, again, in March, 2012, that killed AUS \$10,000 worth of mullet. During the initial fish-kill event in 2009, *Prymnesium parvum* (identified through light and transmission electron microscopy) was successfully isolated and established in culture for later experimental manipulation (PPDW02 strain).

We conducted *Prymnesium* culture experiments under different N:P ratios while measuring toxicity using the RTgill-W1 cell line assay to interpret these field observations. Specialized, lanthanum-modified Phoslock™ clay was applied to both sonicated and live algal cultures to assess effectiveness in removing *P. parvum* cells and their toxic effects. A preliminary account of this work was presented at the 15th International Conference on Harmful Algae [11]. Since then, we screened 14 individual clay minerals belonging to four major clay groups (bentonites, zeolites, kaolins and Korean loess) for cell and ichthyotoxin removal properties at pH 7 and 9 to determine whether a highly modified clay, such as Phoslock™, is required for mitigation purposes or whether certain unmodified, readily available local clays are equally well suited. The zeta potential (approximation of clay surface charge) of the individual clay minerals and their physical characteristics are discussed to facilitate the selection of local clays as an on-farm emergency response tool.

## 2. Methods

### 2.1 Clay Materials

Twelve commercially available Australian clays and two types of Korean loess were obtained and sieved through a 63- $\mu$ m mesh to achieve a comparable size fraction (see Table 1 for details).

**Table 1.** Description of clay types employed in this study as provided by the manufacturer.

Clay group	Clay code	Major clay type	Product name/Company	Cation exchange capacity (CEC)	Suppliers description
Bentonite	B1	Ca-bentonite	B1-AM/Bentonite Products WA, Watheroo, Australia	N.A.	50% A, 50% gypsum; low swelling
	B2	Ca-bentonite	Es/Bentonite Products WA, Watheroo, Australia	95.70	39.02% clay, 9.44% coarse sand, 37.39% fine sand, <14.15% silt
	B3	Modified Na-bentonite	Phoslock™/Phoslock Water Solutions Ltd, Sydney, Australia	N.A.	Lanthanum-modified bentonite (5% La <sup>3+</sup> )
	B4	Ca-bentonite	A/Bentonite Products WA, Watheroo, Australia	64.54	81.9% clay, 2.2% coarse sand, 16% fine sand, <0.01% silt
	B5	Ca-bentonite	Ed/Bentonite Products WA, Watheroo, Australia	85.80	83.4% clay, 4.4% coarse sand, 9.8% fine sand, <2.5% silt
	B6	Na-bentonite	Trubond/Sibelco, Sydney, Australia	82	Montmorillonite as a major component
Kaolin	K1	Kaolin	Fine ceramic clay obtained from potter, Hobart, Australia	N.A.	N.A.
	K2	Kaolin	CA-1/Claypro, Junortoun, Australia	N.A.	Dry milled ball clay (kaolinite as a major component)
	K3	Kaolin	Kaolinite/Zeolite Australia, Werris Creek, Australia	29–39	60%–75% opaline silica, 20%–30% kaolinite, 5% other clays and mica
	K4	Kaolin	Snobrite C/Sibelco, Sydney, Australia	N.A.	70%–95% kaolinite, 5%–15% quartz, <15% other minerals
Zeolites	Z1	Zeolite	Zeolite/Zeolite Australia, Werris Creek, Australia	>120	Clinoptotilite with minor amounts of quartz, smectite
	Z2	Zeolite	ANZ38/Castle Mountain Zeolites, Quirindi, Australia	147	85% clinoptotilite, 15% mordenite), minor amounts of quartz, feldspar, montmorillonite
Korean loess	Kor 1	Korea A	Sourced from Tongyeong, South Korea	N.A.	Korean loess
	Kor 2	Korea B	Sourced from Geoje, South Korea	N.A.	Korean loess

## 2.2. Phoslock™ (B3) Application

### 2.2.1. Live Cultures

Non-axenic *Prymnesium parvum* (UTAS Culture Code PPDW02, isolated from barramundi aquaculture ponds during a harmful algal bloom event, Northern Territory, Australia, in June, 2009, by Gustaaf Hallegraeff) was grown in 6-L conical glass flasks containing 3 L of artificial seawater medium (ASW) [28] with a salinity of 35 and nitrogen:phosphorus ratios of GSe/2 nutrients adjusted to 16:1 (nutrient replete; 400  $\mu\text{M}$   $\text{NO}_3^-$  and 25  $\mu\text{M}$   $\text{PO}_4^{3-}$ ), 24:1 (phosphorus deficient; 400  $\mu\text{M}$   $\text{NO}_3^-$  and 16.7  $\mu\text{M}$   $\text{PO}_4^{3-}$ ) and 8:1 (nitrogen deficient; 200  $\mu\text{M}$   $\text{NO}_3^-$  and 25  $\mu\text{M}$   $\text{PO}_4^{3-}$ ). Cultures were maintained at  $20 \pm 1$  °C and 120  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  of light with a 12:12 h light:dark cycle. Algal growth was monitored by cell counts (Coulter Counter, Beckman Z1, Fullerton, CA, USA). When cultures grown in nutrient-deficient media entered the stationary phase, these cultures and exponentially growing cultures (assumed to be nutrient replete) were diluted with artificial seawater without added nutrients [28] 24 h prior to experimental manipulation to yield  $10^5$  cells  $\text{mL}^{-1}$  (final pH 7.7–8.3). Cultures were divided into 7 replicates of 400 mL per nutrient treatment (500-mL twist-top jars, Labserv) to be randomly allocated to control and clay treatment groups ( $n = 3$ ). One 400-mL replicate was reserved for preparation of lysed cultures (see below). Lanthanum-modified bentonite (Phoslock™, B3) was applied to cultures drop-wise in slurry form (prepared with ASW), such that the addition of 5 mL of clay slurry yielded a final clay concentration of 1.5  $\text{g L}^{-1}$ . Control cultures received 5 mL of ASW, and controls for the effect of Phoslock™ consisted of clay-treated culturing medium. Cell numbers were counted 4 h and 3 days after treatment with a Beckman Coulter Counter (Z1), and the identical subsamples (collected 5 cm below the surface and 3 cm above the bottom of the jars) were tested on the gill cell line RTgill-W1 in semi-permeable inserts (see below). Particle counts from seawater plus Phoslock™ controls were subtracted from particle counts of Phoslock™-treated algal cultures to facilitate interpretation of results without added clay particle interference.

### 2.2.2. Lysed Cultures

*Prymnesium parvum* cells in the remaining 400-mL replicate of each nutrient condition were lysed through sonication (Measuring & Scientific Equipment Ltd., London, England, probe diameter of 2.5 mm at an amplitude level of 9  $\mu\text{m}$  peak to peak, 10 min) and split into six replicates of 40 mL per nutrient treatment. Replicates were assigned to treatment and control groups as described for live cultures above ( $n = 3$ ), and 2 mL of Phoslock™ slurry were applied to yield a final concentration of 1.5  $\text{g L}^{-1}$ . Unfiltered subsamples were tested on the gill cells 4 and 24 h after clay treatment in semi-permeable inserts.

