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Research Article

Protozoa-contaminated microalgae treated with bleach can be safely used as feed for growing oyster larvae

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ABSTRACT. Protozoa contamination of microalgal cultures is a recurring issue in laboratories and bivalve hatcheries, often leading to significant schedule alterations and economic losses. This study evaluated the effectiveness of bleach (sodium hypochlorite) treatment in eliminating *Uronema*-like ciliates from cultures of *Tisochrysis lutea*. It assessed its impact on microalgal survival and integrity, as well as its potential use as feed for oyster (*Crassostrea gigas*) larvae. Growth, pediveliger yield, and metamorphic success of oyster larvae were assessed in 500 L culture tanks using both live and bleach-treated microalgae as feed. Bleach concentration significantly decreased within 4 min of addition to seawater, with the dissipation rate being highest in the presence of *T. lutea*. Aeration had no significant effect on microalgal cell density, regardless of whether the cells were treated with bleach, in both small (1 L) and large (500 L) culture volumes. Notably, 60 or 120 min of exposure to 375 and 750 μL^{-1} bleach L^{-1} effectively diminished the protozoa population without compromising the integrity of the microalgal cells. Oyster larvae fed on bleach-treated microalgae (375 $\mu L\ L^{-1}$) exhibited a slower shell growth rate (7.2 $\mu m\ d^{-1}$) and longer metamorphic period (8 days) than control larvae fed live microalgae (8.5 $\mu m\ d^{-1}$ and 5 days, respectively). In contrast, the yield of metamorphosed larvae was higher in bleach-treated larvae. These results indicate that bleach, when followed by neutralization, can be safely used to eliminate protozoa in microalgal cultures and further use them as feed for rearing oyster larvae, offering a practical, effective, and inexpensive solution for hatcheries facing potential microalgal crashes due to protozoa contamination.

Keywords: *Crassostrea gigas*; larval growth; microalgal chlorination; protozoa contamination; microalgae culture

INTRODUCTION

The productivity of a bivalve hatchery depends on the quantity and quality of live microalgae needed by all developmental stages. Depending on the culture methods and production scale, microalgal production typically accounts for approximately 40% of total operational costs in a hatchery (Coutteau & Sorgeloos 1992, Das et al. 2012). Despite efforts to replace live microalgae with inert feeds, aquaculture still relies on microalgae as the main food source (De Pauw et al. 1984), and maintaining healthy cultures is essential for the success of any bivalve hatchery (Guedes & Malcata 2012). Therefore, in addition to controlling environmental variables such as light, temperature, salinity, pH, and nutrients at optimal levels, the control or eradication of potential predators contaminating the microalgal cultures must be continuously observed (Trotta 1981).

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Parasites, including amoebas, fungi, bacteria, and ciliated protozoa, can contribute to reducing cell concentrations and may even lead to the collapse of microalgal cultures. In this regard, Han et al. (2013) reported fungal contamination (chytrids) in cultures of the microalga *Haematococcus pluvialis* as one of the biggest difficulties in the commercial production of astaxanthin. Significant biomass reduction of the freshwater microalga *Chlorella kessleri*, cultured for biodiesel production, was also reported when it became contaminated with rotifers (Park et al. 2016). Likewise, ciliate contamination of the diatom *Dunaliella salina* in outdoor culture ponds resulted in a significant decrease in cell density within 2 to 3 days (Moreno-Garrido & Cañavate 2001). Ciliates such as *Cohnilembus reniformis*, *Uronema marinum*, *Euplotes* sp., and *E. vannus* are common contaminants of marine microalgal cultures (Day et al. 2012). In general, protozoa are very efficient at destroying microalgal cultures but offer little or no nutritional value to bivalve larvae (Baptist 1993). Ciliates are known to feed primarily on bacteria, but some species, such as *Uronema* sp., also actively prey on small microalgae, including *Isochrysis galbana*, *Pyrenomonas salina*, *Mantoniella squamata*, and *Synechococcus* sp. (Strom & Morello 1998), which notably affects microalgal growth (Schaafsma & Peperzak 2013). When contaminated microalgae are inadvertently used for feeding molluscan larvae, it usually results in massive mortalities and interrupted production schemes (Carney & Lane 2014).

Efforts to prevent or eliminate predation of microalgal cultures include chemical, physical, or biological methods. A simple approach commonly employed when microalgal cultures get contaminated with protozoa is to harvest them and discard them immediately (Baptist 1993). Another alternative is the use of chemical biocides, which have proven effective in eliminating protozoa but are potentially hazardous for human or animal consumption (Molina et al. 2019). Thus, Pouneva (2006) used abscisic acid (ABA), a phytohormone produced by certain plant species and even microalgae, which resulted in the effective eradication of parasites. Likewise, Moreno-Garrido & Cañavate (2001) found that 12 to 14 mg L^{-1} of quinine sulfate applied over 24 h eliminated ciliate protozoa contaminating *D. salina* outdoor cultures without harming the microalgae. Germicidal ultraviolet radiation subtype C (UVC) has also been employed to eliminate parasites; however, its effectiveness in microalgae cultures has not yet been tested (Liebich et al. 2012). Cell filtrates or lysates from the Monodopsidaceae microalga *Nannochloropsis oceanica* were also used as effective growth inhibitors of the ciliates *Uronema marinum*, *Euplotes vannus*, and *Litonotus* sp. (Zhao et al. 2021).

