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Interactive effects of temperature and salinity on the growth and cytotoxicity of the fish-killing microalgal species *Heterosigma akas*hiwo and *Pseudochattonella verruculosa*

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ABSTRACT

Fish-killing blooms of *Heterosigma akashiwo* and *Pseudochattonella verruculosa* have been devastating for the farmed salmon industry, but in Southern Chile the conditions that promote the growth and toxicity of these microalgae are poorly understood. This study examined the effects of different combinations of temperature (12, 15, 18 °C) and salinity (10, 20, 30 psu) on the growth of Chilean strains of these two species. The results showed that the optimal growth conditions for *H. akashiwo* and *P. verruculosa* differed, with a maximum rate of 0.99 day^{-1} obtained at 15 °C and a salinity of 20 psu for *H. akashiwo*, and a maximum rate of 1.06 day^{-1} obtained at 18 °C and a salinity of 30 psu for *P. verruculosa*. Cytotoxic assays ($2 \times 10^1 - 2 \times 10^5$ cell mL^{-1}; cells, filtrates, and cell lysates) performed at salinities of 20 and 30 psu showed a 100% reduction in the viability of embryonic fish cells exposed to intact cells of *H. akashiwo* and a 39% reduction following exposure to culture filtrates of *P. verruculosa*. Differences in the fish-killing mechanisms (direct cell contact vs. extracellular substances) and physiological traits of *H. akashiwo* and *P. verruculosa* explain the recent occurrence of very large blooms under contrasting (cold-brackish vs. hot-salty) extreme climate conditions in Chile.

1. Introduction

Harmful algal blooms (HABs) are a natural phenomenon produced by the proliferation of microalgae. However, some HAB-forming species release potent toxins whose uptake by the food chain can ultimately cause a variety of neurological and gastrointestinal disorders in humans, including paralytic, diarrheic, and amnesic shellfish poisoning. Other algal species form fish-killing HABs that are particularly problematic in fisheries and in the aquaculture industry (Dorantes-Aranda et al., 2015; Lum et al., 2021). The negative socio-economic impacts of HABs on public health, fisheries resources, and coastal commodities have made them a cause for considerable concern (Hallegraeff, 2003).

In the past three decades, there has been an increase in HAB events on a global scale, partly associated with the progressive increase in the exploitation of coastal resources for aquaculture and recreational purposes but also reflecting increased reporting through monitoring programs (Hallegraeff, 1993; Anderson et al., 2008; Hallegraeff et al., 2021). The expansion of HABs, their longer duration, and the

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involvement of previously unaffected areas were already highlighted in 2004 in a publication on marine biotoxins by the Food and Agriculture Organization of the United Nations (FAO, 2004). Nonetheless, most research attention of HAB-forming species has been paid to those that produce biotoxins (Guzmán et al., 2002; Molinet et al., 2003; Díaz et al., 2019; Díaz et al., 2020; Baldrich et al., 2021; Díaz et al., 2021), with few studies of ichthyotoxic microalgae (Mardones et al., 2019).

HABs are also a recurrent problem in the Chilean Patagonian fjords system (Guzmán et al., 1975; Guzmán et al., 2002; Díaz et al., 2019), where 99% of Chile's salmon farms are located. Most of these HABs are caused by the toxic dinoflagellates *Alexandrium catenella*, which produces paralytic shellfish toxins (Guzmán et al., 2002; Molinet et al., 2003; Molinet et al., 2010; Díaz et al., 2014), *Dinophysis acuta* and *Dinophysis acuminata*, producers of the lipophilic toxin okadaic acid but also dinophysistoxins and pectenotoxins (Díaz et al., 2011; García et al., 2012; Contreras and Garcia, 2019; Baldrich et al., 2021; Díaz et al., 2021), and *Protoceratium reticulatum*, which produces yessotoxins (Alves de Souza et al., 2014). HABs of these species have also led to restrictions on the harvesting of natural populations of many bivalve and gastropod species.

Two ichthyotoxic species responsible for HABs in Chile are the phytoflagellates *Heterosigma akashiwo* (Raphidophyceae) and *Pseudochattonella verruculosa* (Dictiochophyceae) (Díaz et al., 2019), studies of either one are very limited. In Chile, the first HAB event caused by *H. akashiwo* (maximum density 100,000 cells mL^{-1}) occurred more than three decades ago (September 1988) and led to the loss of 50% of the national fish production, estimated at 10,000 tons of fish, and an economic loss valued at $11 million USD (Avaria et al., 1999; Díaz et al., 2019). The most recent *H. akashiwo* bloom event (70,000–210,000 cells mL^{-1}) was in the early autumn of 2021 (April–May), at Comau fjord (NW Patagonia), and caused the death of ~3000 tons of cultured salmons across 12 aquaculture facilities, with the loss valued at $4.4 million USD. Blooms of *P. verruculosa* have been recorded since 2004. While most were relatively small (Mardones et al., 2012), in the summer of 2016, an intense bloom of *P. verruculosa* affected 45 aquaculture facilities located at the northern limit (~41°30′S) of the Chilean Patagonian fjord system (Clément et al., 2016; Hernández et al., 2016; León-Muñoz et al., 2018). This event caused the loss of ~39,942 tons of salmons (~27 million fish) and an economic loss valued at $800 million USD (Díaz et al., 2019; Mardones et al., 2021).

Both *H. akashiwo* and *P. verruculosa* have been associated with massive mortalities of wild and cultured fish not only in Chile but also around the world (Hosoi-Tanabe et al., 2007; Imai and Yamaguchi, 2012). *Heterosigma akashiwo* produces compounds directly toxic to fish whereas in *P. verruculosa*, initially identified as *Chattonella* aff. *verruculosa* until molecular confirmation (Hara et al., 1994; Hosoi-Tanabe et al., 2007), secondary metabolites shown to be toxic for planktonic organisms and in cell lines are produced by the microalga's mucocytes (Skjelbred et al., 2011; Chang et al., 2014).

Previous studies reported anomalous meteorological and oceanographic conditions during blooms of *H. akashiwo* and *P. verruculosa* (Garreaud, 2018; León-Muñoz et al., 2018). These findings evidence the need for detailed studies of these two ichthyotoxic species conducted under controlled conditions (Andersen et al., 2015). In this study, we demonstrate the differential effects of temperature and salinity on the physiology and toxicity of *H. akashiwo* and *P. verruculosa* investigated under controlled laboratory conditions.

2. Material and methods

2.1. Origin and maintenance of H. akashiwo and P. verruculosa

Monoclonal *H. akashiwo* (strain CCM-UdeC 225) was originally isolated from the coast of Maullín, Chile (41°37′S; 73°36′W) by the Grupo de Investigación Microalgal, Universidad de Concepción during a bloom in April 2013. Monoclonal *Pseudochattonella verruculosa* (strain REL201603) was originally isolated from Reloncaví Fjord, Chile (41°38′S; 72°20′W) by the Centro i~mar, Universidad de Los Lagos during a bloom in the summer of 2016 (Fig. 1). Cultures of both strains were maintained in 250-mL Erlenmeyer flasks filled with 100 mL of L1 medium (Guillard and Hargraves, 1993), under controlled conditions of a temperature of 12 ± 1 °C, a salinity 34 ± 1 psu, an irradiance of 50 μmol m^{-2} s^{-1}, and a photoperiod of 14:10 (L:D).