### 2.2.3. High pH Culture

*Prymnesium* cultures were grown in GSe medium prepared from filtered coastal seawater (0.2  $\mu\text{m}$ , Pall) with a salinity of 30 to test the efficacy of Phoslock™ to remove ichthyotoxins and algal cells when applied to high density *Prymnesium* cultures at higher pH. Cultures were harvested during the

stationary growth phase ( $8.6 \times 10^5$  cells  $\text{mL}^{-1}$ , pH 9.8), and 30-mL aliquots were transferred to six 40-mL borosilicate test tubes. Three replicates were treated with 2 mL of Phoslock™ slurry prepared in deionized water (DI; MilliQ), with control cultures receiving 2 mL of DI. Four hours after treatment, subsamples were taken just below the surface and 2 cm above the bottom of the tube to be tested on the gill cells in semi-permeable inserts. The particle size distribution (3–10  $\mu\text{m}$  equivalent spherical diameter; ESD) in these identical samples was determined through Coulter Counter analysis (Beckman, Z4), and the removal efficiency of algal cells (RE) was calculated (Equation 1) as:

$$RE = \left[ \left( 1 - \frac{\text{Count}_{\text{treated}}}{\text{Count}_{\text{control}}} \right) \times 100 \right] \quad (1)$$

To account for increased particle counts in clay-treated samples towards the lower end of the size spectrum ( $<4 \mu\text{m}$ ), a further three test-tubes received 30 mL of GSe medium to be treated with Phoslock™.

### 2.3. Clay Screening

#### 2.3.1. Cell Removal

Thirteen different clay types (Table 1) were applied to *Prymnesium* cultures grown in standard GSe medium (N:P 10:1) and harvested during the early stationary phase to evaluate the suitability of different clay types for flocculation purposes. Cultures were diluted with 0.2- $\mu\text{m}$  filtered coastal seawater to yield  $10^5$  cells  $\text{mL}^{-1}$ , and the different clays were applied to 30 mL of culture in 40-mL test tubes as described above for Phoslock™. Four hours after clay treatment, the entire culture supernatant was collected down to a depth of 0.5 cm above the clay pellet and gently mixed prior to measuring chlorophyll a fluorescence of duplicate 2-mL sub-samples in a plate reader at excitation and emission wavelengths of 450 and 485 nm, respectively (Fluostar Optima, BMG Labtech, Ortenberg, Germany). To eliminate background fluorescence, clay added to seawater was employed as a blank. Cell RE was calculated as described above (Equation 1) by comparing fluorescence in clay-treated and control samples. Clay type K4 (employed in later ichthyotoxin removal experiments) was not available to us at the time of cell removal screening.

#### 2.3.2. Ichthyotoxin Removal

To determine the capacity of different clay types to adsorb ichthyotoxins released by lysed *P. parvum* preparations towards the gill cells, fourteen different clay types (Table 1) were evaluated at pH 7 and 9. *Prymnesium parvum* was harvested during the late stationary growth phase and concentrated through centrifugation of 10 mL of culture (10 min,  $3,300 \times g$ , 20 °C, Sorvall Super T21, Du Pont, Delaware, NJ, USA). The upper 9 mL were discarded and the remaining 1 mL immediately sonicated for 1 min on ice. Lysed, concentrated culture was diluted in L-15/ex medium [29] to yield a concentration equivalent to  $10^5$  cells  $\text{mL}^{-1}$ . The pH of the L-15/ex medium was adjusted to 7 and 9 with either 0.1 M HCl or NaOH prior to the addition of lysed culture. It is acknowledged that the final pH would have slightly deviated upwards from the original pH of 7 and 9, due to the addition of clay slurry and culture lysate, but this was not quantified. Clay slurries were prepared, such that the addition

of 0.5 mL of slurry to 5 mL of *P. parvum* preparation yielded a final clay concentration of 1.0 g L<sup>-1</sup>. Toxic controls received an equivalent volume of DI, whereas non-toxic controls consisted of clay addition to pure L-15/ex medium. After treatment, samples were vortexed for 5 s and subsequently centrifuged (1,400× g, 5 min, Hettich Universal 16 A, Tuttlingen, Germany). Without disturbing the clay pellet, 3 mL of supernatant were collected, mixed and tested on the gill cells in conventional 96-well plates.

### 2.3.3. Zeta Potential

The zeta potential of different clay types was determined with a Nanoseries zetasizer Model Zen3600 (Malvern Instruments, Malvern, Worcestershire, U.K.). This instrument calculates the surface charge of particles based on their electrophoretic mobility in an applied electric field. All measurements were conducted at 25 °C and a clay concentration of 1.0 g L<sup>-1</sup> in L-15/ex medium at pH 7 and 9 (refractive index 1.333, viscosity 9.18 × 10<sup>-4</sup> Pa·s and dielectric constant 80.0). Smoluchowski approximation was applied due to large particle size (>1 µm) and high ionic strength (>100 mM) [30].

### 2.3.4. Minimum Effective Dose

The minimum effective dose of Ed (B5) and Phoslock™ (B3) clay was determined during a simulated worst case scenario of complete cell lysis at higher pH likely to be encountered during a dense, natural *Prymnesium* bloom. Lysed preparations from high density cultures (1.2 × 10<sup>6</sup> cells mL<sup>-1</sup>) were prepared as described above and diluted in L-15/ex to yield 4 × 10<sup>5</sup> cells mL<sup>-1</sup> (moderate bloom densities). The pH of the L-15/ex medium was adjusted to the original culture pH of 9.2 and salinity to 30 through NaCl addition. In Eppendorf tubes, 20 µL of clay slurry were added to 980 µL of lysed *P. parvum* preparation to yield a final clay concentration of 0–0.25 g L<sup>-1</sup>. Samples were vortexed and centrifuged as described above, with the final centrifugation step conducted in a microcentrifuge (7,800× g, 1 min, Micromax, Thermo Electron Corporation, Milford, CT, USA). The resulting supernatant was tested with the gill cell line assay in conventional 96-well plates.

## 2.4. Gill Cell Line Assay

The gill cell line viability assays were conducted following the protocols described in detail in [23]. This bioassay allows for the exposure of gill cells to lower salinity samples (<10) in conventional 96-well plates or higher salinity samples in more specialized semi-permeable membrane systems (Corning 3381, Acton, MA, USA). Briefly, for conventional plates, 100 µL of 2 × 10<sup>5</sup> gill cells mL<sup>-1</sup> suspended in L-15 medium were seeded into 96-well plates (Greiner 665180, Kremsmünster, Germany) and allowed to attach for 3 days at 20 °C in the dark. Prior to testing of samples, the L-15 medium was discarded and the cells rinsed twice with 100 µL phosphate buffer saline (PBS). Subsequently, 100 µL of sample was added to each well, and the plates were incubated in the dark for 3.5 h. After exposure, samples were discarded, rinsed twice with PBS, and finally, 100 µL of 5% resazurin (Sigma, 199303, Castle Hill, Australia) in L-15/ex was added. Plates were incubated in the dark for a further 2 h, and gill cell viability was quantified by measuring the metabolic reduction of the

resazurin dye to fluorescent resorufin in a plate reader at excitation and emission wavelengths of 540 and 590 nm, respectively (Fluostar Optima, BMG Labtech, Ortenberg, Germany). Gill cell viability is reported as the percentage of the respective non-toxic control.