Additionally, supplying 400 mg L^{-1} NH_4HCO_3 to protozoa-contaminated algal cultures combined with a 24 h delay for resumption of aeration reduced the amount of ciliated protozoa, including *Uronema* sp., without appreciably affecting the growth in either freshwater (He et al. 2021) or marine microalgal cultures (He et al. 2022). Holm et al. (2008) used sonication to eliminate predators in algal cultures. Although this method could damage cells or destroy algal cell walls, it is not feasible for massive cultures due to economic constraints.

Sodium hypochlorite (bleach) is commonly used in various aquaculture procedures, but it must be applied carefully due to its potential harmful effects on aquatic organisms. Total chlorine refers to the free and combined chlorine. Free chlorine includes the hypochlorite ion (ClO^-) and hypochlorous acid (HClO), the latter exhibiting stronger sterilizing properties. Combined chlorine is formed from mono-, di-, tri-, and organic chloramines. Chlorine demand is defined as the difference between the amount of chlorine required for a specific disinfection procedure and the residual chlorine remaining in the water after disinfection has occurred. Jenkins & Snoeyink (1987) identified factors that influence chlorine demand in water bodies, including the effects of (1) the chemical compounds formed by inorganic elements, (2) those produced with ammonia, and (3) organic compounds that contribute to chlorine ¨assimilation¨. To date, only a handful of studies have investigated the effect of bleach on protozoa commonly present in microalgal cultures or its dissipation pattern in seawater or freshwater. In this regard, Eppley et al. (1976) observed that in both filtered and unfiltered seawater, there was an initial accelerated decrease in free chlorine (35%, from 0.1 to 0.065 mg L^{-1}) and residual chlorine (41%, from 1 to 0.59 mg L^{-1}) within the first 5 min, followed by a slower reduction. They did not detect a reduction of chlorine levels in UV-treated seawater. They concluded that the elimination of organic matter and/or the oxidation of metal ions by UV light removed the molecular species that reacted with chlorine. Park et al. (2016) observed a 42% dissipation of chlorine within 8 min (from 1 to 0.58 mg L^{-1}) due to the presence of freshwater and both microalgal cells and rotifers (*Brachionus calyciflorus*) contaminating the microalgal cultures. Their results indicated that a chlorine dosage of 0.45 to 0.6 mg L^{-1} administered at 2-h intervals maintained the necessary

residual chlorine concentrations to inhibit rotifers without disturbing algal growth in pond cultures, thereby preventing algal crashes.

Due to the need for effective and inexpensive protocols to eradicate protozoans from microalgal cultures and to prevent the loss of entire production batches of bivalve larvae and spat (Carney & Lane 2014), the present study aimed to test the effectiveness of bleach in destroying ciliated protozoa present in microalgal cultures. It also intended to test the adequacy of bleach-treated microalgae as inert feed for the Pacific oyster, *Crassostrea gigas*, throughout larval development and metamorphosis. The working hypothesis was that while bleach-treated microalgae contaminated with protozoa may lose motility and viability, they can still be filtered out, digested, and their nutrients absorbed and incorporated by molluscan larvae, allowing for sustained healthy growth and development.

MATERIALS AND METHODS

Microalgal cultures and chemicals

Parallel cultures of *Tisochrysis lutea* (*Tl*), *Pavlova pinguis* (*Pp*), and *Chaetoceros calcitrans* (*Cc*) were maintained in 500 L plastic bags using the flow-through SeaCAPS® system. Cultures identified as "clean" (free of protozoa) were continuously harvested at a rate of 30-40 L d^{-1} and used for preliminary experiments involving aeration and bleach concentration (exps. 1 and 2). Some culture bags became contaminated with a *Uronema*-like ciliated protozoa (Day et al. 2012, Zhao et al. 2021, He et al. 2022) and were labeled "contaminated". The latter cultures were employed, alongside clean ones, to evaluate the effects of bleach on protozoa/microalgae integrity (assay 3) and to assess the suitability of bleach-treated microalgae as larval feed (exp. 4). The first three experiments described below were conducted using *Tl* as the test microalga model. In contrast, all three species were included in the final experiment. Notably, visual assessments revealed that all three species maintained their cell integrity when used as feed in experiment 4.

Bleach-treatment assays were conducted using commercially available sodium hypochlorite (Clorox®, 5.25% chlorine). Following each exposure period in experiments 1-4, chlorine was neutralized by adding 0.3 mL of 1N sodium thiosulfate ($Na_2S_2O_3$) per each milliliter of bleach (Castagna et al. 1996). Chlorine concentrations were measured colorimetrically (exp. 2) in 2.5 mL vials using diethyl-p-phenylenediamine reagent powder pillows (Hach method 8167, 0-2 mg L^{-1} range), with appropriate dilutions. Absorbance was read in a HACH DR/890 spectrophotometer, and deionized water was used for calibration. The actual chlorine concentration of the commercial bleach solution was confirmed to be 5% (w/v). All microalgal cell densities (exps. 1-4) were quantified using a Multisizer 3 Coulter Counter (Beckman Coulter, CA, USA).