2.2. Morphological characterization and strain variability

Detailed morphological analyses of *H. akashiwo* and *P. verruculosa* were performed using confocal microscopy. Living cells were observed by mounting culture samples on glass-bottomed chambers (35 mm) using ibidi mounting medium (IMM). Cell nuclei were visualized by fixing the samples in 2% formalin (10 min) followed by centrifugation at 1000 ×*g* for 1 min and resuspending the pellet in 2.5 mL of cold methanol. After an incubation of at least 12 h at 4 °C to facilitate pigment extraction, the cells were washed in PBS (pH 7, Sigma-Aldrich, St. Louis, MO, USA) using the same centrifugation conditions. The cells in the resulting pellet were resuspended in 300 μL of propidium iodide (Sigma-Aldrich, 60 μg·mL^{-1}) and 30 μL of RNaseA (Sigma-Aldrich, 100 mg·mL^{-1} in PBS) and incubated for at least 2 h in the dark prior to their observation. They were then mounted on the slides using IMM. Both living and fixed, stained cells were observed using a confocal LEICA SP8 microscope equipped with three laser lines (405, 488, and 552). Imaging was performed at 63× magnification using the super-resolution mode LIGHTNING. The images were optimized for best contrast and brightness and then analyzed using LASX software (LEICA Microsystems, Wetzlar, Germany).

2.3. Acclimation of HAB strains

Cultures of *H. akashiwo* and *P. verruculosa* were started using an initial inoculum of 10^3 cells mL^{-1} and were maintained using the same culture medium and controlled parameters described in Section 2.1. They were first acclimated to the experimental conditions for 1 month and then to the different salinity and temperature conditions for 2 months, following the methodology of Yamaguchi et al. (1997). Aliquots of each strain (10^3 cells mL^{-1}) were transferred to 250-mL Erlenmeyer flasks filled with 100 mL of L1 medium adjusted to a salinity of 10, 20, or 30 psu. The cultures were maintained at temperatures of 12, 15, or 18 °C under an irradiance of 50 m^{-2} s^{-1} and a photoperiod of 14:10 h (L:D).

2.4. Growth experiments

The combined effect of salinity and temperature on the growth of *H. akashiwo* and *P. verruculosa* was examined in triplicate cultures of each strain maintained for 15 days as described above. Two-mL aliquots were taken daily, fixed with 10% Lugol, and the cell densities (cells mL^{-1}) were determined using a Sedgewick-Rafter chamber and an inverted microscope (Olympus, CKX41). Every 3 days, 6 mL of L1 medium of the appropriate salinity was added to each culture. Growth curves were plotted and the growth rate (*k*, i.e., the doubling of biomass per day), described by Eq. (1) (Guillard, 1973), was calculated for each sample during the exponential phase as follows:

$$k = \frac{3.322}{t2 - t1} \times log\frac{N2}{N1} \quad (1)$$

where N_1 and N_2 are the cell density (cell mL^{-1}) at the beginning ($t1$) and end ($t2$) of the incubation period (days), respectively. While, the value 3.322 is the conversion factor to transform base 10 logarithm (log_{10}) in base 2 logarithm (log_2). The optimal growth rate under the combined temperature-salinity conditions was determined by calculating the maximum growth rate (*k*) reached by exponentially growing *H. akashiwo* and by *P. verruculosa*.

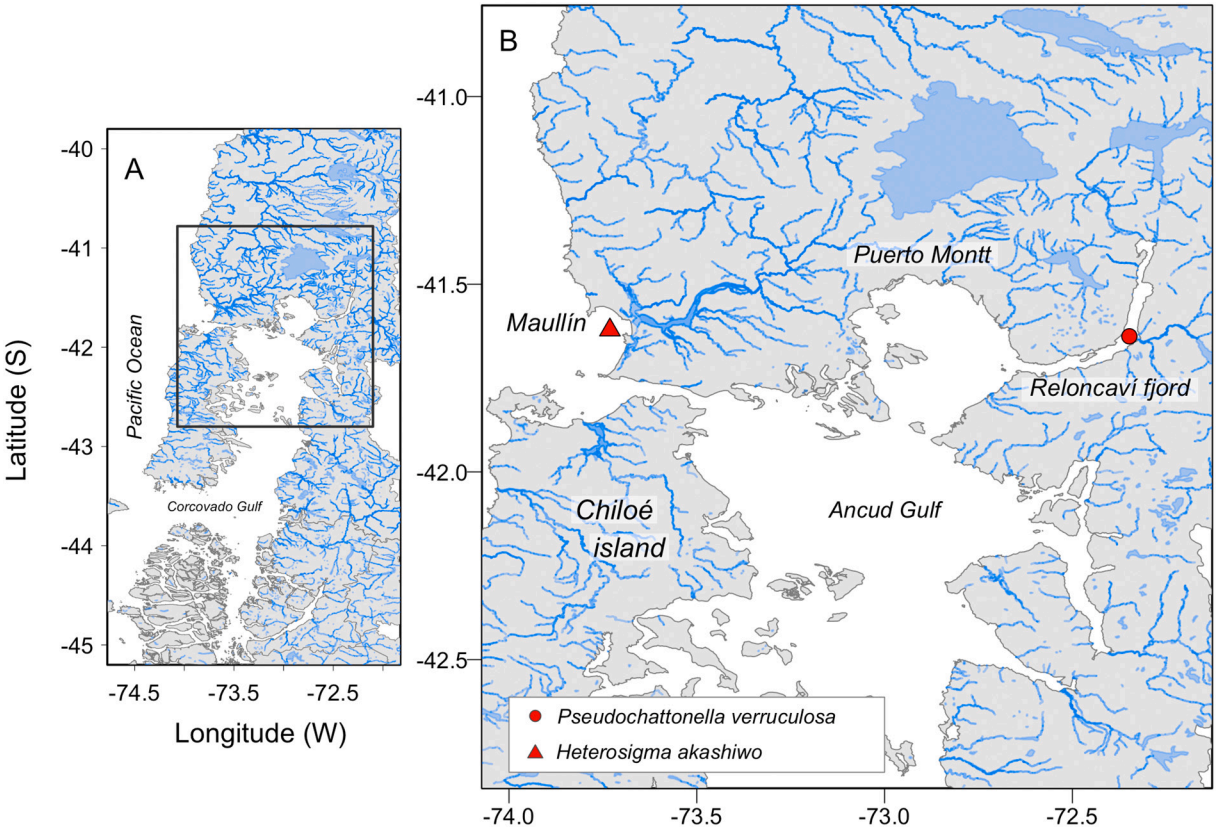

Fig. 1. Isolation site of *Heterosigma akashiwo* and *Pseudochattonella verruculosa*. A) Map of the Los Lagos and Aysén regions, Southern Chile; B) Areas of *H. akashiwo* and *P. verruculosa* blooms in 2013 and 2016, respectively.

