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Effects of thermal disinfection and autoclave sterilisation on the quality of microalgae concentrates

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Abstract

Microalgae concentrates are important for hatchery operations but have been found to contain high concentrations of bacteria. Therefore, this study investigated the effects of four thermal disinfection and sterilisation treatments, low-temperature long-time (LTLT; 62–65°C, 30 min), high-temperature short-time (HTST; 72–75°C, 15 s), ultra-high-temperature (UHT; 120–150°C, 1–3 s) and high-temperature high-pressure (HTHP; 121°C, 0.2 MPa, 15 min), on concentrates of the microalgae, *Chaetoceros gracilis*, *Chlorella vulgaris* (fortified with eicosapentaenoic acid and docosahexaenoic acid) and *Nannochloropsis oculata*. The concentrates had reduced bacterial counts following LTLT, HTST and UHT and were completely sterilised by HTHP. Furthermore, cell dispersibility was retained in all treatment groups except HTHP-treated *C. gracilis*. Rotifers that were cultured with HTHP-treated *C. vulgaris* and *N. oculata* experienced significant population increases, whereas the survival rate and growth of *Artemia* nauplii significantly improved when supplied with HTHP-treated *N. oculata*, indicating that HTHP enhances the digestibility of this microalga. HTHP did not affect the total lipid content and fatty acid composition of the microalgae. These results suggest that HTHP-treated *C. vulgaris* and *N. oculata* could be used for the nutritional enrichment of rotifers and *Artemia*, while avoiding the risk of bacterial contamination, and would have an increased shelf life at room temperature.

KEYWORDS

Artemia nauplii, *Chlorella vulgaris*, disinfection, *Nannochloropsis oculata*, rotifer, sterilisation

1 | INTRODUCTION

Microalgae are essential for the production of juvenile fish, molluscs and crustaceans in hatchery operations for aquaculture and stock enhancement (Borowitzka, 1997a; Brown, Jeffery, Volkman, & Dunstan, 1997; Conceição, Yúfera, Makridis, Morais, & Dinis, 2010; Muller-Feuga, 2000). It is widely accepted that microalgae affect both the biotic and abiotic conditions in the larval culture. The filter-feeding planktonic larvae of most mollusc and echinoderm species, as well as some crustaceans, can utilise microalgae directly as a food source (Borowitzka, 1997a; Brown et al., 1997; Muller-Feuga, 2000). Furthermore, although the carnivorous larvae of fish and crustaceans cannot digest microalgae directly, they have a great reliance on the nutrition they provide to the herbivorous zooplankton on which they feed. For example, rotifers are usually cultured using microalgae as food (Conceição et al., 2010; Dhert, Rombaut, Suantika, & Sorgeloos, 2001; Fu, Hada, Yamashita, Yoshida, & Hino, 1997; Muller-Feuga, 2000; Reitan, Rainuzzo, Øie, & Olsen, 1997; Watanabe, Kitajima, & Fujita, 1983; Yoshimura, Tanaka, & Yoshimatsu, 2003), and the nutritional quality of *Artemia* nauplii can be fortified by supplying them with microalgae before they were added to larval culture tanks (Conceição et al., 2010; Dan, Oshiro, Ashidate, & Hamasaki, 2016; Seixas,

Rey-Méndez, Valente, & Otero, 2008). Furthermore, supplementation of the rearing tank with microalgae can improve the nutritional quality of both rotifers and *Artemia* spp. and thus the growth and survival of the carnivorous larvae (Conceição et al., 2010; Dan & Koiso, 2008; Dan et al., 2016; Reitan et al., 1997; Takeuchi, 1997, 2001). Microalgae also increase the turbidity of the rearing water and thus the contrast in the water column, which helps visual feeding by the larvae (Conceição et al., 2010; Reitan et al., 1997; Stuart, Rotman, & Drawbridge, 2016). Moreover, they improve the water quality; dissolved oxygen level is enhanced by their photosynthetic oxygen releasing, and the pH is stabilised due to bicarbonate uptake for photosynthesis (Ge et al., 2016; Muller-Feuga, 2000; Spolaore, Joannis-Cassan, Duran, & Isambert, 2006).

Several genera of microalgae are used intensively in hatchery operations, such as *Chlorella*, *Chaetoceros*, *Isochrysis*, *Nannochloropsis*, *Pavlova*, *Skeletonema*, *Tetraselmis* and *Thalassiosira* (Borowitzka, 1997a; Brown et al., 1997; Conceição et al., 2010). These microalgae are traditionally cultured in large outdoor ponds (open-air systems) or indoor plastic bags and tubes (closed systems) (Borowitzka, 1997a, 1997b; Conceição et al., 2010). However, in outdoor culture systems, the productivity of microalgae fluctuates greatly depending on the meteorological conditions, such as temperature and the amount of sunshine, and contamination by harmful organisms (Borowitzka, 1997b). By contrast, indoor culture systems can eliminate these variables (Borowitzka, 1997b) but require relatively expensive facilities to provide high levels of artificial lighting and temperature control.