Short-term effects of aeration on microalgae exposed to bleach (exp. 1)

The effect of aeration (bubbling at bottom) on short-term cell density changes (initial 3,883 cells μL^{-1}) of clean *Tl* cultures exposed to 188 µL bleach L^{-1} (ca. 9.4 mg chlorine L^{-1}) was evaluated in six 1 L plastic jars. This initial bleach concentration was chosen based on previous studies, which indicated that a concentration of 10 mg chlorine L^{-1} eliminates approximately 80% of the ciliate *Colpoda* sp. after 24 h while maintaining the growth capacity of the microalga *Chlorella vulgaris* (Wang et al. 2017). The appropriate amount of sodium thiosulfate was added to each jar after 30 min to neutralize the bleach. Following homogenization with a plastic stirrer, aeration was applied to three jars, while the remaining jars were left without aeration (control). After 30, 60, and 90 min, microalgal samples were collected to assess cell density using the Multisizer 3 Coulter Counter.

Short-term effects of bleach concentration on seawater and microalgal cell density (exp. 2)

Time-dependent changes of microalgal density and bleach concentration in seawater were assessed without aeration in bleach-treated *Tl* cultures in 9 plastic jars (1 L) containing clean *Tl* cultures (initial density = 5,227 cells μL^{-1}). An amount of 188 µL of bleach was added to each of the three microalgal jars, and three received 375 µL L^{-1}, to evaluate whether a higher bleach concentration affected microalgal cell density. The three remaining jars contained only seawater (control) and received 188 µL of bleach. All jars were gently homogenized with a perforated stirrer after the addition of bleach and left undisturbed at room temperature without aeration. Microalgal (treatments) and water samples (control) from all jars were obtained after 4, 30, 60, and 120 min from the beginning of the experiment, and chlorine concentration was determined for each sampling point as detailed above.

Bleach effects on protozoa integrity (exp. 3)

An exploratory assay was implemented in 1-L jars to qualitatively (visually) assess the integrity of both

protozoa and microalga after treatment with three bleach concentrations (188, 375 and 750 µL L^{-1}) and three exposure periods (30, 60, and 120 min) without replication of protozoa-contaminated (ca. 10,800 ± 0.700 protozoa mL^{-1}) *Tl* cultures (3,500 ± 44 cells μL^{-1}). Jars were neutralized with Na-thiosulfate following each exposure period. The integrity of protozoa and microalgae was evaluated under a light microscope (10x) using a 1 mL sample from each jar and a Sedgwick-Rafter chamber after 24 h. Protozoa present in five randomly selected squares of 1×1×1 mm (length×width×depth) were scored as digested/empty (incomplete cell or empty cytoplasm) or undigested (undisrupted cell). At the same time, microalgae were considered active if they maintained their motility. Numbers were translated into percentages by dividing the number of organisms of each category by the total number of organisms observed in the chamber.

Crassostrea gigas growth assay (exp. 4)

Microalgae

Based on the previous microalgal and protozoa assays, it was decided to evaluate the long-term integrity of bleach-treated microalgae in six 500-L cylindroconical fiberglass tanks. Three tanks containing protozoa-contaminated microalgae (73 ± 1 *Tl* cells μL^{-1}) were exposed to 375 µL bleach L^{-1} and neutralized with sodium thiosulfate after 1 h. Aeration was introduced to all experimental tanks, including three control (clean) microalgal treatments (77 ± 2 *Tl* cells μL^{-1}) with no bleach treatment. Cell counts were recorded in samples obtained from each tank at times 0, 24, and 48 h. The temperature was kept at 19.2 ± 0.3°C during the experiment. Relative algal cell density was estimated at each sampling time as follows:

$$\text{Relative algal cell density (\%)} = C_t \times 100 / C_0 \quad (1)$$

where C_0 and C_t are the algal cell density (cells μL^{-1}) at time zero and time t, respectively.

Larval growth, survival, and metamorphosis

Once the integrity of *Tl* in 500 L tanks was validated, a last assay was implemented using all three algal species (*Tl*, *Pp*, *Cc*) as feed for oyster larvae (*C. gigas*) as follow: six 500-L cone-bottom cylindrical fiberglass tanks with 1µm filtered seawater were stocked at an initial density of 10 "D" oyster larvae mL^{-1} (24 h post-fertilization) obtained from a temperature-induced spawn (Helm et al. 2004) of 24 adult *C. gigas* oysters. Three tanks were used to test the effects of chlorine-treated microalgal feed (Ch) on larval survival, growth, and metamorphic success, while three additional tanks served as controls (Ctrl) and were fed with live microalgae. Microalgal cell densities and proportions of species given to *C. gigas* larvae are detailed in Table 1. Before feeding, the microalgal species with known cell densities were daily harvested and combined in two 20 L plastic containers. One container was treated for one hour with 375 µL L^{-1} of commercial bleach (Clorox), followed by neutralization with sodium thiosulfate, while the other remained untreated. After 1 h of neutralization, the appropriate volume of both bleach-treated and non-treated algae was added as feed for *C. gigas* larvae in the 500-L cylindroconical rearing tanks, according to the daily target microalgal cell densities. The larval feeding regime (Table 1) consisted of a 50:50 mix of *Tl* and *Pp* for the first 12 days, a 47:47:6 mix of *Tl* + *Pp* + *Cc* from days 13 to 26, and a 33:33:33 mix of all three species for the final eight days.