2.5. Fish cell cultures

The cell line CHSE-214, derived from embryos of Chinook salmon, *Onchrhynchus tshawytscha*, was obtained from the Laboratorio de Biotecnología, Fundación Chile/Fraunhofer Chile, Puerto Montt (Collection of Authenticated Cell Culture, ECACC 91041114). The cells were cultured as described by Davoren et al. (2005) in 25-cm^2 culture flasks filled with L15 Leibovitz medium containing L-glutamine, 10% fetal bovine serum (v/v), and 100 IU penicillin/streptomycin (30-002-CI, Corning®) mL^{-1}. The cultures were incubated in the dark at 20 ± 1 °C.

In the microalgal exposure experiments, 48 h prior to their use CHSE-214 cells were detached from the culture flasks using 0.25% trypsin-2.21 mM EDTA and seeded in 96-well culture plates at a density of 5.0 × 10^4 cells mL^{-1}. The cell monolayer was adjusted to 2.5 × 10^5 cells mL^{-1}. To assess cell viability, 100 µL of a cell suspension (initial volume: 500 µL) was transferred to 1.7-mL microcentrifuge tubes and incubated with 100 µL of Trypan Blue. The sample was carefully mixed and a 12-µL aliquot of the cells was transferred a hemocytometer (Neubauer chamber). The number of living (non-dyed) cells was then counted.

2.6. Microalgal and cell lysate treatments

To determine the toxicity of *H. akashiwo* and *P. verruculosa* in cultured Chinook salmon cells, the microalgae were prepared at cell densities of 2 × 10^1; 2 × 10^2, 2 × 10^3, 2 × 10^4, and 2 × 10^5 cells mL^{-1} in 15-mL test tubes by serial dilution of the highest culture density (2 × 10^5 cells mL^{-1}) using L1 culture medium with a salinity of 20 or 30 psu. Cell lysates were also prepared from serial dilutions of the highest density culture. The cells were centrifuged at 4 × 10^3 rpm for 10 min and filtered using a Millipore Sigma filter 0.22 µm pore size.

2.7. Fish cell exposure

The viability of CHSE-214 cells exposed to *H. akashiwo* and *P. verruculosa* was determined using the methyl thiazole tetrazolium (MTT) assay as described by Mosmann (1983). The culture medium of CHSE-214 cells cultured for 48 h was discarded and replaced with 100 µL of each algal exposure treatment in quadruplicate samples. In the negative control wells, the culture medium was replaced with 100 µL of hydrogen peroxide (H_2O_2, 7.8 mM) and 100 µL of L1 medium. After a 1-h exposure, the content of the wells was replaced with 100 µL of Modified Eagle Medium (MEM with glutamine, sodium bicarbonate and no phenol red 11935-046; Gibco®) containing 10 µL of MTT reagent (Vybrant® MMT Cell Proliferation Assay kit V-13154, Invitrogen) at 5 mg mL^{-1} and incubated for 3 h at 20 °C. SDS-HCl (100 µL) solution was added to each well to dissolve the formazan salts and the plates were further incubated in the dark for 12 h at 20 °C. The absorbance at 570 nm was then measured with a microplate reader. A blank control, prepared in wells without a cell monolayer but containing MEM, MTT, and SDS-HCl, was used to adjust the absorbance. Cell viability was expressed as the percentage of live cells, calculated according to Eq. (2):

$$\text{Cell viability} = \%\frac{D.Ot}{D.Onc} \times 100 \quad (2)$$

where $D.O_t$ corresponds to the adjusted absorbance of the treatment (D·O 570 nm of the treatment–D·O 570 nm of the blank) and $D.O_{nc}$ to the adjusted absorbance of the negative control (D·O 570 nm of the growth control–D.O 570 of the blank).

2.8. Statistical analysis

To evaluate the effect of each experimental condition (salinity and temperature) on the growth pattern of *Herosigma akashiwo* and

Pseudochattonella verruculosa we fitted a logistic model according to Eq. (3):

$$n(t) = \frac{K'}{1 + \left(\frac{K'}{n0} - 1\right)e^{-rt}} \quad (3)$$

where K' is the carrying capacity, $n0$ the value at $t = 0$, and r the per capita growth rate (Bolker, 2008). The models were fitted using the "nlme" package (Pinheiro et al., 2020). To assess differences in the growth function between experimental conditions, a set of hypotheses corresponding to the different values of the parameters of the logistic function were assessed using the likelihood ratio test, implemented in the "lmtest" library available in R.2.14.0 (Zeileis and Hothorn, 2002). Preliminary we evaluated the following four hypotheses (H1-H4) for both species and the following two (H5-H6) only for P. verruculosa:

H1. All the experimental conditions show the same logistic growth parameters.

H2. All the experimental conditions show different logistic growth.

H3. Temperature experimental conditions show different logistic growth.

H4. Salinity experimental conditions show different logistic growth.

H5. Experimental condition 30 psu show the same growth at three temperature.

H6. Experimental condition 30 psu show dicerent growth at three temperatures.

The Akaike Information Criterion (AIC) to select the most informative model (Akaike, 1974), where the preferred model presented the minimum AIC value, considering a ≥2 unit difference (Burnham and Anderson, 2004), was applied.

The theoretical surfaces for the temperature and salinity conditions optimal for growth, defined as the values resulting in the maximum growth rate (k) obtained from Eq. (1) and maximum cell density of *H. akashiwo* and *P. verruculosa*, were evaluated using the response surface from the "rsm" package (Lenth, 2009). This package provides functions useful for designing and analyzing sequentially conducted experiments aimed at identifying optimal response surfaces. If no growth was detected, the experimental response was considered to be zero. All statistical procedures were carried out using the statistical and programming software R 2.1.12 (R Development Core Team, 2018).

3. Results

3.1. Morphological characterization of H. akashiwo and P. verruculosa

Heterosigma akashiwo cells were round to elongated, measuring 10–16 μm in length and 8–13 μm in width (Fig. 2). They contained numerous triangular to elongated chloroplasts located at the cell periphery (Fig. 3A, B, autofluorescence and overlay) as well as one forwardly directed flagellum (Fig. 3A, transmitted light and overlay). A large, round nucleus (diameter: 5–5.5 μm) was positioned at the center of the cell (Fig. 3A, B, transmitted light, and overlay) and contained a central, less condensed area with spherical borders (Fig. 3C, D, nuclear staining, and overlay).

Pseudochattonella verruculosa cells were ovoid to elongated, measuring 8–14 μm in length and 6–13 μm in width (Fig. 2). Thy contained numerous round to ovoid chloroplasts at the cell periphery and a forwardly directed flagellum (Fig. 4A, B). A rounded nucleus (diameter: 3.5 μm) was seen at the anterior end of the cell (Fig. 4A, transmitted light and overlay) and included a less condensed area in its center (Fig. 4C, D, nuclear staining and overlay). Cells with two nuclei were commonly observed in exponentially growing cultures (Fig. 4D) and presumably indicated a dividing cell or the presence of mobile zygotes (Chang et al., 2014).