### 2.5. Statistics

Model I, two-way analyses of variance (ANOVA) were conducted on the nutrient manipulation data (main effects: nutrient status and treatment). Significant interactions of the main effects were followed by the *post hoc* Tukey HSD test. Where no significant interactions were observed, Welch's T-test was employed to test for treatment (Phoslock™) effect. To satisfy ANOVA assumptions of homogeneity of variance and normality, cell removal data were squared and gill viability data square root transformed. A significance level of 95% ( $\alpha = 0.05$ ) was employed in all analysis, performed with the statistical package R (<http://www.r-project.org/>). Where given in the text, mean values are reported as  $\pm 1$  standard deviation, and error bars in figures represent the standard error ( $n = 3$ ).

## 3. Results

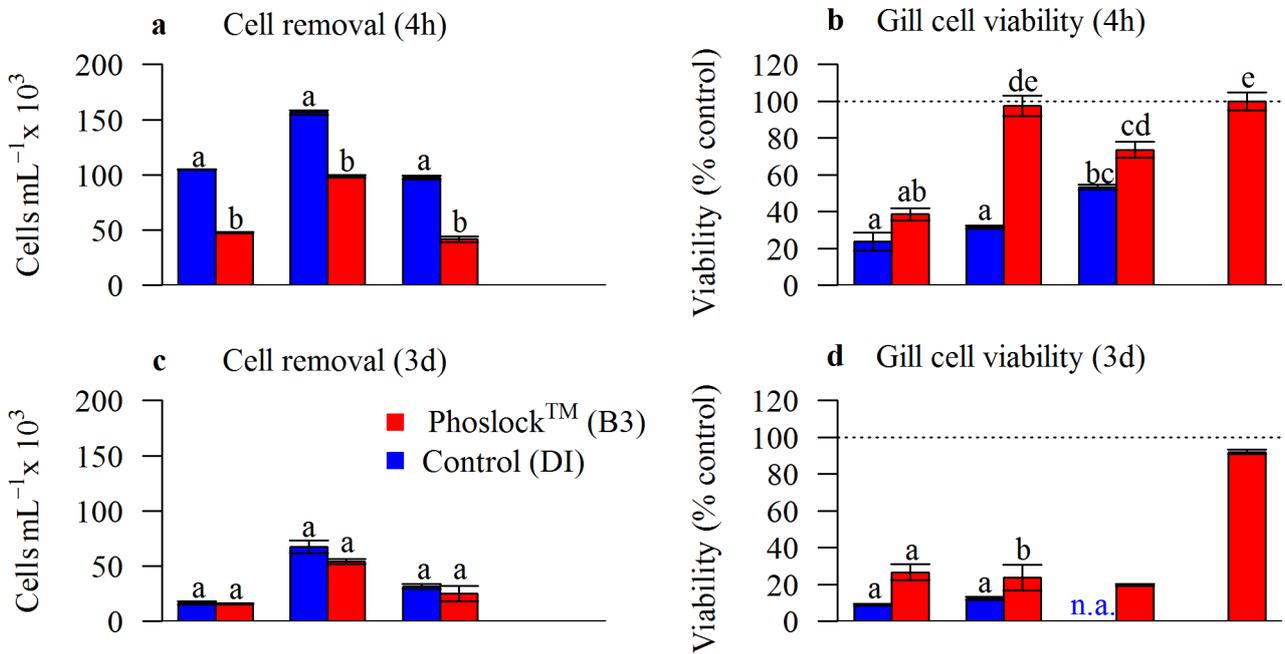
### 3.1. Phoslock™ (B3) Application

#### 3.1.1. Live Cultures

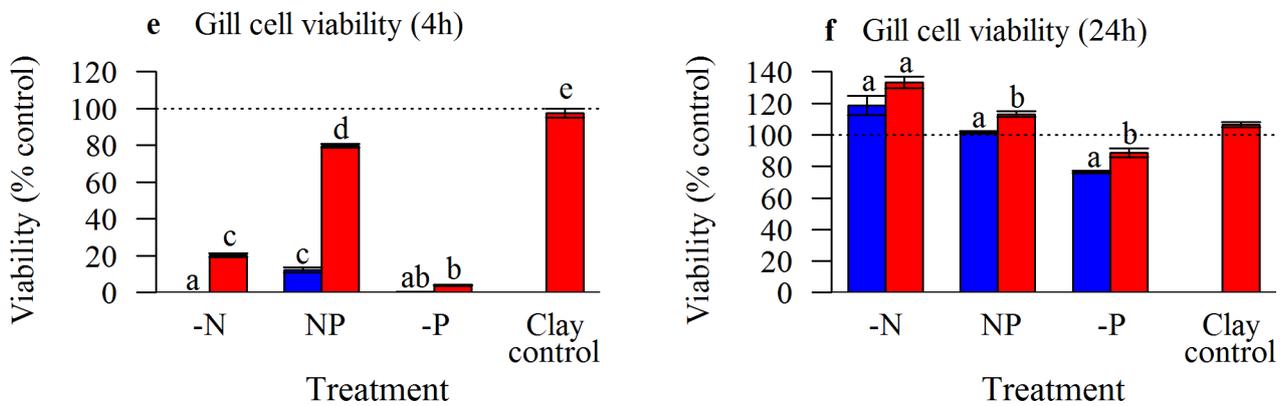
Application of Phoslock™ clay to live *Prymnesium parvum* cultures significantly reduced cell numbers across all nutrient conditions when compared to control cultures after 4 h (Figure 1a). Dilution of experimental cultures 24 h prior to the experiment to  $10^5$  cells  $\text{mL}^{-1}$  did not negatively influence algal cells, as evident from continued exponential growth of cultures, therefore assumed to be nutrient replete ( $1.5 \times 10^5$  cells  $\text{mL}^{-1}$  at the start of the experiment). Cell concentrations in cultures grown in phosphorus- and nitrogen-deficient media remained stationary during the acclimation phase. While nutrient status and initial cell concentration did not influence the extent of cell removal ( $5.7 \times 10^4 \pm 550$  cells  $\text{mL}^{-1}$  removed across all nutrient treatments), they significantly influenced toxicity towards the gill cell line RTgill-W1 (Figure 1b). Control cultures grown in nitrogen-deficient media proved the most toxic towards the gill cells, followed by controls of the nutrient-replete and phosphorus-deficient treatments (with only  $24 \pm 8$ ,  $32 \pm 2$  and  $53 \pm 3\%$  of viable gill cells remaining, respectively). Four hours after Phoslock™ application, no toxicity was detected in the nutrient-replete cultures, and gill viability increased by 15 and 21% compared to the untreated controls in the nitrogen- and phosphorus-deficient treatments, respectively (Figure 1b). However, three days after clay treatment, toxicity was significantly increased in both controls and treated cultures across all nutrient conditions, although the viability of gill cells exposed to Phoslock™-treated samples was still higher than for untreated controls in the case of the nutrient-replete treatment (Figure 1d). Cell concentrations three days after clay application were significantly lower when compared to those 4 h after treatment, with no differences detected between cultures of the control and treatment groups within each nutrient condition (Figure 1c).