To reduce the risk of there being insufficient microalgae during hatchery operations and the costs associated with their production, there has been an increased use of industrially produced microalgae concentrates, pastes and freeze-dried powders in the last few decades (Borowitzka, 1997a). The microalgae in these products are cultured intensively in huge outdoor ponds or indoor closed systems and are processed using concentrators and/or spray dryers (Conceição et al., 2010). Industrially produced concentrates of *Chlorella vulgaris* and *Nannochloropsis oculata* have been used in Japanese hatcheries since 1990s (Maruyama, Nakao, Shigeno, Ando, & Hirayama, 1997; Yoshimura, Hagiwara, Yoshimatsu, & Kitajima, 1996), the suspensions of which contain over 10 billion cells ml^{-1}. The *C. vulgaris* concentrate has been found to significantly improve the rotifer culture technology, resulting in a very high harvest density of >3,000 individuals ml^{-1} (Fu et al., 1997; Maruyama et al., 1997; Yoshimura et al., 1996, 2003). By contrast, the *N. oculata* concentrate is commonly used for the nutritional enrichment of rotifers before they are fed to larvae and for supplementation of the larval rearing tanks because *N. oculata* cells contain an abundance of eicosapentaenoic acid (EPA), which is an essential fatty acid for many marine animals (Maruyama et al., 1997; Watanabe et al., 1983). Concentrates of diatoms such as *Chaetoceros calcitrans* and *Chaetoceros gracilis* are also commercially available, but their suspensions contain <1 billion cells ml^{-1} due to their cell fragility (Kato, Okauchi, & Nakagami, 2004).

It is well known that microalgae suspensions contain a substantial amount of bacteria because of the high organic load in the culture medium (Conceição et al., 2010; Salvesen, Reitan, Skjermo, & Øie, 2000). It appears that microalgae concentrates contain even greater numbers of bacteria because the bacteria that are attached to the microalgal cells and bacterial flocs may also be condensed during the concentration process. Several studies have shown that microalgae have positive effects on microflora diversification in the larval rearing water and the digestive tract (Conceição et al., 2010; Olsen et al., 2000; Reitan et al., 1997). However, in general, it is widely accepted that a bacterial load in the larval rearing water should be avoided as much as possible to prevent the accidental proliferation of pathogenic and/or opportunistic bacteria that might cause larval mortality. Indeed, reducing the bacterial load associated with microalgae has been shown to improve the survival rate of fish larvae while keeping harmful bacteria at a low level (Stuart et al., 2016). In addition, the bacteria that occur in microalgae suspensions have been known to degrade the algal cells, resulting in the further proliferation of putrid bacteria and a short life of the products (commonly less than 1 month) (Afi et al., 1996; Kato et al., 2004). Although technologies for sterilising or disinfecting seawater, *Artemia* cysts, rotifers and fish eggs have been intensively studied from the perspective of controlling the risk of epidemic larval disease (Douillet, 1998; Skjermo & Vadstein, 1999; Sorgeloos, Bossuyt, Laviña, Baeza-Mesa, & Persoone, 1977; Watanabe, Shinozaki, Koiso, Kuwada, & Yoshimizu, 2005), little attention has been paid to reducing the bacterial load associated with microalgae and their concentrates.

The aim of this study was to develop a technique for disinfecting or sterilising microalgae concentrates. Four different treatments were tested: three thermal (i.e., pasteurisation) treatments that have traditionally been used to treat milk and drinks, and an autoclave treatment that attains both high-temperature and pressure using steam. Given the crucial roles of microalgae in hatchery operations, it is important that any treatment maintains their dispersibility in seawater, dietary effects on rotifers and *Artemia* nauplii and nutritional composition, particularly n-3 unsaturated fatty acid contents, at an acceptable level. Therefore, the effects of the treatments on each of these parameters were also evaluated.

2 | MATERIALS AND METHODS

2.1 | Thermal disinfection and autoclave sterilisation methods

This study was conducted in 2013 at the Tamano Laboratory, National Research Institute of Fisheries and Environment of Inland Sea, Japan Fisheries Research and Education Agency, Tamano, Okayama, Japan. Commercially available concentrates of *C. gracilis* (Yanmar Co. Ltd., Osaka, Japan), *C. vulgaris* fortified with EPA and docosahexaenoic acid (DHA) (Super Chlorella V12; Chlorella Industry Co. Ltd., Tokyo, Japan) and *N. oculata* (Yanmarine K-1; Chlorella Industry Co. Ltd., Tokyo, Japan) were obtained, all of which are commonly used in Japanese hatcheries. Three thermal disinfection methods that are traditionally used to process milk and drinks for human

consumption were tested (Tamime, 2009; Table 1): (a) a low-temperature long-time (LTLT) treatment, which was carried out by heating the microalgae concentrates in vials or Erlenmeyer flasks in a heating water bath at 62–65°C for 30 min; (b) a high-temperature short-time (HTST) treatment, which was performed by passing the concentrates through a silicon tube (φ1 mm) in a heating water bath at 72–75°C for 15 s; and (c) an ultra-high-temperature (UHT) treatment, which was applied by passing the concentrates through a silicon tube (φ1 mm) in a heating oil bath at 120–150°C for 1–3 s. During the LTLT treatment, the temperature of the concentrates was monitored using a digital thermometer. For the HTST and UHT treatments, the treatment time was adjusted by controlling the speed at which the concentrates passed through the tube using a tube pump (EYELA MP-3; Tokyo Rikakikai Co. Ltd., Tokyo, Japan), and the samples were immediately cooled following the treatment by placing the tube in cold water at 0°C. In addition, a HTHP sterilisation treatment was performed by autoclaving the concentrates at 121°C and 0.2 MPa for 15 min (BS-325; TOMY SEIKO Co. Ltd., Tokyo, Japan). The treatments were carried out on the first day of each experiment, and the samples were then preserved at 4°C.