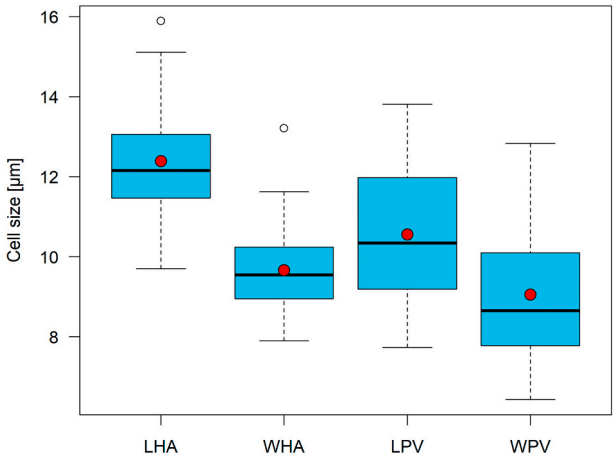

Fig. 2. Boxplot of the cell size (μm) of *H. akashiwo* and *P. verruculosa*. LHA: Length of *H. akashiwo*; WHA: width *H. akashiwo*; LPV: length of *P. verruculosa*; WPV: width of *P. verruculosa*. The red point indicates the median value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Growth experiments

Heterosigma akashiwo grew exponentially under all combinations of temperature and salinity (Fig. 5A–C). The growth rate (k) ranged between 0.57 and 0.99 day^{-1}, and the cell density between 1.22×10^5 and 3.09×10^5 cells mL^{-1}. The highest growth rate and cell density were obtained at 15 °C and a salinity of 20 psu (Fig. 5B), and the lowest growth rate and cell density at 12 °C and a salinity of 10 psu (Fig. 5A). Overall, however, the growth rate was highest (0.67–0.99 day^{-1}) at a salinity of 20 psu, regardless of the incubation temperature, and lowest at a salinity of 10 psu at all temperatures except 18 °C, at which growth was lowest at a salinity of 30 psu (0.8 day^{-1}). The most informative model (lower AIC) was the one that considered that all growth curves were different (Table 1). Only the growth curve of experiment (temperature 12 °C and salinity 10 psu) showed a carrying capacity (K') with high standard error, suggesting excessive uncertainty in the parameter estimation (even with a probability not different from 0). Thus, the highest K' values were observed at 12 °C, which contrasted with low per capita growth rate (r) values in the same experimental conditions. On the other hand, the higher r values (~5–7) were observed at temperatures of 15 and 20 °C and at all salinities. The optimal values of r were observed at 15 °C at 20 and 30 psu (Table 2).

High growth rates were reached by *P. verruculosa* only at a salinity of 30 psu (Fig. 5). The growth rate (k) of this strain was between 0.47 and 1.06 day^{-1}, and the cell density between 2.1×10^4 and 1.02×10^6 cell mL^{-1}. The highest growth rate was obtained at a temperature of 18 °C and a salinity of 30 psu (Fig. 5C) but the highest cell density was obtained at 15 °C and a salinity of 30 psu (Fig. 5B). The lowest growth rate and cell density were measured in cells cultivated at 12 °C and a salinity of 20 psu (Fig. 5A). Growth was inhibited at a salinity of 10 psu under all experimental temperatures (Fig. 5). For the evaluation of *P. verruculosa* growth models, the values with growth 0 (salinity 10 psu) were discarded, for which six experiments were evaluated. Since the H2 model did not converge (due to the shape of the curves of the experiment at salinity 20 psu), two alternative models (H5 and H6) were evaluated. These models only considered the experiments with salinity 30 psu and whose results for *P. verruculosa* indicated that the most informative model was the one that assumed the growth curves are different at temperatures of 12, 15 and 18 °C at 30 psu salinity (Table 1). The highest K' value was obtained at 15 °C, while the highest r value was observed at 18 °C (Table 3).

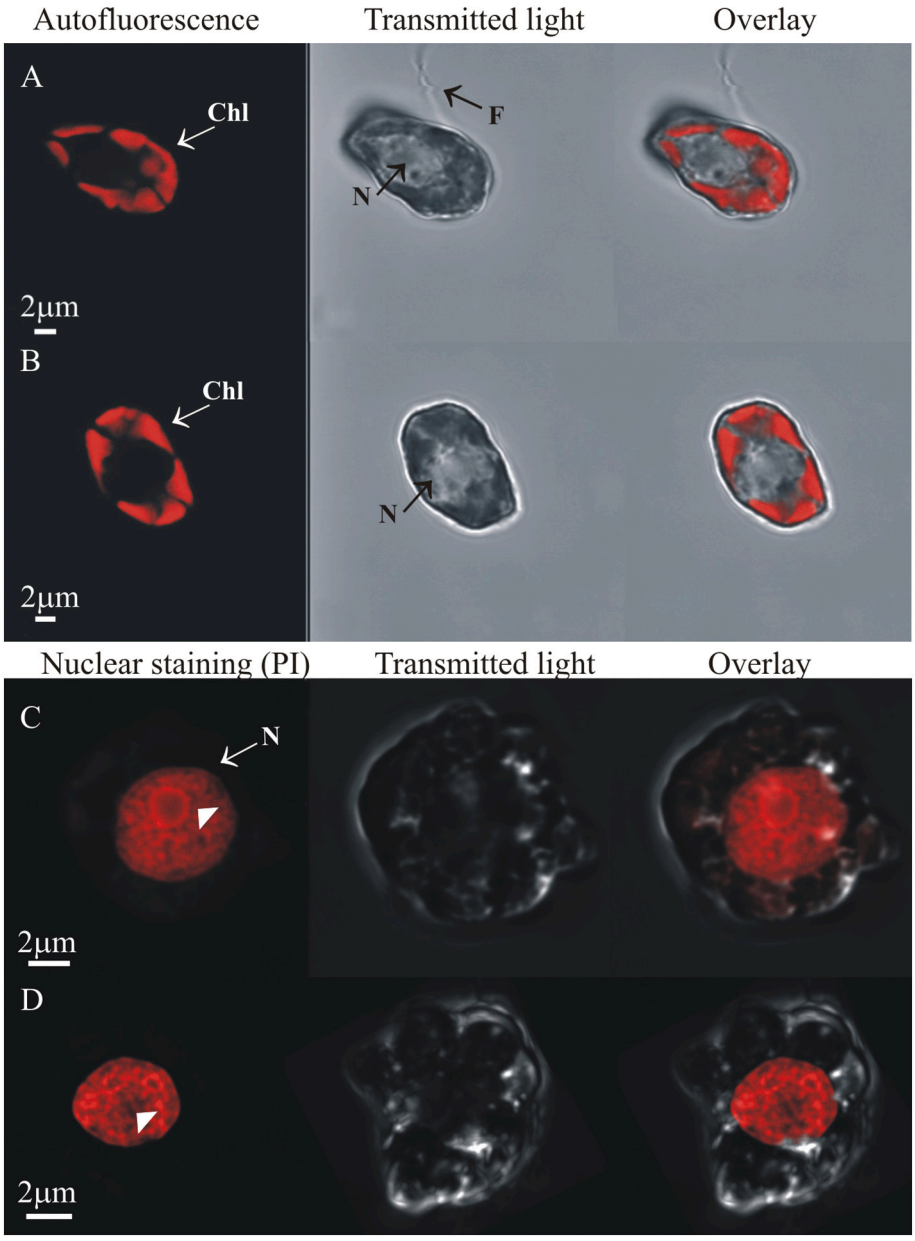

Fig. 3. Microscopy images of *H. akashiwo* cells. Living cells (A, B) and fixed cells with propidium-iodide-stained nuclei (C, D) are shown. (A, B) Autofluorescence (excitation 488 nm) reveals the shape and position of chloroplasts (Chl), and (C, D) transmitted light the flagella (F), nucleus (N), and cell morphology. Nuclear staining shows a roundish to oval nucleus (N) with a less-condensed area at its center (arrowhead), detected in all cells observed.