Phoslock™ (B3) application to *P. parvum* cultures grown at different N:P

Live Cultures



Lysed Cultures

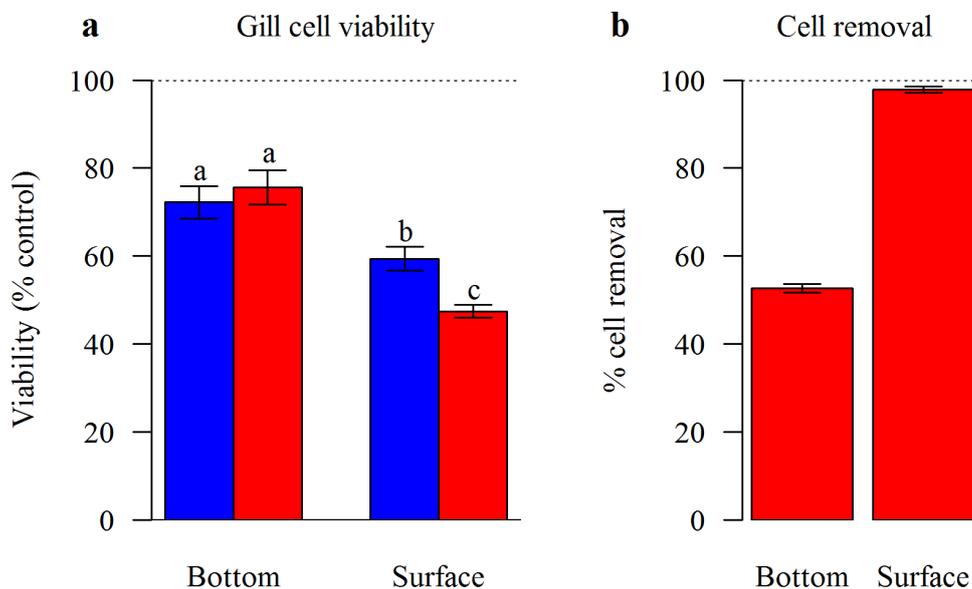


**Figure 1.** Lanthanum-modified bentonite clay (Phoslock™, B3) application to live and lysed *P. parvum* cultures grown under different nutrient conditions (nutrient replete, NP; nitrogen deficient, -N; phosphorus deficient, -P). Phoslock™-treated cultures received 1.5 g L<sup>-1</sup> of clay slurry (red bars) and controls an equivalent volume of deionized water (DI, blue bars). (a) Cell concentration and toxicity towards the gill cells (b) in live cultures 4 h after clay application. (c) Cell concentration in live cultures and toxicity towards the gill cell line RTgill-W1 (d) three days after clay treatment. (e,f) Toxicity of lysed *P. parvum* towards the gill cells 4 and 24 h after clay treatment. Letters represent significant differences between control and clay treatments within each nutrient condition (a,c,d,f, Welch’s T-test) or between all treatment conditions (b,e; Tukey’s HSD). n.a.: not available.

### 3.1.2. Lysed Cultures

Lysis of algal cells through sonication significantly increased toxicity towards the gill cells, with only  $13 \pm 2\%$  viable gill cells remaining in the nutrient replete treatment and no viable gill cells detected after exposure to control cultures of the nutrient-deficient treatments (Figure 1e). Once again, significantly higher gill cell viability was detected in Phoslock™-treated samples, with viability increased to  $20 \pm 2$  and  $80 \pm 2\%$  in the nitrogen-deficient and nutrient-replete treatments, respectively. Gill cell viability in the phosphorus-deficient treatment only increased marginally in response to clay addition ( $+4 \pm 1\%$ ). Twenty four hours after clay application, toxicity in control and clay-treated cultures of the phosphorus-deficient treatment was reduced ( $77 \pm 2$  and  $89 \pm 5\%$  viability, respectively), and no toxicity was detected in algal cultures of the nutrient-replete or nitrogen-limited nutrient treatments (Figure 1f).

Phoslock™ (B3) application to dense, high pH *Prymnesium* cultures

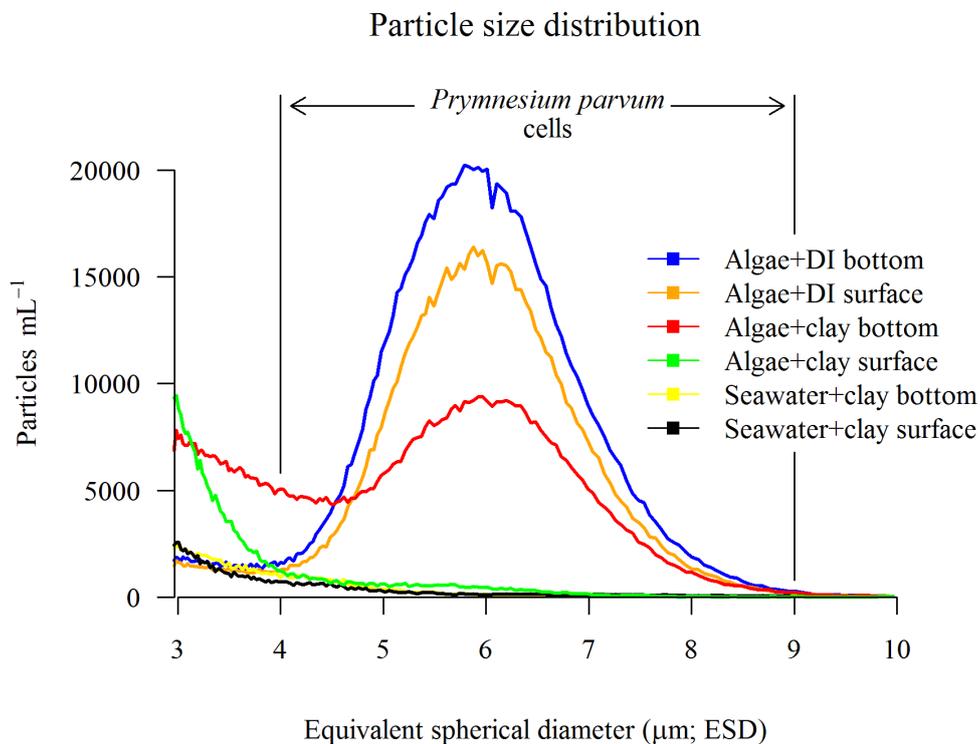


**Figure 2.** (a) Gill cell viability after exposure to high density (surface:  $7.6 \times 10^5$ ; bottom:  $9.8 \times 10^5$  cells  $\text{mL}^{-1}$ ), high pH (9.8) *P. parvum* cultures treated with lanthanum-modified bentonite clay, Phoslock™ (red bars) and deionized water (control; blue bars). Letters indicate statistically significant treatment groups, as in Figure 1 (Tukey’s HSD). (b) Percentage of cell removal efficiency in identical samples, collected 1 cm below the surface and 2 cm above the bottom of a 40-mL test tube.