2.2 | Effect of thermal disinfection and autoclave sterilisation on microalgal cell dispersibility

The cell densities in the concentrates of *C. gracilis*, *C. vulgaris* and *N. oculata* were counted using a Burker–Turk haemocytometer and were found to be 0.238, 12.4 and 13.7 billion cells ml^{-1} respectively. The LTLT and HTHP treatments were applied to vials containing 5 ml of concentrate, with four replicates per microalga. For the HTST and UHT treatments, each concentrate was infused directly into four sterilised vials from the outlet of a treatment tube. Subsamples of the treated microalgae were loaded into a haemocytometer, and their cells were observed microscopically at ×400 magnification using a binocular microscope (Eclipse 55i; Nikon Co. Ltd., Tokyo, Japan). The cell dispersibility of each microalga in the water column was then assessed by counting the number of aggregated cells (more than two cells attached to each other) using a haemocytometer grid and dividing this by the total number of cells observed to obtain a percentage.

2.3 | Effect of thermal disinfection and autoclave sterilisation on bacterial count

Non-treated and treated microalgae concentrates were serially diluted with sterilised seawater and plated on marine agar 2216 (MA; Difco Laboratories Inc., Detroit, USA) immediately after treatment (0 hr). Viable bacterial colony counts on MA were enumerated as colony-forming units (CFUs) after 48 hr incubation at room temperature (c. 25°C). Bacterial counts were also carried out for treated microalgae samples that had been stored at 4°C for 48 hr after treatment.

2.4 | Dietary effect of HTHP-treated microalgae on rotifer cultures

On the basis of the cell dispersibility and bacterial count results, the dietary effects of the HTHP-treated concentrates of *C. vulgaris* and *N. oculata* on cultures of the rotifer *Brachionus plicatilis* were compared with non-treated concentrates and no-microalgal addition. *Brachionus plicatilis* were stocked in 15 10 L circular plastic tanks containing 10 L of sterilised seawater at a density of 94.8 ± 8.4 (mean ± standard deviation) individuals ml^{-1}. Three replicate tanks were assigned to each of five treatment groups: non-treated and HTHP-treated *C. vulgaris* and *N. oculata*, and no-microalgal addition. Each tank was aerated with an air stone placed in the centre of the tank to maintain sufficient oxygen. The tanks were immersed in a water bath, and the water temperature was maintained at 25°C using a heater connected to a thermostat. The microalgae concentrates were supplied as food for the rotifers at concentrations of 3.0 million cells ml^{-1} for *C. vulgaris* and 6.0 million cells ml^{-1} for *N. oculata* once per day at 8 a.m. (half the amount of *C. vulgaris* was supplied because their cells are approximately twice as heavy as those of *N. oculata*; Dan & Koiso, 2008). These supply densities are generally adopted for hatchery operations (Dehert et al., 2001). The rotifers were cultured for 5 d with no renewal of the culture water during the culture period. The density of the rotifers was estimated every morning by examining three 2.0 ml samples collected from each tank, and the relative growth rate of each rotifer population was calculated as the estimated rotifer density/initial rotifer density ×100.

TABLE 1 Thermal disinfection and sterilisation methods used to treat concentrates of the microalgae *Chaetoceros gracilis*, *Chlorella vulgaris* and *Nannochloropsis oculata*

Treatment	Abbreviation	Temperature (°C)	Time	Pressure (MPa)	Manipulation
Low-temperature long-time	LTLT	62–65	30 min	0.1	Hold in vials or Erlenmeyer flasks in a heating water bath
High-temperature short-time	HTST	72–75	15 s	0.1	Pass through a φ1-mm silicon tube in a heating water bath and then cool in cold water
Ultra-high-temperature	UHT	120–150	1–3 s	0.1	Pass through a φ1-mm silicon tube in a heating oil bath and then cool in cold water
High-temperature high-pressure	HTHP	121	15 min	0.2	Hold in vials or Erlenmeyer flasks in a programmed autoclave

2.5 | Dietary effect of HTHP-treated microalgae on *Artemia* cultures

There are various methods of culturing *Artemia* depending on target species (Seixas et al., 2008; Sorgeloos, Dhert, & Candreva, 2001). In this experiment, to examine the effect of treated or non-treated microalgae on growth and survival of *Artemia*, relatively a long-term (6 d) culture trial was carried out. *Artemia* nauplii (Utah Strain; Kitamura Co., Ltd., Kyoto, Japan) were hatched in seawater at 25°C for 24 hr and stocked in aerated 1 L plastic beakers at a density of 10.7 ± 0.7 individuals ml^{-1}. They were then cultured for 6 d in stagnant seawater. As in the rotifer culture trial, the dietary effects of HTHP-treated *C. vulgaris* and *N. oculata* on *Artemia* were compared with non-treated microalgae and no-microalgal addition in three replicate beakers per treatment, giving a total of 15 beakers. *Chlorella vulgaris* and *N. oculata* were supplied twice per day at 9 a.m. and 4 p.m. at densities of 1.0 and 2.0 million cells ml^{-1} respectively. The numbers of surviving *Artemia* were estimated by collecting three 10 ml samples from each beaker. In addition, the total lengths of 30 *Artemia* individuals from each beaker were measured every 2 d.

To investigate the consumption of non-treated and HTHP-treated microalgae by *Artemia* nauplii, newly hatched nauplii were stocked in 1 L plastic beakers at a density of 10.0 individuals ml^{-1}. Non-treated and HTHP-treated *C. vulgaris* and *N. oculata* were then supplied to three replicate beakers (total 12 beakers) once at the start of the trial at densities of 1.5 and 3.0 million cells ml^{-1} respectively. The microalgal cell density in each beaker was determined every 3 hr up to 24 hr after the start of the trial using a Burker–Turk haemocytometer.