The theoretical surfaces for temperature and salinity, and thus the responses of the dependent variables, for the growth rate (k) and maximum density are shown in Fig. 6. For both microalgal species, the theorical response was non-linear and fitted and corresponded to a second-order model (Fig. 6). In the case of *H. akashiwo*, the theoretical growth rate response followed a dome-shaped curve with a maximum value at 15 °C (Fig. 6A), while for *P. verruculosa* the curve of the theoretical growth rate response had an inverted-dome shape with a minimum value at 15 °C (Fig. 6B). The curves of the theoretical cells densities of both microalgae were similar to those of the growth rate (Fig. 6C, D).

3.3. Fish cell exposure

The viability of CHSE-214 cells following a 1-h exposure to *H. akashiwo* differed depending on the microalgal cell density as well as the temperature and salinity of the microalgal culture medium (Fig. 7). Direct exposure to *H. akashiwo* at the lowest cell density (2×10^1 cell mL^{-1}) and cultured at a salinity of 30 psu induced a 100% decrease in the viability of the fish cells (Fig. 7A). Among the tested supernatants, that prepared from *H. akashiwo* cells cultured under the same conditions induced the highest reduction in cell viability (62% vs. the control). The viability of cells exposed to *H. akashiwo* cultured at a salinity of 20 psu was 45% of the respective control (Fig. 7C). Exposure to a lysate prepared from cells cultured at salinities of 30 and 20 psu reduced viability by 56% and 46%, respectively, compared to the controls (Fig. 7E). In all treatments, the toxicity of *H. akashiwo* for CHSE-214 cells was highest when the microalga was grown at a salinity of 30 psu; however, viability did not exceed 72% when the fish cells were exposed to the microalga cultured at a salinity of 20 or 30 psu.

The viability of CHSE-214 cells directly exposed to *P. verruculosa* cultured at a temperature of 18 °C and a salinity of 30 psu decreased by 23% (Fig. 7B). The largest decrease in cell viability was observed in

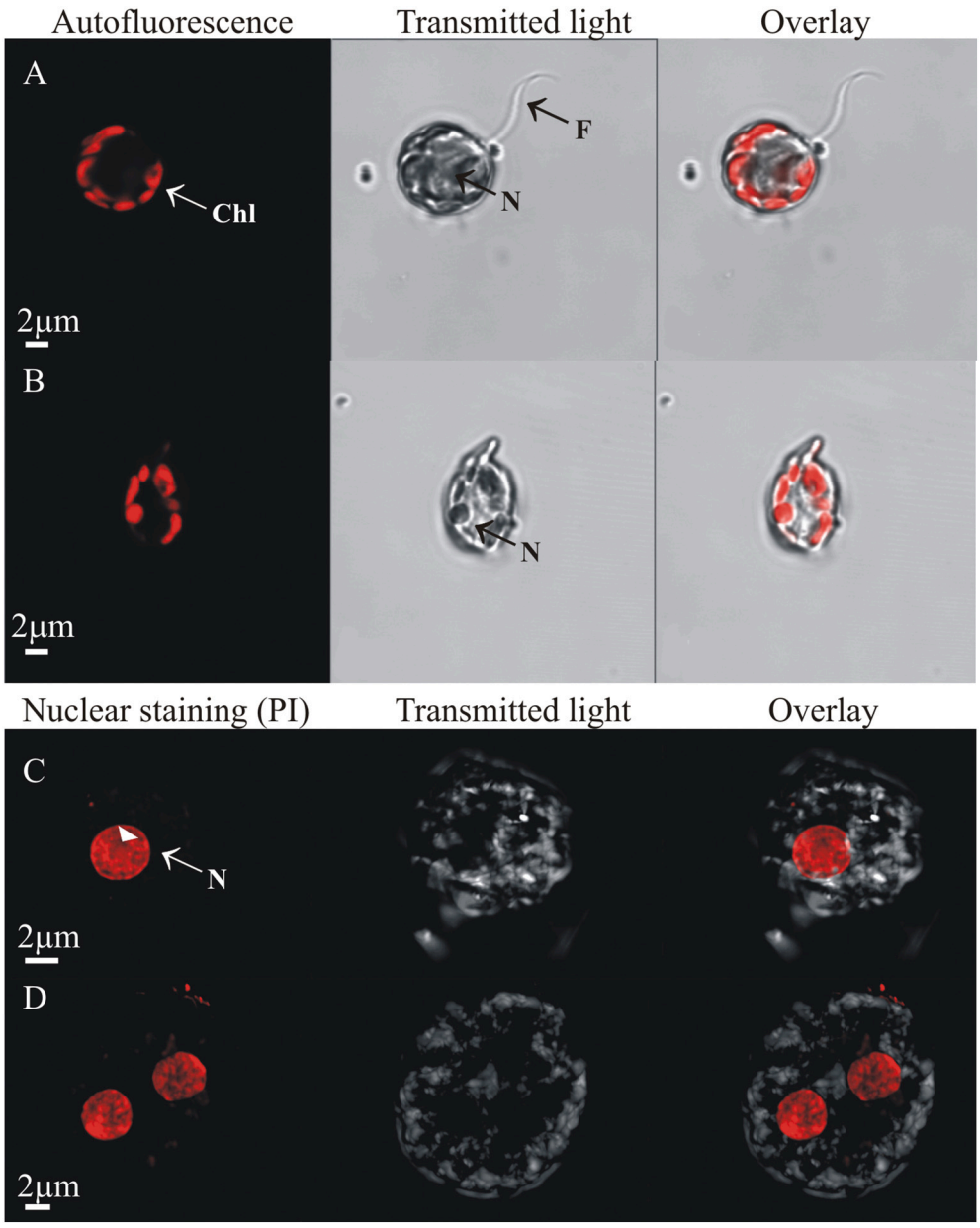

Fig. 4. Microscopy images of *P. verruculosa* cells. Living cells (A, B) and fixed cells with propidium-iodide-stained nuclei (C, D) are shown. (A, B) Autofluorescence (excitation 488 nm) reveals the shape and position of chloroplasts (Chl), and (C, D) transmitted light the flagella (F), nucleus (N) and cellular morphology. (C, D) Nuclear staining shows an almost spherical nucleus. In some cells, a less condensed central area (arrowhead) was detected.

treatments with a *P. verruculosa* supernatant (Fig. 7D). Treatments with a lysate of *P. verruculosa* grown under the same conditions induced a 28% decrease in fish cell viability (Fig. 7F). In all exposure experiments with *P. verruculosa*, the decrease in cell viability was proportionate to the increase in microalgal cell density.