Larval food density was adjusted daily based on cell counts from experimental tanks and varied according to larval stage, ranging from 20 cells μL^{-1} for D-larvae to 70 cells μL^{-1} for the pediveliger stage (Table 1). Seawater was maintained at 25°C using digitally controlled heaters, and full water exchanges were conducted three times a week. Larvae were retained on screens of appropriate mesh size (55-240 µm) for 35 to 38 days. On day 24, the larvae were culled out, and those retained on the 125-µm mesh screen were returned to the experimental tanks at an adjusted density of 0.6 larvae mL^{-1}. Meanwhile, those smaller than 125 µm were transferred to non-experimental tanks.

Larvae from each tank were sampled daily during the first five days, on day 10, and then every three days thereafter. The shell length (antero-posterior axis parallel to the hinge line) of 15 to 30 organisms was recorded under a light microscope (10x). The mean shell length from three replicates was plotted against time, and the larval growth rate was estimated as the slope of the linear regression line of shell length *vs*. time. Estimation of live larvae was assessed on days 6, 14, and 31 by concentrating and transferring larvae from each tank to 20-L graduated plastic containers. The number of live larvae was scored under a light microscope (10x) using one mL subsamples and Sedgewick-Rafter chambers. Percent survival (S) was calculated from direct counts of each tank using the following equation:

$$S = (Nt / Ni) \times 100 \quad (2)$$

where N_t and N_i are the number of larvae in the tanks at time t and the initial number, respectively.

Table 1. Microalgal densities fed to Pacific oyster, *Crassostrea gigas*, larvae reared in 500-L cylindroconical tanks. Live and bleach-treated combinations of *Tisochrysis luthea* (*Tl*), *Pavlova pinguis* (*Pp*), and *Chaetoceros calcitrans* (*Cc*) were offered at different proportions during larval development: 50*Tl*: 50*Cc* (days 1-11); 47*Tl*: 47*Cc*: 6*Pp* (days 13-26); 33*Tl*: 33*Cc*: 33*Pp* (days 27-35).

	Cell density (cells µL^{-1}) of live microalgal mixture (control)							
	20	25	35	50	60	30	40	60
Larval age (days)	1-3	4-6	7-11	12-16	17-22	23-25	26-29	30-34
	Cell density (cells µL^{-1}) of bleach-treated microalgal mixture							
	20	30	35	45	70	80	40	70
Larval age (days)	1-3	4-6	7-10	11-16	17-21	22-23	24-27	28-37

Settlement/metamorphosis of pediveliger larvae was induced with freshly prepared 10^{-4} M ± epinephrine hydrochloride ($C_9H_{13}NO_3 \cdot HCl$, product E4642 Merck Millipore), applied for 2 h. Responsive, metamorphosing larvae/postlarvae were transferred to a floating PVC tray covered with nytex screen (bottom) and maintained under a downwelling water flow produced with air lifts (Helm et al. 2004) inside a rectangular tank. Non-responsive pediveliger larvae were transferred back to cylindroconical tanks and treated again the following day with epinephrine. Epinephrine treatment continued on consecutive days until a minimal amount of remaining non-responsive larvae was discarded.

Statistics

Samples from all experimental treatments were tested for normality and homogeneity of variance. In cases where normality assumptions were not met, the data were log-transformed to the base 10. Algal concentration percentages were arcsine transformed before conducting the statistical tests. The effects of chlorine concentration and exposure period were analyzed using a two-way ANOVA. When no significant differences were found, treatments at each time point were compared using an independent *t*-test. If significant differences were detected, Tukey's test was applied to assess differences between treatments. The slope of the linear regression line obtained between shell length (µm) and age (days) was considered as the shell growth rate (Ferreira-Arrieta et al. 2015). The statistical software Jamovi, version 2.5 (The Jamovi project, 2023), was used to perform statistical tests and analyses.

RESULTS

Short-term effects of aeration on microalgae exposed to bleach (exp. 1)

Both aerated and control (no air) jars exhibited the same short-term pattern (ANOVA = 1.15, $P > 0.05$) and magnitude of mean cell density changes in the presence of bleach (188 µL bleach L^{-1}). Only time had a significant effect (ANOVA = 97.60, $P < 0.001$) over microalgal concentration with a sharp 13% decrease observed after 30 min (Fig. 1). Cell density remained relatively constant (3,316 ± 83 cells µL^{-1}) in non-aerated (control) jars afterwards, while continuously decreased at a rate of ca. 110 cells µL^{-1} h^{-1} in aerated jars over the next hour (Fig. 1).