2.6 | Total lipid content and fatty acid composition analysis

Non-treated and HTHP-treated *C. vulgaris* and *N. oculata* were centrifuged at 10,000× *g* for 10 min and the resulting precipitates were stored at −80°C until analysis (n = 2 for each concentrate).

The total lipid content of each sample was determined by the chloroform–methanol (2:1, v/v) method (Folch, Lees, & Stanley, 1957). To analyse the fatty acid composition, the total lipids were saponified with 50% KOH in ethanol and the saponifiable matter was esterified with BF_3-methanol. The resulting fatty acid methyl esters were then diluted in n-hexane and analysed with a gas–liquid chromatography (GC-17A; Shimadzu Co., Ltd., Kyoto, Japan) equipped with a silica capillary column (24080-U; Supelco Inc., Bellefonte, PA, USA; 30 m × 0.32 mm × 0.25 μm film thickness). Helium was used as the carrier gas, the pressure was adjusted to 120 kPa, and the injection port and detector temperatures were 250 and 270°C respectively. The column temperature was initially held at 170°C and then increased at a rate of 2°C/min to a final temperature of 230°C. The individual fatty acids were identified by comparison with commercial standards (Supelco 37 Component FAME Mix; Supelco) and quantified with a C-R8A Chromatopac Data Processor (Shimadzu Co., Ltd., Kyoto, Japan).

2.7 | Data analysis

All statistical analyses were performed in R (R3.4.4; R Core Team, 2017) with a 5% significance level. Differences in microalgal cell dispersibility were assessed using a generalised linear model (GLM) with the *glm* function and the quasi-binomial family (logit link) to account for overdispersion in the error distribution (Everitt & Hothorn, 2009; McCullagh & Nelder, 1989). The numbers of aggregated or single cells were included as a two-vector response variable, and treatment was included as the explanatory variable (as a categorical fixed factor). Because no aggregated cells were found in the non-treated *C. gracilis* samples, these data were eliminated from the analysis to avoid error. The survival rate of *Artemia* nauplii was also assessed using a GLM with the quasi-binomial family (logit link), in which the numbers of live or dead individuals were included as a two-vector response variable and the type of microalgae supplied (species and treatment) was included as the explanatory variable.

To examine differences in the bacterial counts of the microalgae, a linear model (LM) was applied using the *lm* function. The log-transformed bacterial count (CFU) was included as the response variable, and the treatment and stored time (0 or 48 hr) after treatment were included as explanatory variables. An LM was also used to evaluate the effect of each treatment on rotifer proliferation and *Artemia* growth, in which the rotifer density and total length of *Artemia* at the end of the culture trials were included as response variables and the type of microalga was included as the explanatory variable. The nutritional contents of the microalgae concentrates were also compared using an LM, in which with the total lipid content and fatty acid composition were included as response variables and the species of microalga and treatment were included as explanatory variables.

To examine differences in the consumption of the different concentrates by *Artemia* nauplii, a linear mixed-effects model (LMM) was applied using the *lmer* function in the lme4 package (Bates, 2010; Everitt, 2005; Everitt & Hothorn, 2009; Zuur, Ieno, Walker, Saveliev, & Smith, 2009), in which the cell density remaining was included as the response variable, and treatment (non-treated or HTHP-treated) and observation time (hours after microalgae supply) were included as explanatory variables. *C. vulgaris* and *N. oculata* were analysed separately because they were supplied at different densities. Because these data were collected by collecting samples from the same beaker every 3 hr, the beaker identity number was also included in the LMM as a random intercept effect to account for any potential autocorrelation in the repeated measures data (Zuur et al., 2009).

To evaluate the statistical significance of the explanatory variables, the *F* test was performed using the *ANOVA* function (type II) in the car package (Fox & Weisberg, 2011). Differences between treatments were then evaluated using Tukey's method with the *glht* function in the multcomp package (Hothorn, Bentz, & Westfall, 2008).

3 | RESULTS

3.1 | Effect of thermal disinfection and autoclave sterilisation on microalgal cell dispersibility

Microalgal cell aggregations were observed in the non-treated *C. vulgaris* and *N. oculata* concentrates at a low rate (1.1%) but were not found in the non-treated *C. gracilis* concentrate (Table 2, Figure 1). During the UHT treatment, it was observed that the concentrates evaporated as they passed through the tube in the heating bath but then returned to a liquid state immediately upon cooling. It was also noted that some of the treatments appeared to change the colour of the concentrates: from brown to green for *C. gracilis* after all treatments; and from green to brown for *C. vulgaris* and *N. oculata* after the HTHP treatment (Figure S1).

There was a significant difference in the proportion of aggregated cells among treatments for all microalgae (*C. gracilis*: $F = 110.1$, $df = 3, 10$, $p < 0.0001$; *C. vulgaris*: $F = 3.791$, $df = 4, 15$, $p = 0.0253$; *N. oculata*: $F = 45.86$, $df = 4, 15$, $p < 0.0001$). For *C. gracilis*, the proportion of aggregated cells in the LTLT, HTST and UHT treatments ranged from 1.2% to 12.7% and the cells were homogeneously dispersed in the suspension. However, the HTHP-treated *C. gracilis* showed a significantly higher proportion of aggregated cells than the other treatments and floc formation was observed, resulting in precipitation (Figure 1). For *C. vulgaris*, the proportion of aggregated cells was high in the HTHP treatment (5.5%) but the cells remained dispersed in the suspension and there was no significant difference between treatments. For *N. oculata*, significantly higher proportions of aggregated cells were observed for the three thermal disinfection treatments (LTLT, HTST and UHT) than for the non-treated concentrates and, again, the highest proportion of aggregated cells was observed after the HTHP treatment. However, these aggregations were composed of less than five cells and maintained their dispersibility in the suspension (Figure 1).