### 3.1.3. High pH Culture

Phoslock™ (B3) application to high cell density (surface:  $7.6 \times 10^5$ ; bottom:  $9.8 \times 10^5$  cells  $\text{mL}^{-1}$ ), high pH (pH 9.8), live *P. parvum* cultures did not significantly improve gill cell viability (Figure 2a). The change in gill cell viability in response to Phoslock™ treatment was significantly influenced by sample location, as evident from a significant interaction effect between the main effects, treatment and location ( $F_{1,7} = 6.33, p = 0.04$ ). Viability of gill cells exposed to samples collected near the bottom

of the test tube was reduced to an average of  $74 \pm 6\%$ , with no significant differences observed between control or clay treatments (Tukey’s HSD:  $p = 0.88$ ). Surface samples proved more toxic, with the control treatments reducing gill viability to  $59 \pm 5\%$ , and Phoslock™ application decreased gill cell viability to  $48 \pm 3\%$ , despite complete cell removal in these samples ( $98 \pm 1\%$  RE; Figure 2b). A cell removal efficiency of only  $53 \pm 2\%$  was recorded in bottom samples. The particle size distribution in surface and bottom samples followed a normal distribution in the size range of 4–9  $\mu\text{m}$  ESD (equivalent spherical diameter) with a peak at 6  $\mu\text{m}$ , corresponding to the *P. parvum* population (Figure 3). In the lower size range ( $<4 \mu\text{m}$ ), particle counts were considerably higher in the Phoslock™-treated samples (bottom:  $(189 \pm 14) \times 10^3$ , surface:  $(126 \pm 11) \times 10^3$  particles  $\text{mL}^{-1}$ ) than controls (bottom:  $(65 \pm 10) \times 10^3$ , surface:  $(55 \pm 1) \times 10^3$ ).



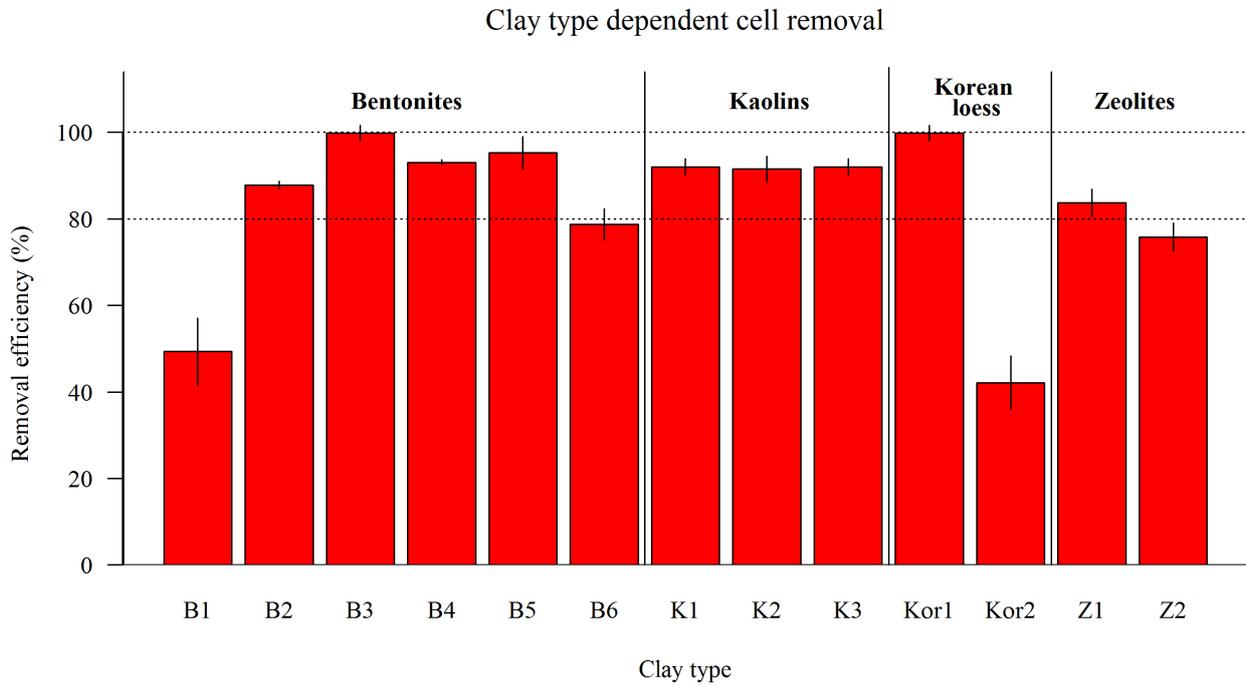
**Figure 3.** Particle size distribution in surface and bottom samples 4 h after Phoslock™ (B3) addition to *P. parvum* cultures. Cultures were treated with  $1 \text{ g L}^{-1}$  Phoslock™ slurry (black and orange lines represent top and bottom samples, respectively) and control cultures with an equal amount of deionized water (DI; red and blue lines represent top and bottom samples, respectively). To facilitate the interpretation of the results, a further control of  $1 \text{ g L}^{-1}$  Phoslock™ clay in seawater (no algae) was employed (yellow and black lines represent bottom and surface samples, respectively). The particle size range corresponding to *P. parvum* cells is indicated (4–9  $\mu\text{m}$  ESD).

### 3.2. Clay Screening for Cell and Ichthyotoxin Removal

#### 3.2.1. Cell Removal

*Prymnesium parvum* cell removal efficiency (RE) differed significantly between individual clay types (ANOVA,  $F_{12,26} = 29.03$ ,  $p < 0.001$ ; Figure 4). While no obvious trend in the clays’ capacity to

remove cells was observed between clay groups, most clays achieved high cell removal ( $\geq 80\%$  RE). Only Korean yellow loess type B (Kor2) and the gypsum-bentonite composite B1-AM (B1) performed poorly (approximately 50% RE).



**Figure 4.** Removal efficiency of *P. parvum* cells by different clay types.

### 3.2.2. Ichthyotoxin Removal

Significant differences between and within clay groups were detected when exposing gill cells to lysed *P. parvum* preparations at pH 7 and 9 (Figure 5), with a two-way ANOVA indicating the significant influence of both main effects and their interaction on gill cell viability (Table 2).

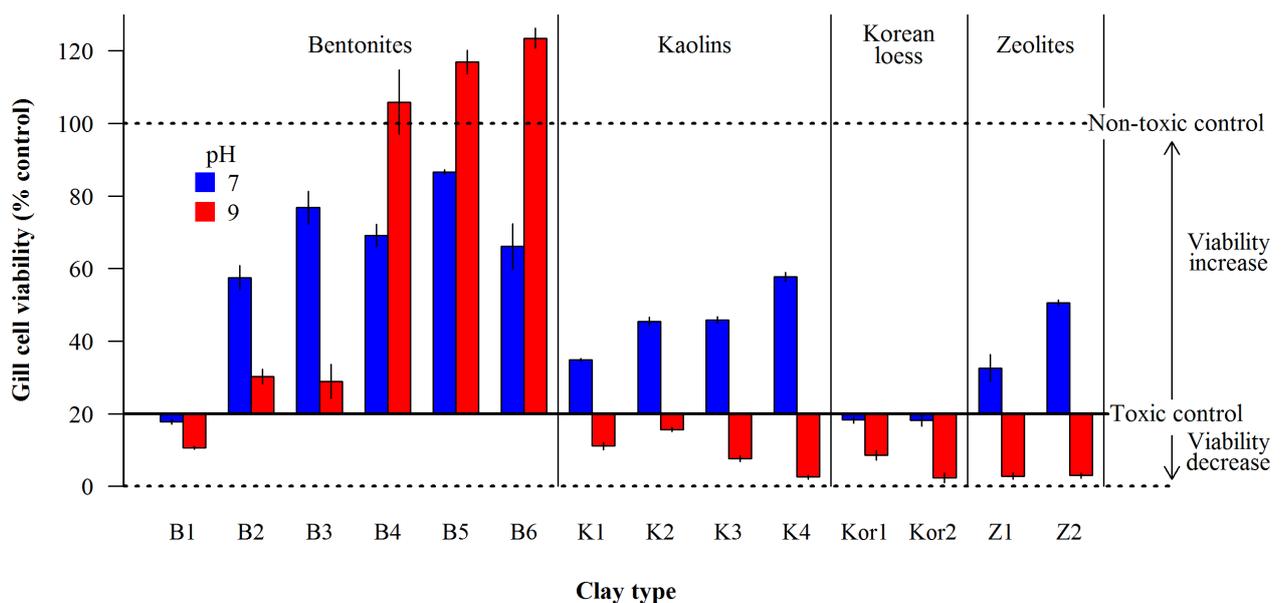
**Table 2.** Two-way analysis of variance (ANOVA) conducted on the main effects of clay type, pH and their interaction on gill cell viability. Degrees of freedom (Df), sum of squares (SS) and mean square values (MS) are provided.