Short-term effects of bleach concentration on seawater and microalgal cell density (exp. 2)

Both dosage (ANOVA = 1879, $P < 0.001$) and exposure time (ANOVA = 9965, $P < 0.001$) had a significant effect on the chemical behavior of chlorine. Chlorine concentration in seawater containing microalgae (5,227 cells µL^{-1}) significantly decreased within the first 4 min of bleach addition (Fig. 2a). It decreased by 65% (6.50 ± 0.32 mg chlorine L^{-1}) at the highest dose, followed by 56% (4.13 ± 0.17 mg chlorine L^{-1}) at lowest dose and 25% (7.01 ± 0.00 mg chlorine L^{-1}) at lowest dose without microalgae (control). Microalgal cell density matched the temporal pattern described above and chlorine exposure time had a significant effect (ANOVA = 119.02, $P < 0.001$) on microalgal density, such that within 4 min a significant 22% decrease (4,062 ± 120 cells µL^{-1}) was recorded at the highest bleach concentration (375 µL bleach L^{-1}), followed by a 13% reduction (4,555 ± 490 cells µL^{-1}) at 188 µL bleach L^{-1} (Fig. 2b). Both bleach concentration and

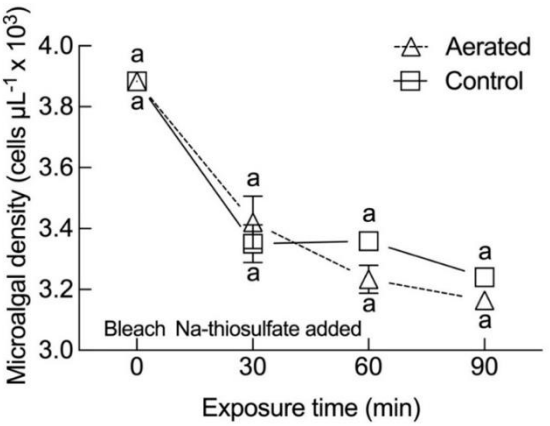


Figure 1. Short-term changes of mean microalgal (*Tisochrysis lutea*) cell density in aerated 1-L jars exposed to 188 µL bleach L^{-1}. Standard error bars are shown in each case. Initial algal concentration was 3,883 cells μL^{-1}. Symbols with the same letter above/below indicate no statistical differences between treatments within a given exposure period.

microalgal cell density reached mean asymptotic values afterwards (Fig. 2a-b). It is noteworthy that a color gradient was observed in jars containing microalgae after treatment with bleach (Fig. 2c), with lighter brownish tones developing as the bleach concentration increased (Fig. 2c).

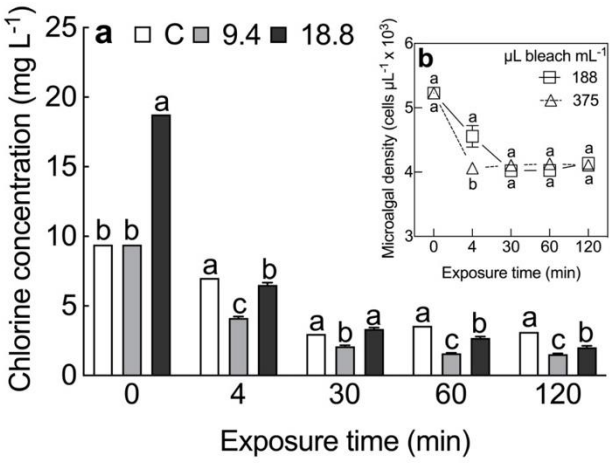
Protozoa assay (exp. 3)

Protozoa evaluations carried out after 24 h in treatments using all three bleach concentrations and exposure period of 30 min resulted in 50 to 60% digested/empty organisms in treatments with 188 and 375 µL bleach L^{-1} (Fig. 3a). At the same time, 80% protozoas were digested/empty with 750 µL bleach L^{-1} (Table 2). Longer exposure periods (60 or 120 min) increased the number of digested/empty protozoans to 80-85% at all three bleach concentrations. They caused the formation of a few too many lumps of dead microalgae (Fig. 3b, Table 2). Non-digested protozoans accounted for 15 to 20% of the total, with most remaining static (without movement) at the 24-h evaluation period.

Crassostrea gigas growth assay (exp. 4)

Microalgae

Microalgae exposed to bleach for one hour (375 µL L^{-1} seawater) in 500 L cylindroconical tanks followed by neutralization and continuous aeration exhibited a non-significant reduction of cell density after one or two days when compared to the control treatment (*t*- Student,



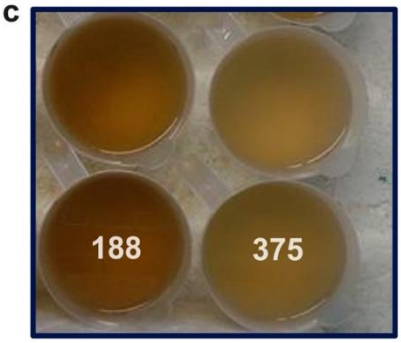


Figure 2. Short-term changes of a) chlorine concentration, b) microalgal cell density after exposure to 188 and 375 µl bleach L^{-1} (9.4 and 18.8 mg chlorine L^{-1}, respectively) seawater in 1-L jars containing the microalgae *Tisochrysis lutea* (5,227 cells μL^{-1}), and c) comparative color intensity exhibited by *T. luthea* immediately after exposure to 188 and 375 µl bleach L^{-1}. White and grey bars (a) represent control (188 µl bleach L^{-1}, no microalgae) and 188 µl bleach L^{-1} treated microalgal jars respectively, while black bars represent microalgal jars treated 375 µl bleach L^{-1}. Different letters on top of the bars (a) or symbols (b) indicate statistical differences among treatments within a given exposure period ($P < 0.05$). Standard error bars are shown in each case.