3.2 | Effect of thermal disinfection and autoclave sterilisation on bacterial counts

Each of the non-treated microalgae concentrates contained similar amounts of bacteria (7.23–7.85 log CFU ml^{-1}; Figure 2). All of the treatments significantly reduced the bacterial counts for all microalgae (*C. gracilis*: $F = 79.54$, $df = 4, 30$, $p < 0.0001$; *C. vulgaris*: $F = 39.55$, $df = 4, 30$, $p < 0.0001$; *N. oulata*: $F = 285.1$, $df = 4, 30$, $p < 0.0001$). The HTHP treatment completely sterilised the concentrates, resulting in bacterial counts of zero, whereas the HTST and UHT treatments exhibited a significantly higher disinfection potency than the LTLT treatment. Storage at 4°C for 48 hr after treatment did not affect the bacterial counts in any of the microalgae concentrates (*C. gracilis*: $F = 1.281$, $df = 1, 30$, $p = 0.2667$; *C. vulgaris*: $F = 0.0833$, $df = 1, 30$, $p = 0.7748$; *N. oculata*: $F = 2.0988$, $df = 1, 30$, $p = 0.1578$).

3.3 | Dietary effect of HTHP-treated microalgae on rotifer cultures

The rotifer density was significantly affected by the different microalgae concentrates and treatments after 5 d of culture ($F = 464.9$, $df = 4, 10$, $p < 0.0001$; Figure 3). With no-microalgae addition, the rotifer density began to decrease on day 3 and reached 18.5 individuals ml^{-1} on day 5. By contrast, the other treatment groups that were supplied with microalgae exhibited a constant increase to reach 264–316 individuals ml^{-1} by day 5. However, the group that was supplied with HTHP-treated *N. oculata* reached a significantly lower density than the other groups.

3.4 | Dietary effect of HTHP-treated microalgae on *Artemia* cultures

There was a significant difference in the survival of *Artemia* nauplii supplied with different microalgae concentrates and treatments ($F = 36.62$, $df = 4, 10$, $p < 0.0001$; Figure 4a). In the no-microalgae addition and the non-treated *C. vulgaris* and *N. oculata* groups, nearly all of the *Artemia* died on day 4 after hatching. The group that was supplied with HTHP-treated *C. vulgaris* showed a higher survival rate than the control group, but this declined after day 4 to eventually reach 31.0% on day 6. The highest survival was achieved in the group supplied with HTHP-treated *N. oculata*, which was still 75.1% on day 6.

The total length of *Artemia* nauplii at the end of the culture trial (day 6) was also affected by the microalgal supply ($F = 53.23$, $df = 4, 10$, $p < 0.0001$; Figure 4b), with *Artemia* that were cultured with

TABLE 2 Effect of the disinfection and sterilisation treatments on cell aggregation in the microalgae *Chaetoceros gracilis*, *Chlorella vulgaris* and *Nannochloropsis oculata*

	Proportion of aggregated cells (%)				
Microalga	Control	LTLT	HTST	UHT	HTHP
C. gracilis	0.0	1.2 ± 1.9^{a}	1.3 ± 1.3^{a}	12.7 ± 20.3^{a}	46.5 ± 8.0^{b}
C. vulgaris	1.1 ± 1.3^{a}	1.9 ± 1.4^{a}	1.6 ± 1.3^{a}	3.4 ± 3.8^{a}	5.5 ± 3.1^{a}
N. oculata	1.1 ± 0.7^{a}	16.1 ± 1.5^{b}	19.5 ± 6.8^{b}	21.5 ± 17.1^{b}	44.2 ± 2.3^{c}

Note. Data are means ± standard deviations ($n = 4$). Different superscript letters in the same row indicate a significant difference between treatments (generalised linear model with Tukey's method, $p < 0.05$). LTLT: low-temperature long-time; HTST: high-temperature short-time; UHT: ultra-high-temperature; HTHP: high-temperature high-pressure.