Source	Df	SS	MS	F	p
Clay type	13	452.40	34.80	227.22	<0.001
pH	1	90.60	90.60	591.51	<0.001
Clay type * pH	13	141.00	10.85	70.83	<0.001
Residuals	56	8.60	0.15		

While individual kaolins (K1–4), zeolites (Z1–2) and Korean loess (Kor1–2) behaved similarly within their clay group, large variations in the effect of the different bentonite types on gill cell viability were observed, particularly in response to pH. At pH 7, clay treatment generally increased gill cell viability, with the exception of the Korean loess group (Kor1–2) and the bentonite type B1 (no viability changes). The largest improvements in gill viability relative to the toxic control at pH 7 were

observed when ichthyotoxic preparations were treated with clays from the bentonite group (B2–6; average of +51%), followed by kaolins (K1–4; average of +26%) and zeolites (Z1–2; average of +22% gill viability). While no significant differences in the toxicity of controls were observed between pH 7 and 9 (one-way ANOVA:  $F_{3,1} = 0.69$ ,  $p = 0.468$ ), the influence of clay treatment on the gill cells differed significantly at these two pH values. At pH 9, kaolins, zeolites and Korean loess exacerbated toxicity towards the gill cells, leading to complete viability loss in some cases (K4, Kor2, Z1–2). Similarly, bentonite type B2 (Es) and B3 (Phoslock™), which significantly improved gill viability at pH 7 (+38% and +57% gill cell viability increase, respectively), displayed only minor improvements in gill cell viability at pH 9 (+10% and +9%, respectively). Conversely, bentonite types B4–B6 performed best at pH 9, resulting in complete removal of toxicity towards the gill cells. The viability of gill cells exposed to toxic samples treated with B5 and B6 actually exceeded that of non-toxic controls (seawater + clay). This appears to be due to an interaction of clay and culture medium conditioned during algal growth as opposed to pure seawater (no algae); an observation only recorded for samples that had been rendered non-toxic through clay application.

pH and clay type dependent *Prymnesium parvum* ichthyotoxin adsorption

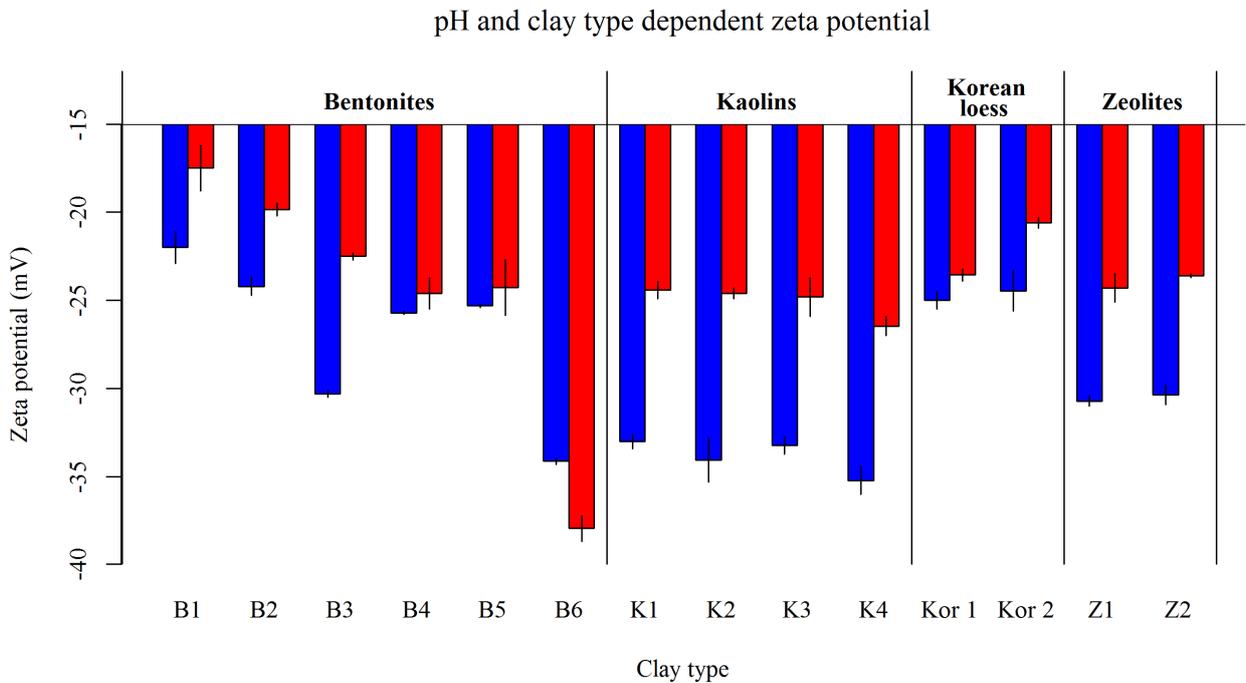


**Figure 5.** Gill cell viability after exposure to ichthyotoxic *P. parvum* preparations treated with different clay types at pH 7 (blue bars) and 9 (red bars). Arrows indicate the direction of fish gill viability change compared to the toxic control (solid black line, 20% viability).