$P > 0.05$) (Fig. 4). Noteworthy, lumps of microalgae and organic matter were grouped within hours of aeration forming a fine ring on the wall of the tanks treated with bleach. The ring was manually removed with a cloth to avoid contamination. Notably, visual assessments showed that all three microalgal species used as feed in the larval rearing experiment lost some color during bleach treatment but maintained their cell integrity. At the same time, they remained aerated in the 500-L experimental tanks.

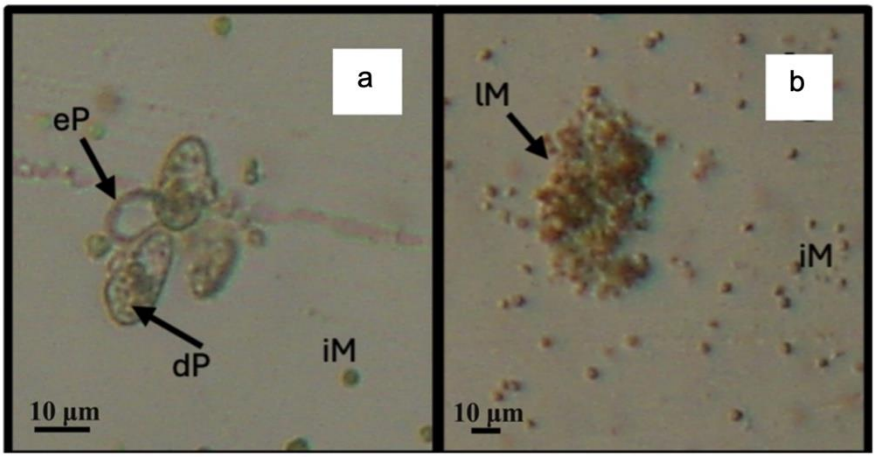


Figure 3. View of a) protozoa and b) microalgae *Tisochrysis lutea* after bleach treatment. dP: partially digested protozoan, eP: empty protozoan, lM: microalgal lump, iM: individual microalgae.

Table 2. Relative amount of digested and undigested protozoa (% of total present in 1 mm^2) observed after exposure to three bleach concentrations (188, 375, and 750 µL L^{-1} seawater) and three exposure periods (30, 60, 120 min) in jars containing protozoa-contaminated microalgae. Jars were neutralized after exposure, and samples were evaluated after 24 h in terms of the relative amount (%) of active microalgae (M), digested/empty and undigested protozoa (P).

Exposure period (min)	Relative amount (%)	Bleach concentration (µL L^{-1})		
		188	375	750
30	Active M	5	1	0
	Digested/empty P	50	60	80
	Undigested P	50	40	20
60	Active M	0	L, many lumps	0, many lumps
	Digested/empty P	85	80	85
	Undigested P	15	20	15
120	Active M	0	0, many lumps	0, many lumps
	Digested/empty P	85	80	80
	Undigested P	15	20	20

Larval growth, survival, and metamorphosis

Both groups of larvae exhibited similar percent survival through the 14-day age range (62 to 69%). However, larvae fed on bleach-treated microalgae showed a significantly higher survival percentage (*t*-Student, $P < 0.05$) by day 24 (Table 3). No detectable mortality occurred once the density of umbonate larvae was reduced to 0.6 larvae mL^{-1} on day 24, resulting in an overall yield of pediveliger larvae of 84% in both groups (Table 3). It is noteworthy that larvae fed on live algae reached the pediveliger stage at an age of between 31 and 33 days, in contrast to the 31-38 days required by larvae fed on bleach-treated algae (Table 3).

Changes in the shell length of *C. gigas* larvae was linear through day 24 when the cull out took place and density was reduced in all the experimental tanks (Fig. 5). For this reason, the growth rate was considered representative of all larvae from day 1 through 24 and showed significant differences (*t*-Student = 21.738, $P < 0.01$) between larval groups feeding on live or bleach-treated microalgae, such that those fed with live food exhibited a faster growth rate (8.5 ± 0.04 µm d^{-1}) than those fed with chlorinated microalgae (7.1 ± 0.03 µm d^{-1}).

DISCUSSION

The present study showed that the addition of bleach to protozoa-contaminated microalgal cultures not only produced swift chemical changes in the seawater but also digested and/or killed the *Uronema*-like ciliates, leaving most microalgae suitable for consumption by

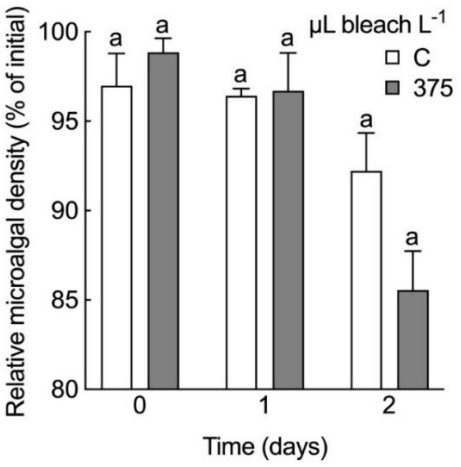


Figure 4. Relative changes of *Tisochrysis lutea* of mean cell density (% of initial) in aerated 500-L cylindroconical tanks exposed to 375 µL bleach L^{-1} (gray bars) or without bleach (control, white bars). Bleach-treated tanks were neutralized with sodium thiosulfate after 1 h of exposure, and both treatments were continuously aerated afterwards. Initial cell density was 73 ± 1 cells μL^{-1} (bleached) and 77 ± 2 cells μL^{-1} (control). Different superscripts represent significant differences ($P < 0.05$) among treatments within a given sampling day. Standard error bars are shown in each case.