4. Discussion

Changes in the environmental conditions of the Patagonian fjords system have favored the growth of *H. akashiwo* and *P. verruculosa*. Blooms of both species have caused the mass mortality of farmed salmon and thus important economic losses for the salmon industry. The bloom of 2016 was the largest fish-killing bloom on record (Díaz et al., 2019; Mardones et al., 2021).

4.1. Effects of temperature and salinity on the growth of H. akashiwo and P. verruculosa

Reloncaví Fjord and its sound comprise the northern limit of the system of fjords and channels of Patagonia and the area where most of Chilean aquaculture is located. However, in recent years this same area has suffered recurrent HABs (León-Muñoz et al., 2018; Díaz et al., 2019; Mardones et al., 2021). The salinity range of its waters is broad and mainly regulated by freshwater inputs from the Puelo River, with an annual flux of 650 m^3 s^{-1} (Castillo et al., 2016; León-Muñoz et al., 2018). Large-volume inflows of water are attributable to rainfall in winter and to meltwaters in spring. Together they produce a strong vertical stratification of the water column that limits the exchange of nutrients between the surface (mainly from rivers) and the deep layer (oceanic origin) (Silva et al., 1995). In 2016, a significant decrease in the flow of the Puelo River and a decrease in precipitation in the Los Lagos

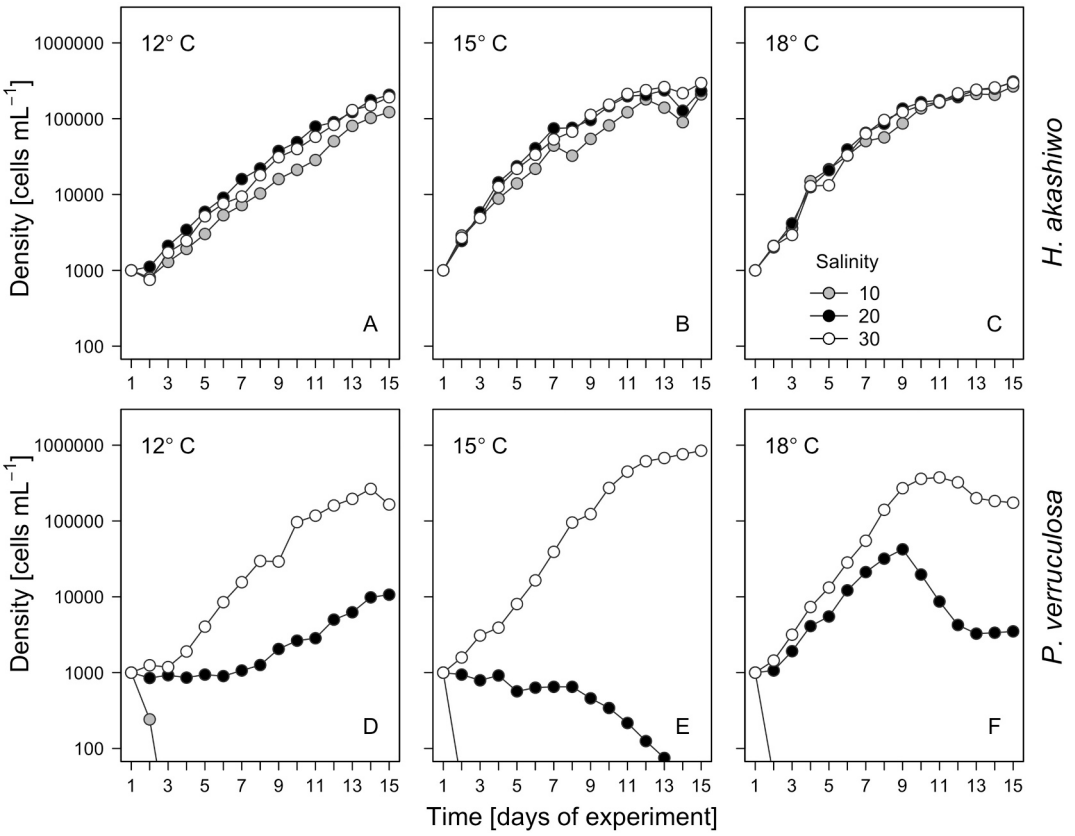

Fig. 5. Growth of *H. akashiwo* (upper panel) and *P. verruculosa* (lower panel) under three combinations of temperature (12, 15, 18 °C) and salinity (10,20,30). Values are expressed as the mean.

Table 1
Evaluation of different hypotheses for the logistic function parameters for *H. akashiwo* and *P. verruculosa*. The best fit model was determined using the lowest Akaike's information criterion (AIC) and showed by asterisk.

Hypotheses	Model	AIC
Heterosigma akashiwo		
H1	All the experimental conditions show the same logistic growth parameters	9852
H2	All the experimental conditions show different logistic growth	9258*
H3	Temperature experimental conditions show different logistic growth	9450
H4	Salinity experimental conditions show different logistic growth	9814
Pseudochattonella verruculosa		
H1	All the six experimental conditions in which growth was detected show the same logistic growth parameters	7233
H2	All the six experimental conditions show different logistic growth	Model did not converge
H3	Temperature experimental conditions show different logistic growth	7181
H4	Two salinity experimental conditions show different logistic growth	Model did not converge
H5	Experimental condition 30 psu show the same growth at three temperatures	3624
H6	Experimental condition 30 psu show different growth at three temperatures	3427*

The best fit model is showed by asterisk.

Table 2
Growth parameters of *Heterosigma akashiwo* obtained from logistic model.