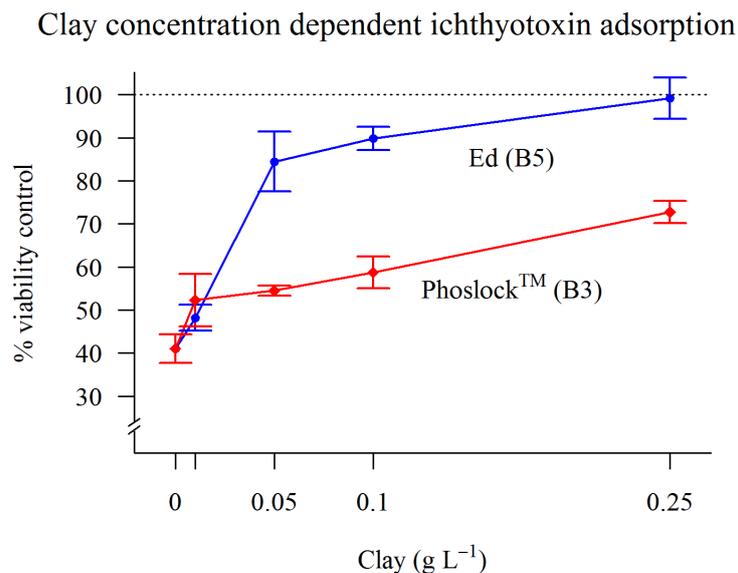
### 3.2.3. Zeta Potential

Zeta potential measurements of clay suspensions revealed negative charges ( $>-15$  mV) for all clay types (Figure 6), with a two-way ANOVA indicating significant interactive effects of clay type and pH on zeta potential ( $F_{13,29} = 11.44$ ,  $p \leq 0.001$ ). Individual kaolins (K1–4), zeolites (Z1–2) and Korean loess (Kor1–2) exhibited similar zeta potentials within their groups and comparable effects of pH on the extent of that charge. At pH 7, kaolins ( $-33.9 \pm 1.0$  mV) displayed the largest negative potential, followed by zeolites ( $-30.5 \pm 0.3$ ) and Korean loess ( $-24.7 \pm 0.4$ ). At pH 9, the extent of the negative

charge was reduced in all three groups when compared to pH 7 (kaolins: +7.8; zeolite: +6.6; Korean loess: +1.9 mV). As previously observed for ichthyotoxin adsorption, the bentonite group showed non-coherent behavior at the two tested pH values. Similarly to the other clay groups, bentonite types B1–3 exhibited less negative charges at pH 9 when compared to pH 7, whereas the zeta potential of B4–5 ( $-25 \pm 0.7$  mV) remained unchanged and the negative charge of B6 increased ( $-34.1$  to  $-38$  mV at pH 9).



**Figure 6.** Zeta potential (mV) of different clay types in L-15/ex medium at pH 7 (blue bars) and pH 9 (red bars).



**Figure 7.** Gill cell viability after exposure to lysed *P. parvum* preparations treated with different concentrations (0–0.25 g L<sup>-1</sup>) of Ed clay (blue circles) and lanthanum-modified bentonite (Phoslock™; red diamonds) at pH 9.2.

### 3.2.4. Minimum Effective Dose

Treatment of lysed *P. parvum* preparations (pH 9.2) with increasing concentrations of Phoslock™-(B3) and Ed- (B5) type clays significantly increased gill cell viability relative to the toxic control ( $41 \pm 7\%$  viability, Figure 7). Ed clay-treated preparations increased gill cell viability to  $85 \pm 14\%$  at a clay loading of only  $0.05 \text{ g L}^{-1}$ , with no viability loss observed at  $0.25 \text{ g L}^{-1}$ . Gill cell viability increased linearly with increasing Phoslock™ concentration, with the highest clay loading of  $0.25 \text{ g L}^{-1}$  only increasing gill cell viability to  $73 \pm 5\%$  (compared to complete removal of toxicity by Ed clay at the same concentration).

## 4. Discussion

### 4.1. Phoslock™ (B3) Application

Nutrient manipulation experiments, conducted to better understand field observations at the Arda-Tek barramundi farm in Northern Australia, proved the tropical *Prymnesium parvum* strain, isolated from these ponds (PPDW02), to be highly toxic towards the gill cell line RTgill-W1. Similar to European *P. parvum* strains [10,12], our tropical strain proved most toxic during the stationary phase when exposed to nutrient limitation. While we cannot exclude the contribution of other environmental factors to the observed increase in toxicity in the nutrient-deficient treatments [6,31] and did not conduct C:N:P analysis, we have shown that lysed cells temporarily released large amounts of toxins that rapidly degraded even in the dark (Figure 1, compare 4- and 24-h toxicity). The increased toxicity of live algal cultures three days after clay application in both treated and control cultures is likely due to the release of ichthyotoxins during culture senescence (Figure 1, compare 4-h and three-day cell counts), highlighting the requirement for complete removal of algal cells during the initial flocculation step. Previous investigations on clay flocculation reported *P. parvum* cell removal to be dependent on the nutrient condition of cells, hence attributing removal of toxicity directly to the removal of algal cells and their intracellular toxins (toxicity measured as hemolytic activity [12,16]). However, employing the sensitive gill cell line assay in the present work, we found no evidence of such a correlation, with identical decreases in cell density, but not toxicity observed across all nutrient conditions. Rather than removing *P. parvum* toxicity by flocculating cells, Phoslock™ (B3) clay appeared to actively adsorb water-soluble *P. parvum* ichthyotoxins, thus presenting a much greater benefit than by just achieving cell or phosphorus removal. While Sengco *et al.* [16] and Hagström and Granéli [12] reported increases in the hemolytic activity of *P. parvum* cultures in response to clay treatment, presumably a stress response of live cells to flocculation, we did not observe such an increase during our nutrient manipulation experiments with diluted cultures ( $1\text{--}1.5 \times 10^5 \text{ cells mL}^{-1}$ , pH 7.7–8.3). However, we did observe significantly increased toxicity in surface samples when high density ( $10^6 \text{ cells mL}^{-1}$ ), high pH (9.8) *Prymnesium* cultures were treated with Phoslock™ clay, despite complete cell removal at the surface (Figure 2). This indicates the release of intracellular toxins. Physical contact between clay particles and algal cells has previously been demonstrated to cause lysis in a range of harmful algal species, ranging from fragile raphidophytes to armored dinoflagellates [19–22]. Observed higher particle counts in the lower size range ( $<4 \mu\text{m}$ ) that neither corresponded to *P. parvum* cells nor clay particles support this suggestion, as these smaller-sized

particles likely represent cell fragments and/or aggregates of cell fragments and clay particles. The reduced toxin adsorption by Phoslock™ clay at higher pH explains its failure to remove intracellular toxins released through algal cell lysis, and therefore, the observed increase in toxicity after clay application. By comparison, the pH of the Australian barramundi ponds, to which Phoslock™ (B3) was successfully applied, was adjusted to below 7.7 [2].

#### 4.2. Clay Screening

Flocculation of *P. parvum* cells presents an important benefit of clay application, since continuous ichthyotoxin production of cells remaining after treatment would require subsequent clay additions. Previous clay screening for flocculation purposes of *P. parvum* cells by Sengco *et al.* [16] showed that bentonite-type clays were best suited for *P. parvum* cell removal, albeit with a removal efficiency of only 17.5% by natural clays at cell concentrations comparable to our present work ( $10^5$  cells mL<sup>-1</sup>). While the authors were able to achieve complete removal through the addition of polyaluminum chloride [12,16], nearly all representatives of the four clay groups assayed in the present work were able to achieve high removal efficiencies ( $\geq 80\%$ ) without the addition of chemical flocculants. Critically, cell removal proved to be a poor predictor of ichthyotoxin removal for which significant differences between clay groups at pH 7 and 9 were observed (Figure 6).

The practical implications of the pH dependence of ichthyotoxin adsorption are clearly reflected in the comparative minimum effective dosages of Phoslock™- (B3) and Ed- (B5) type clay required to completely remove the ichthyotoxic effects of lysed *P. parvum* preparations at the ecologically relevant pH of 9.2 (Figure 7). Previously discussed observations of continuous ichthyotoxin production and release of intracellular toxins through clay particle collision-mediated cell lysis highlight the importance of screening clays not just for cell flocculation, but also their ichthyotoxin adsorptive properties.