C. gigas larvae. This bleach treatment not only prevented the typical need for disposing of protozoa-contaminated, near-crashing microalgal cultures but also resulted in a sustainable and efficient method for maintaining healthy larval cultures of *C. gigas*, which may be used in other bivalve species.

The rapid (4 min) decrease of bleach concentration in seawater observed in the present study (exp. 2) most likely reflects the swift reaction that takes place between hypochlorite and seawater, giving rise to the formation of hypobromous acid and hypobromite ion, chemical species that together with chlorine and other residual compounds contribute to the oxidation of organic matter (Eppley et al. 1976). Seawater characteristics, such as organic matter, salinity, pH levels, temperature, and microbial activity, also contribute to decreasing chlorine concentrations through chemical reactions and degradation processes that neutralize the disinfectant properties of chlorine (Jenkins & Snoeyink 1987). Chlorine concentration in seawater decreased significantly in the presence of microalgae and followed the same temporal pattern at all bleach concentrations tested in the present study, suggesting that chlorine-derived chemical species were able to oxidize at least partially the dissolved and particulate organic matter present in the tanks, including the microalgae *Tl*, which was partially evidenced by the discoloration gradient observed with increasing bleach concentrations and exposure time. It is known that the decrease in chlorine concentration is partially controlled by the density and metabolic activity of the microalgae present, resulting in a faster dissipation of chlorine than in free-alga seawater (Ma et al. 2024). Chlorine concentration typically decreases in the presence of microalgae due to adsorption through their cell surface, oxidation, and chemical reactions with organic compounds released by damaged microalgae, as well as degradation by microbial activity (Ma et al. 2024). Decreased cell viability and damage of algal cells exposed to chlorine were previously reported (Sukenik et al. 1987, Garoma & Yazdi 2019), while leakage of extracellular components and collapse of the reticulate layer of the diatom *Scenedesmus* sp. were observed when the cells were exposed to 10 and 20 mg chlorine L^{-1} (Sukenik et al. 1987). For this reason, it is not surprising that microalgal growth (Park et al. 2016, Vannoni et al. 2018) and photosynthesis (Eppley et al. 1976, Ebenezer et al. 2012) are negatively impacted by chlorine in seawater. On the other hand, in the present study, aeration (exp. 1) had no apparent negative effects on algal density and integrity once the bleach was neutralized with Na-thiosulfate in small (1 L) or large (500 L) culture volumes. This result guaranteed the availability of microalgae as feed during the rearing period of oyster larvae.

It is noteworthy that the concentrations of 188 to 750 µL bleach L^{-1} used in the present study are roughly equivalent to 9 to 38 mg chlorine L^{-1}, respectively. A small percentage of *Tl* remained active (alive) after 24 h when bleach was applied for 30 min at its lowest concentration (188 µL L^{-1}). In contrast, the rest of concentrations and exposure periods resulted in inactive microalgae and/or formation of lumps, thus suggesting that most microalgae died at all concentrations tested when the bleach treatment was applied for ≥60 min (exp. 3). Previous studies showed that chlorine concentration in seawater as low as 1.5 mg L^{-1} dropped the cell density of the diatoms *Navicula pellicullosa* (8% of control), and *Achnanthes* spp. (30% of control) after 2 h of exposure (Vannoni et al. 2018). The viability of *Scenedesmus* sp. also decreased by 50% and the chlorophyll concentration by 30% with chlorine concentrations of 2.5 mg L^{-1} applied for 10 min; however, chlorophyll remained stable (at 50% of the initial concentration) with chlorine levels of 10 and 20 mg L^{-1} (Sukenik et al. 1987). Eppley et al. (1976) showed that microalgal photosynthesis present in seawater samples was not affected by residual chlorine

Table 3. Cumulative percent survival of Pacific oyster, *Crassostrea gigas*, larvae during development through the pediveliger (P) stage. Larvae were reared in cylindroconical tanks, fed untreated (control) and chlorine-treated microalgae. GGR: gross growth rate, D-larva: early veliger stage. Mean values with different superscripts indicate significant differences (*t*-Student test) at $\alpha = 0.05$.

	Cumulative survival (%)	
Day	Live	Treated
1	100	100
14	$62^{a} \pm 2.0$	$69^{a} \pm 2.0$
24	$14^{a} \pm 1.0$	$33^{b} \pm 3.7$
	Mean No. larvae/tank after 24 days	
	676,933 ± 36,671	1,672,666 ± 375,725
	No. larvae left in tanks on D-24	
	300,000	300,000
	Percent of D-24 larvae reaching the P stage	
31	23 ± 13.7	2 ± 0.8
33	22 ± 1.2	
35	39 ± 5.4	35 ± 3.0
38	0.0	47 ± 7.3
Total	85%	84%
Not set	15 ± 0.3	16 ± 4.7
Yield (No. P)	265,283 ± 26,597	315,883 ± 10,920
GGR (µm d^{-1})	$8.5^{a} \pm 0.04$ µm d^{-1}	$7.1^{b} \pm 0.03$ µm d^{-1}