Experiment (Temp_Sal)	Parameter	Estimate	Std. Error	t value	Pr(>|t|)
12_10	*K′1*	8.14E+05	1.72E+06	0.474	0.635458
12_20	*K′2*	4.14E+05	1.10E+05	3.748	0.000205
12_30	*K′3*	4.46E+05	1.63E+05	2.735	0.006521
15_10	*K′4*	1.73E+05	1.07E+04	16.1	<2e−16
15_20	*K′5*	2.09E+05	7.45E+03	28.036	<2e−16
15_30	*K′6*	2.76E+05	9.05E+03	30.479	<2e−16
18_10	*K′7*	2.58E+05	1.18E+04	21.904	<2e−16
18_20	*K′8*	2.81E+05	1.01E+04	27.705	<2e−16
18_30	*K′9*	2.88E+05	1.08E+04	26.588	<2e−16
12_10	*r1*	3.35E−01	2.18E−02	15.346	<2e−16
12_20	*r2*	4.03E−01	1.33E−02	30.334	<2e−16
12_30	*r3*	3.89E−01	1.43E−02	27.237	<2e−16
15_10	*r4*	5.85E−01	2.17E−02	26.947	<2e−16
15_20	*r5*	7.06E−01	1.99E-02	35.498	<2e−16
15_30	*r6*	6.62E−01	1.39E−02	47.746	<2e−16
18_10	*r7*	5.53E−01	1.29E−02	42.967	<2e−16
18_20	*r8*	5.87E−01	1.20E−02	49.029	<2e−16
18_30	*r9*	5.76E-01	1.16E−02	49.753	<2e−16

region resulted in one of the driest years of the past 70 years (Aguayo et al., 2019). In March 2016, the flow of the Puelo River decreased to less than half of the average historical flow recorded between 1950 and 2016 (León-Muñoz et al., 2018). The reductions in freshwater flow and precipitation caused the salinity of the surface water to increase (>31 psu), such that the concentration of nutrients, notably nitrate, increased as well (Rivera et al., 2017). These conditions favored the growth of *P. verruculosa* and resulted in a massive bloom (Clément et al., 2016). Similarly, in the present study the growth of *P. verruculosa* was highest at the highest tested salinity (30 psu). Under the usual conditions governing the Reloncaví Fjord system, the environmental barrier established by freshwater inputs would have hindered the growth of *P. verruculosa*, although not of *H. akashiwo*, which is able to grow over a wide salinity range (in this study, 10–30 psu). Martínez et al. (2010)

Table 3
Growth parameters of *Pseudochattonella verruculosa* obtained from logistic model.

Experiment (Temp_Sal)	Parameter	Estimate	Std. Error	t value	Pr(>|t|)
12_30	*K′1*	2.58E+05	6.31E+04	4.092	4.80E−04
15_30	*K′2*	8.96E+05	4.53E+04	19.78	<2e−16
18_30	*K′3*	2.73E+05	2.08E+04	13.166	<2e−16
12_30	*r1*	4.94E−01	4.51E−02	10.97	<2e−16
15_30	*r2*	6.71E−01	1.38E−02	48.61	<2e−16
18_30	*r3*	7.71E−01	5.32E−02	14.478	<2e−16

showed little or no growth of *H. akashiwo* at a salinity of 5 psu. In line with that result and with ours, Yamochi and Abe (1984) reported that *H. akashiwo* has the capacity to grow at low salinity (5.7 psu). The tolerance of this euryhaline flagellate of a salinity amplitude ranging between 5 and 30 psu would enhance its bloom-forming potential and allow it to escape predation (by ciliates), by moving to waters of higher or lower salinity (Strom et al., 2013).

The main factor controlling microalgal growth is thus far unknown. Kirst (1990) suggested that microalgal growth depends mainly on irradiance and temperature, whereas according to Yamochi and Abe (1984) temperature and salinity are the main determinants as they control the availability of nutrients such as nitrogen, phosphorus, and iron. Skjelbred et al. (2013) suggested that the growth of *Pseudochattonella* strains is light-dependent and optimal temperature and salinity conditions. Mardones et al. (2012) showed that the ecological niches of *H. akashiwo and P. verruculosa* are similar, such that both species are present at water temperatures of 11.2–17.3 °C and salinities of 11.4–33.3 psu, with a higher abundance of *H. akashiwo* at a salinity of 25 psu, consistent with the results of the present study. Reports in the international literature have described large blooms formed by members of the Raphidophyceae at environmental temperatures >15 °C but much smaller blooms at temperatures <15 °C and no blooms at temperatures <10 °C (Steidinger and Meave del Castillo, 2018). In a study of a Chilean strain of *P. verruculosa*, Mardones et al. (2019) reported a maximum cell density (8.43×10^4 cells mL^{-1}) at 15 °C and a salinity of 30 psu, and a growth rate of 1.44 day^{-1} at 15 °C and a salinity of 20 psu. However, the authors did not study the combined effect of temperature and salinity, which under natural conditions can influence toxin production (e.g. Paz et al., 2006) and other physiological functions (Figueroa et al., 2011), as shown for other microalgal species.

The cell densities measured in our study were higher than those determined in other studies, and at a water temperature of 15 °C and a salinity 30 psu they exceeded those reported by Mardones et al. (2019) by an order of magnitude (8.8×10^5 cells mL^{-1}). The corresponding growth rate at 18 °C and a salinity of 30 psu was 1.06 day^{-1} (Fig. 5). The fact that Mardones et al. (2019) used the same strains of *P. verruculosa* implies that the differences in cell density and the growth rate were not intrinsic, as both parameters are likely influenced by temperature, salinity, culture medium, photoperiod, and irradiance, all of which differed between the two studies. Instead, we showed that temperature and salinity are interdependent factors controlling the growth and ichthyotoxicity of harmful microalgae such as *H. akashiwo and P. verruculosa*.

Clément et al. (2016) reported that during the 2016 bloom event in the Los Lagos region, the water temperature was 15.1 °C and the salinity 32.5 psu, conditions similar to those tested in the present study