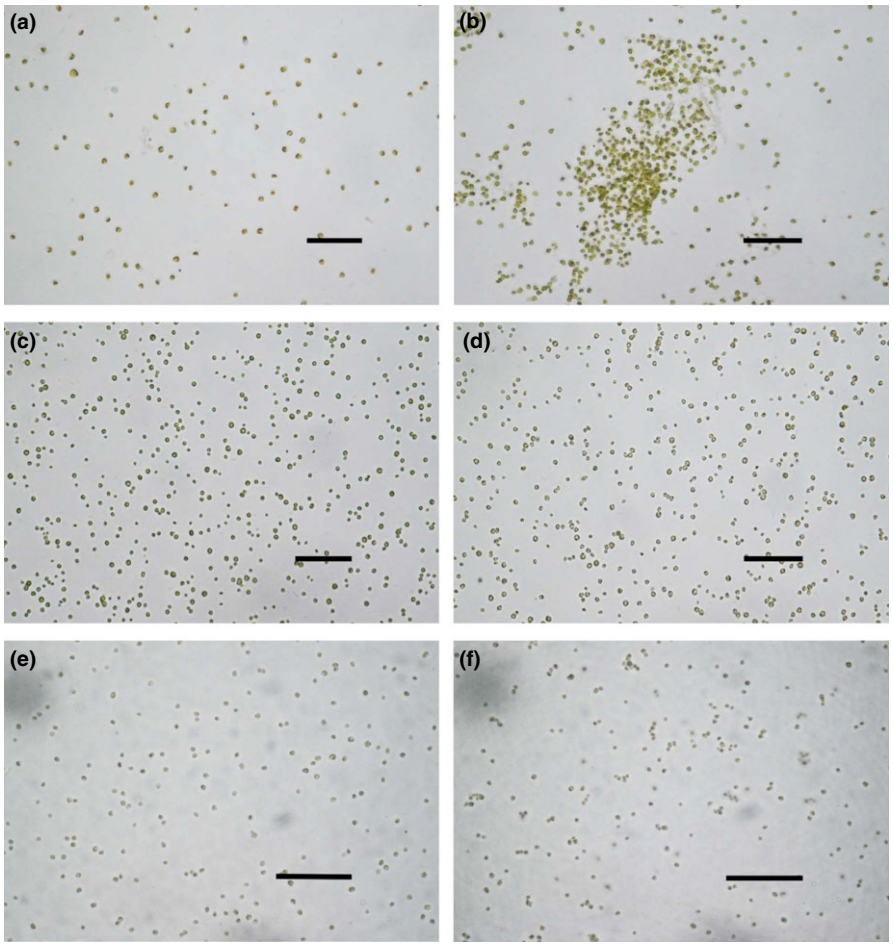

FIGURE 1 Photographs of non-treated (a, c, e) and high-temperature high-pressure (HTHP)-treated (b, d, f) concentrates of the microalgae *Chaetoceros gracilis* (a, b), *Chlorella vulgaris* (c, d) and *Nannochloropsis oculata* (e, f)[Colour figure can be viewed at wileyonlinelibrary.com]

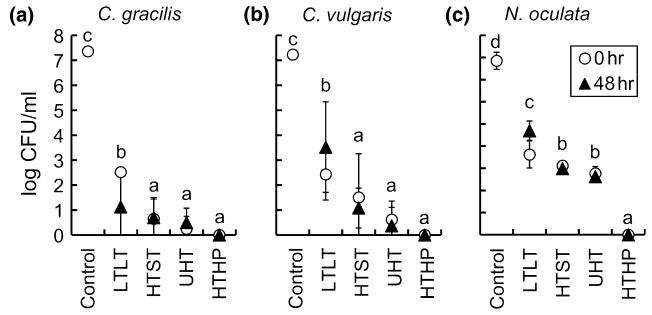

FIGURE 2 Effects of disinfection and sterilisation of the microalgae concentrates of *Chaetoceros gracilis* (a), *Chlorella vulgaris* (b) and *Nannochloropsis oculata* (c) on the number of colony-forming units (CFUs) of bacteria immediately after treatment (0 hr) or after 48 hr storage at 4°C. Vertical bars indicate standard deviations. Differences among treatment groups are indicated by different letters ($p < 0.05$). LTLT: low-temperature long-time; HTST: high-temperature short-time; UHT: ultra-high-temperature; HTHP: high-temperature high-pressure

HTHP-treated *N. oculata* being significantly larger than those in the other groups.

The numbers of microalgal cells remaining in the *Artemia* culture water were also significantly affected by treatment in the *N. oculata*-supply groups ($F = 116.3$, $df = 1, 4$, $p = 0.0004$) but not in the *C. vulgaris*-supply groups ($F = 0.1294$, $df = 1, 4$, $p = 0.1294$) (Figure 5). Although a slight time-dependent decrease was detected for both microalgae (*C. vulgaris*: $F = 112.1$, $df = 1, 47$, $p < 0.0001$; *N. oculata*: $F = 39.26$, $df = 1, 47$, $p < 0.0001$), a dramatic decrease was observed at 9 hr from the start of cultivation in the HTHP-treated *N. oculata*-supply group, with no-microalgal cells remaining at 24 hr.

3.5 | Effect of HTHP treatment on the total lipid content and fatty acid composition

There was a significant difference in the total lipid content and fatty acid composition between *C. vulgaris* and *N. oculata* (total lipid: $F = 187.4$, $df = 1, 5$, $p < 0.0001$; EPA: $F = 2{,}852$, $df = 1, 5$, $p < 0.0001$; DHA: $F = 340.9$, $df = 1, 5$, $p < 0.0001$; *n*-3 highly unsaturated fatty acids (*n*-3 HUFA): $F = 268.5$, $df = 1, 5$, $p < 0.0001$; Table 3). The total lipid, EPA and *n*-3 HUFA contents were higher in *N. oculata*, whereas levels of DHA were higher in *C. vulgaris*. The HTHP treatment did not affect the total lipid content and fatty acid composition of either microalga (total lipid: $F = 3.659$, $df = 1, 5$, $p = 0.1140$; EPA: $F = 1.620$, $df = 1, 5$, $p = 0.2591$; DHA: $F = 3.185$, $df = 1, 5$, $p = 0.1344$; *n*-3 HUFA: $F = 0.2152$, $df = 1, 5$, $p = 0.6622$). The levels of each of the detected fatty acids are summarised in the supporting information (Table S1).