The toxin adsorptive characteristics of clay have previously only been described for bentonite-type clays [11,16–18], employed because of their high cell removal rather than ichthyotoxin adsorptive capabilities. Our detailed screening of different clay groups revealed that in the case of *P. parvum* ichthyotoxins, these toxin adsorptive properties are not only restricted to bentonite, but also encompass to a certain extent zeolite- and kaolin-type clays at a pH of 7. The weaker adsorption capacity of kaolin compared to bentonite has been attributed to the comparatively higher cation exchange and swelling capacity of bentonites, which not only allows molecules to be incorporated into the interlayer spaces, but also greatly increases the surface area available for absorption [25,26]. While zeolites, similarly to bentonites, exhibit a high cation exchange capacity and possess a large surface area due to their porous structure, they are non-swelling. Interestingly, the low swelling bentonite preparation (B1) also failed to remove any ichthyotoxins. However, when considering the large variation in cell flocculation efficiencies reported in the literature between major clay types [20,22,32], it becomes increasingly clear that the performance of individual clays does not reflect the general characteristics of the clay group. A large proportion of this observed variation may be due to the different levels of impurities present in available commercial clays employed in most studies (e.g., Table 1). Based on the lower ichthyotoxin removal at pH 9 by all clay minerals tested here (except B4–6), it appears likely that pH-dependent changes in the protonation status of toxin molecules and/or the pH-dependent surface

charge of clay minerals is driving their adsorption kinetics [25–27,33]. Unfortunately, due to the poorly understood chemical nature of *P. parvum* ichthyotoxins, the contribution of the ionization state of toxin molecules cannot yet be fully resolved.

Valuable insight into the pH dependence of the adsorption process can be gained through interpretation of zeta potential measurements, an approximation of surface charge that governs the behavior of clay particles in suspension [34–36]. Clay particles are inherently negatively charged due to isomorphic substitutions in their crystal lattice and exhibit pH-dependent charges on their edges [37,38]. Deprotonation of edge sites with increasing pH gives rise to a more negative zeta potential of clays in water [39]. However, the presence of multiple charged cations of alkali earth and/or heavy metals can result in the opposite trend (in pH ranging from 7 to 10), if such cations are present either in the dispersant or on the clay surface [34,35,39]. The reason for this behavior is increased particle aggregation due to the surface coating of clay particles with insoluble carbonates at higher pH. Therefore, the drop in the absolute zeta potential value between pH 7 and 9 was expected for the majority of clays and provided by the presence of CaCl<sub>2</sub> in the L-15/ex medium [29]. The zeta potential of sodium-rich clays has been shown to decrease with pH (7 to 10) due to higher dispersal of individual clay particles [34]. Accordingly, the zeta potential of the Na-bentonite B6 was observed to be greater at pH 9 than at pH 7. Another Na-bentonite, Phoslock™ (B3), has been modified with La<sup>3+</sup> by the manufacturer, a trivalent cation that is known to form insoluble compounds under alkaline conditions [15]. This accounts for the large observed difference in zeta potential between pH 7 and 9 (+8 mV) in this special case.

The three bentonites B4–B6 were the only clays to consistently achieve high ichthyotoxin removal at both pH 7 and 9. Interestingly, the calcium-bentonite B2, which originates from the same clay deposit as B4 and B5, failed to efficiently reduce toxicity and exhibited a much reduced surface charge at pH 9. The only difference between these clays is the depth from which they were mined. B4 and B5 originated from the lower layers of the deposit, whereas B2 was mined from the surface and subject to weathering processes that appear to have significantly reduced its capacity to adsorb *P. parvum* ichthyotoxins.

Similarly to B2, all other clays, including Phoslock™ (B3), which exhibited lower zeta potentials at pH 9, displayed lower toxin adsorption (B3) or even exacerbated ichthyotoxicity (kaolins, zeolites and Korean loess) at pH 9, despite exhibiting zeta potentials comparable to the high adsorbing bentonites (B4–6). While we observed no direct correlation between the extent of zeta potential and ichthyotoxin adsorption, changes in clay charge at pH 7 and 9 appear to be consistent with changes in *P. parvum* ichthyotoxin adsorption.

## 5. Conclusion

Our detailed screening of fourteen different individual clays, representing four major clay mineral groups, demonstrates that the potential of clays as mitigation agents of fish-killing *Prymnesium parvum* blooms in aquaculture ponds extends beyond their phosphorus removal (valid for Phoslock™) and cell flocculating properties. Ichthyotoxin adsorption offers an important additional benefit to clay application through the removal of extracellular, as well as intracellular ichthyotoxins. This presents an important addition to the global effort to mitigate ichthyotoxic effects of *P. parvum*, not only in

Australia, but also abroad. Unweathered, unmodified bentonites proved to be better suited for ichthyotoxin adsorption purposes than Phoslock™ at higher pH (9–9.8), since lower concentrations were required for complete removal of ichthyotoxicity. The removal of *P. parvum* cells remains imperative to eliminate the source of continued ichthyotoxin production, but care should be taken to select clays not solely based on their flocculating, but also their ichthyotoxin adsorptive properties to efficiently combat extracellular, as well as intracellular ichthyotoxins released during the initial flocculation event through physical damage by clay particles. While reduction of aquaculture pond pH through the stimulation of microbial growth by the addition of molasses could greatly increase the ichthyotoxin adsorption efficiency of Phoslock™ clay, unmodified bentonites represent a more cost-effective alternative to the specialized lanthanum-modified Phoslock™ clay. Due to their world-wide application in a range of commercial applications, unmodified, unweathered bentonite-type clays are easily accessible to local fish farmers and represent a great potential on-farm emergency response tool.

Cell flocculation proved to be a poor indicator of ichthyotoxin adsorption, the extent of which cannot be explained by the major clay mineral group present, but rather through understanding of the physical and chemical characteristics of individual clays. Zeta potential measurements proved to be a good first step towards a more detailed understanding of ichthyotoxin adsorptive properties of clay minerals, revealing that pH-induced changes in clay zeta potential can be indicative of changes in *P. parvum* ichthyotoxin adsorption. More detailed knowledge of the chemical composition of clay minerals and well-characterized ichthyotoxins (e.g., karlotoxins, brevetoxins) will potentially provide valuable insight into the adsorption kinetics of different clay types to facilitate an efficient selection of local clays for cost-effective mitigation purposes in the future.

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### **Author Contributions**

Ideas and experiments were conceived of by A.S. and G.H. and conducted by A.S. and J.J.D.-A. Statistical analyses were performed and the manuscript was written by A.S. A.B. and T.G.P. provided valuable discussion of practical experiences and applications regarding aquaculture pond management and clay application in the field. A.P. supervised the zeta potential measurements and, together with A.R.P., provided valuable discussion of clay/ichthyotoxin kinetics. M.N.M. supervised the Coulter Counter measurements and contributed to the experimental design and data interpretation. All authors discussed the results and their implications and commented on the manuscript at all stages.

### **Conflicts of Interest**

The authors declare no conflict of interest.

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