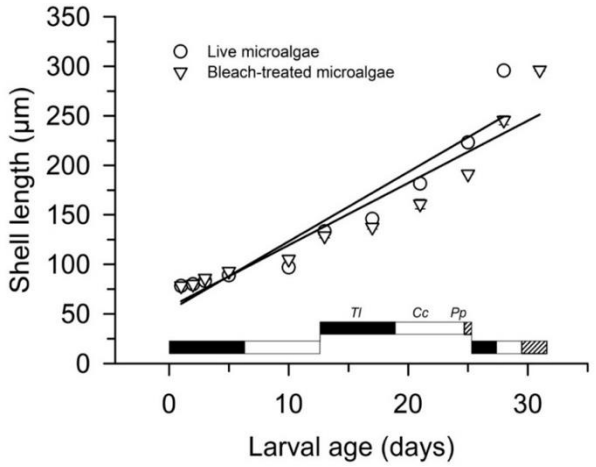


Figure 5. Larval shell growth of the Pacific oyster, *Crassostrea gigas*, fed daily with a mixture of three species of live microalgae or the same microalgae treated with 350 µL bleach L^{-1} for 1 h, followed by neutralization with Na-thiosulfate.

concentrations of 3 mg L^{-1}. However, concentrations of 6 mg L^{-1} significantly decrease the viability of the freshwater green alga *Chlorella vulgaris* by 90% within 6 min, while lower effects were observed with concentrations <6.5 mg L^{-1} (Garoma & Yazdi 2019). Together, these studies suggest that phytoplankton growth is not compromised by chlorine exposure to concentrations up to 3 mg L^{-1}. In contrast, higher concentrations (6 mg L^{-1}) may result in cell death or significantly decreased growth. Specific research showed that while *C. vulgaris* was able to maintain its growth rate at chlorine concentrations of 6 mg L^{-1}; the rotifer *Brachionus calyciflorus* contaminating the cultures exhibited an LD50 (median lethal dose) of 0.2 to 0.4 mg chlorine L^{-1}. Long-term experiments (>24 h) also demonstrated that the growth of this ciliate could not recover at concentrations greater than 0.6 mg L^{-1} (Park et al. 2016). Conversely, experiments carried out with *C. vulgaris* contaminated with the ciliate *Colpoda* sp. showed that 24 h exposure to concentrations of 10 mg bleach L^{-1} eliminated approximately 80% of ciliates and inhibited the growth of *C. vulgaris*, while 20 mg L^{-1} killed both prey and predators (Wang et al. 2017).

To our knowledge, this is the first study to demonstrate that ciliate-contaminated microalgae, treated for 60 min with 375 µL bleach L^{-1} in the absence of air, followed by neutralization, can be successfully used as food for rearing any of the oyster larval stages without affecting their development and growth. While 750 µL bleach L^{-1} produced a more intense discoloration of microalgae than 375 µL L^{-1} (equivalent to ca. 19 mg chlorine L^{-1}) and killed *Tl*, this higher chlorine concentration did not significantly increase the formation of microalgal lumps. Although all three chlorine concentrations appeared to produce similar effects on both algal concentration and protozoa elimination, it was decided to expose the ciliate-

contaminated microalgal cultures to 375 µL bleach L^{-1} before being used as food in the long-term feeding assay in oyster larvae, based on the assumption that such concentration did not exert significant damage to the integrity of microalgae and its nutrient contents. While no quantitative assessments were conducted to test the effect of bleach on *Pp* and *Cc*, visual observations revealed that both species exhibited a pattern similar to *Tl* in terms of discoloration and cell integrity. However, further research is still needed on this issue. The use of parallel assays control (live) treatments helped to demonstrate that while growth rate and the time needed to reach the metamorphic stage of *C. gigas* where slightly slower in organisms fed the bleach-treated microalgae, total yield of pediveliger larvae or the percent settlement/metamorphosis where not affected (exp. 4). Furthermore, the apparent low survival observed in larvae fed live microalgae from D through the umbonate stages (days 14 to 24) most likely resulted from deficient maintenance and/or accidental handling and for this reason the larval density of both treatments was readjusted on day 24.

The main drawback of this work is that the concentration of bleach required for the elimination of protozoans does not allow for the resumption of microalgal growth, so they must be harvested and used as food after the bleach treatment. The approach used in the present study may be effectively applied to batch-cultured protozoa-contaminated microalgae that are currently discarded in hatcheries during bivalve larval rearing. Thus, microalgae grown in columns, tanks, or batch culture systems can be harvested, bleached, and used for growing bivalve larvae. Bleach, selectively used as a treatment against ciliates in contaminated microalgal production systems, may represent a low-cost, short-term solution for retaining high-quality live feeds for bivalve larvae. The possibility of cold storage of bleach-treated microalgae remains to be investigated.

Author's credit contribution

E. Zepeda: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing-original draft, writing-reviewing & editing; Z. Garcia-Esquivel: conceptualization, methodology, validation, investigation, resources, writing-reviewing & editing, supervision, project administration, funding acquisition; A. Braga: methodology, writing-reviewing & editing; S. Castellanos: methodology, writing-reviewing & editing; T.N. Olivares-Bañuelos: writing-reviewing & editing.

Conflict of interest

The authors declare no competing or financial interests in this manuscript.

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