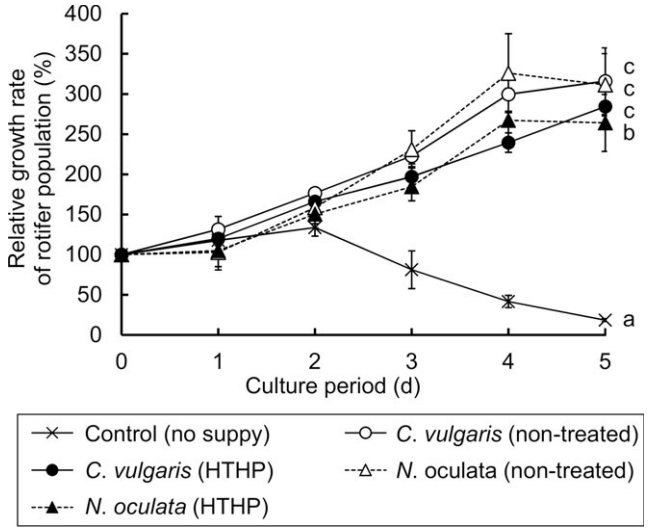

FIGURE 3 Changes in the mean relative growth rate of populations of the rotifer *Brachionus plicatilis* cultured for 5 d with a supply of non-treated or high-temperature high-pressure (HTHP)-treated *Chlorella vulgaris* or *Nannochloropsis oculata* or with no-microalgae addition. Vertical bars indicate standard deviations. Differences among treatment groups are indicated by different letters ($p < 0.05$)

4 | DISCUSSION

It is widely recognised that the composition of the bacterial flora in larval rearing water is closely related to the success or failure of juvenile production because a diverse bacterial flora that is established by non-opportunists may inhibit the proliferation of opportunistic pathogenic bacteria (Skejermo & Vadstein, 1999). Consequently, the unintentional addition of a bacterial load that will likely disturb this flora to the larval rearing water should be avoided as much as possible. In the present study, the non-treated microalgae concentrates contained high densities of bacteria (7.23–7.85 log CFU ml^{-1}), indicating that the disinfection or sterilisation of microalgae concentrates may help to stabilise the larval condition without disturbing the bacterial flora in the rearing water. The LTLT, HTST and UHT treatments successfully reduced the bacterial counts in the microalgae concentrates while retaining the cell dispersibility for *C. gracilis*, *C. vulgaris* and *N. oculata*. However, the HTHP treatment attained complete sterilisation while maintaining the cell dispersibility, the levels of important fatty acids and dietary effects on rotifers for *C. vulgaris* and *N. oculata*. Thus, it appears that HTHP-treated *C. vulgaris* and *N. oculata* concentrates could be used for the nutritional enrichment of rotifers before they are fed to larvae and for supplementation of the larval rearing water while reducing the risk of bacterial diseases. The cell aggregations that were observed in HTHP-treated *C. gracilis* could have been caused by the fragility of the cell walls: diatoms have characteristic cell walls (frustules) that are composed of hydrous amorphous silica and amino acids, the structure of which can be denatured by heating (Arasuma & Okuno, 2018).

Early *Artemia* nauplii lack the ability to digest microalgae with rigid cell walls such as *C. vulgaris* and *N. oculata* (Dan et al., 2016).

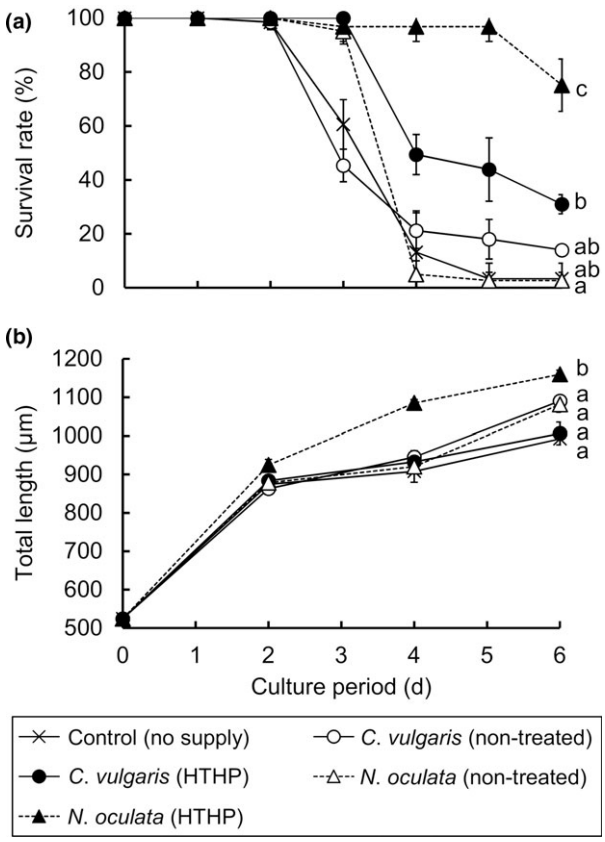

FIGURE 4 Changes in the mean survival rate (a) and mean total length (b) of newly hatched *Artemia* nauplii cultured for 6 d with a supply of non-treated or high-temperature high-pressure (HTHP)-treated *Chlorella vulgaris* or *Nannochloropsis oculata* or with no-microalgae addition. Vertical bars indicate standard deviations. Differences among treatment groups are indicated by different letters ($p < 0.05$)

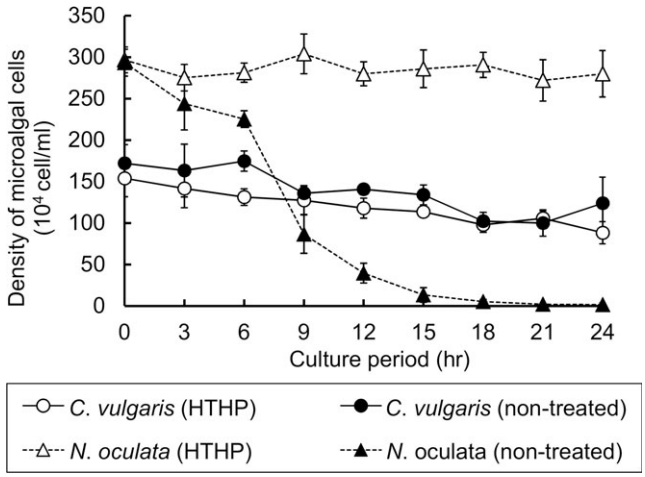

FIGURE 5 Changes in the mean density of microalgal cells remaining in the culture water of newly hatched *Artemia* nauplii provided with a supply of non-treated or high-temperature high-pressure (HTHP)-treated *Chlorella vulgaris* or *Nannochloropsis oculata*. Vertical bars indicate standard deviations

TABLE 3 Total lipid, eicosatetraenoic acid (EPA), docosahexaenoic acid (DHA) and *n*-3 highly unsaturated fatty acids (*n*-3HUFA) contents of non-treated and high-temperature high-pressure (HTHP)-treated *Chlorella vulgaris* and *Nannochloropsis oculata*

	C. vulgaris		*N. oculata*	
Lipid, fatty acids	**Non-treated**	**HTHP**	**Non-treated**	**HTHP**
Total lipid content (% dry matter)				
	15.7 ± 1.04	15.7 ± 0.60	25.6 ± 0.22	28.8 ± 0.08
Fatty acid composition (% area)				
EPA	4.7 ± 0.23	5.1 ± 0.10	43.9 ± 0.41	41.6 ± 1.07
DHA	10.0 ± 1.01	12.2 ± 0.54	*n*.d.	*n*.d.
n-3HUFA	15.4 ± 2.22	19.1 ± 0.54	43.9 ± 0.41	41.6 ± 1.07

Note. Data are means ± standard deviations (n = 2). *n*.d.: not detected. *n*-3HUFA includes 20:5n-3, 22:5n-3 and 22:6n-3.

Indeed, nearly all of the *Artemia* that were cultured with non-treated *C. vulgaris* and *N. oculata* died on day 4 after hatching, as well as those without microalgal supply, implying that they were not able to digest the microalgae and fell into starvation. However, surprisingly, HTHP treatment resulted in *N. oculata* becoming digestible by *Artemia*. Notably, there was a distinct decrease in the density of the supplied HTHP-treated *N. oculata* cells from 9 hr after the *Artemia* hatched, which correlates well with the growth of *Artemia* (within 6–8 hr of hatching, *Artemia* moult into second-stage larvae, which are able to feed; Sorgeloos et al., 2001). Therefore, it appears that the *Artemia* were able to digest the HTHP-treated *N. oculata*. This is likely due to the *N. oculata* cell walls being destroyed or denatured under the HTHP conditions. It is currently unknown why the *C. vulgaris* cells did not also become digestible under the same conditions, but this is likely related to differences in the structure and composition of the cell walls between the two species (Bernaerts et al., 2018). Because *N. oculata* contains large amounts of EPA, which is an essential fatty acid for the larvae of many marine species (Brown et al., 1997; Watanabe et al, 1983), HTHP-treated *N. oculata* have high potential for the nutritional enrichment of *Artemia* before they are fed to larvae and for the supplementation of larval rearing water during the period of *Artemia* feeding. Indeed, the EPA content of *Artemia* was greatly improved by supplying HTHP-treated *N. oculata*, as shown in the supporting information (Table S2).

It was also confirmed that rotifers were able to proliferate when supplied with HTHP-treated *C. vulgaris*. In Japanese hatcheries, the rotifer culture technology has been developed using *C. vulgaris* concentrates, which achieve stable proliferation and a high harvest density of >3,000 individuals ml^{-1} (Fu et al., 1997; Maruyama et al., 1997; Yoshimura et al., 1996, 2003). However, in a preliminary high-density rotifer culture trial that used over 500 individuals ml^{-1}, growth of the rotifer population stalled after being supplied with HTHP-treated *C. vulgaris* (Dan unpublished data). This result was presumably due to some factor(s) that is essential for a high-density rotifer culture and contained in *C. vulgaris* cells becoming defective or denatured during treatment. Therefore, further research is required to identify this factor and to develop a high-density rotifer culture technique using HTHP-treated *C. vulgaris*. In addition, the dietary effects of HTHP-treated microalgae on mollusc and echinoderm larvae should be investigated in the future, as this technique also has the potential to reduce the cost and increase the stability of hatchery operations for these species.

Securing a stable microalgae supply during the larval culture period is a major issue associated with the use of microalgae in hatcheries (Borowitzka, 1997a). Because HTHP-treated microalgae concentrates were completely sterilised, it may be possible to store them for over a year at room temperature in sealed containers (i.e., retortable pouches) without bacterial degradation. This may also reduce hatchery costs, as no refrigeration would be required during storage or transport. Thus, this technology has the potential to improve the usability of microalgae concentrates, particularly in remote areas.

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CONFLICT OF INTEREST

The authors have no conflicts of interest directly relevant to the content of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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