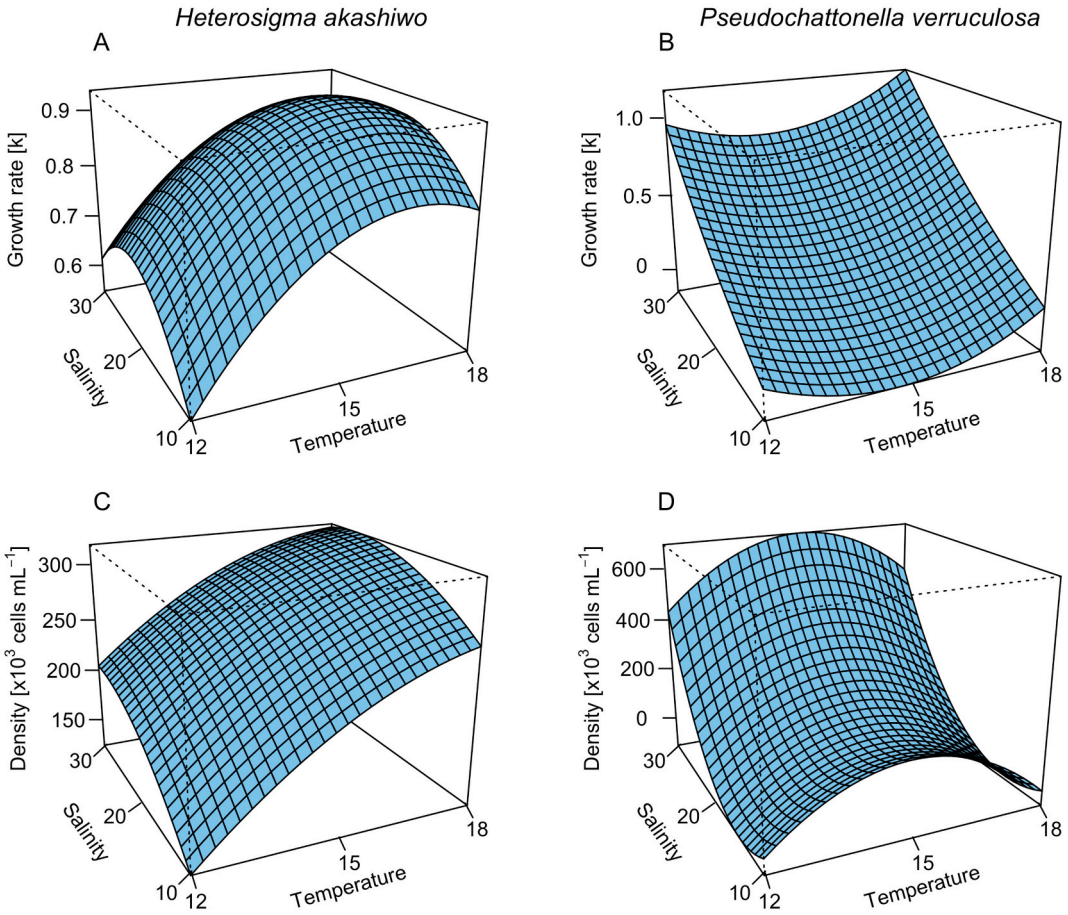

Fig. 6. Theorical responses to the combined effects of temperature and salinity on the growth rate (upper panel) and cell density (lower panel) of *H. akashiwo* (left panels) and *P. verruculosa* (right panels) as determined in a response surface analysis.

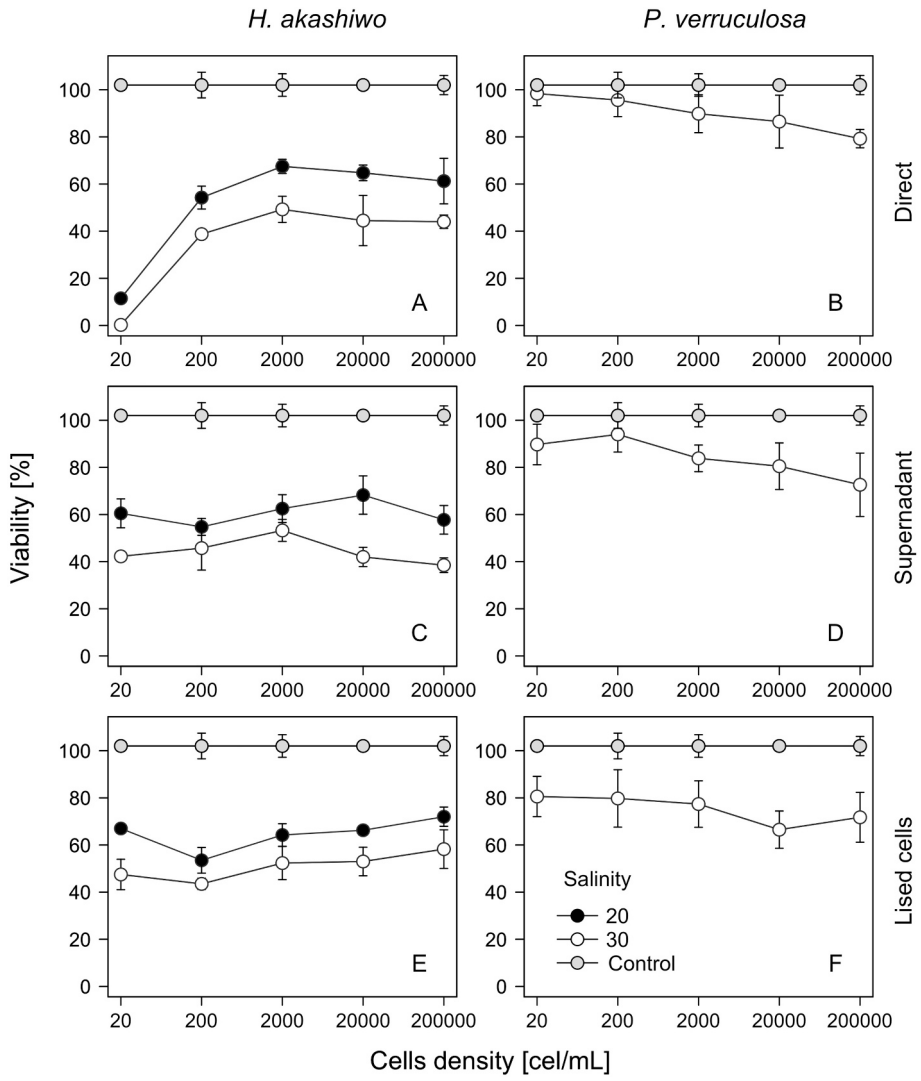

Fig. 7. Viability of Chinook salmon cells (CHSE-214) following a 1-h exposure to three treatments with *H. akashiwo* (left panels) and *P. verruculosa* (right panels) at five cell densities and two combinations of temperature and salinity (15 °C and salinities of 20 and 30). Values are expressed as the mean ± S.D.

(temperature of 15 °C and a salinity of 30 psu) and supporting high cell densities of *P. verrculosa*. Eckford-Soper and Daugbjerg (2017) measured the growth parameters of *Pseudochattonella* in the laboratory and demonstrated growth over a temperature range of 10–20 °C and salinities of 19–25 psu.

The results of our study provide additional evidence that *H. akashiwo and P. verruculosa* exploit different ecological niches. While *H. akashiwo* is able to reach high abundances over a wide range of salinities and temperatures, *P. verruculosa* requires a high salinity (30 psu) and a lower temperature (15 °C) for its optimal growth.

4.2. Ichthyotoxicity of H. akashiwo and P. verruculosa

Several fish cell lines, such as RTgill-W1, EPC, and especially the Chinook salmon embryo cell line CHSE-214, have been used to evaluate ichthyotoxicity (Davoren et al., 2005; Skjelbred et al., 2011; Astuya et al., 2018). The utility of the MTT colorimetric assay to determine the effects of certain metals (Srikanth et al., 2017), fatty acids (Rubio Mejia, 2015), and harmful microalgae (Astuya et al., 2018) has also been demonstrated.

Both CHSE-214 cells and the MTT assay were used to examine the lytic activity of *H. akashiwo and P. verruculosa*. A low density (2×10 cells mL^{-1}) of *H. akashiwo* cells cultured at a salinity of 30 psu caused a 100% loss of fish cell viability. As microalgal cell density increased, fish cell viability increased but it did not exceed 50% at microalgal cell densities $>2 \times 10^3$ cells mL^{-1}. Similarly, the viability of CHSE-214 cells directly exposed to a low density of *H. akashiwo* cells (2×10 cells mL^{-1}) cultured at a salinity of 20 psu was reduced by 90% and viability did not exceed 65% at *H. akashiwo* cell densities $>2 \times 10^3$ cells mL^{-1}. These results are in line with field studies, which determined that the mortality of salmon produced in southern Chile was as high as 40% following exposure of the fish to *H. akashiwo* at low cell abundances, with a very high level of toxicity determined at a cell density as low as 40 cells mL^{-1}. The reasons for the paradoxical higher toxicity of low-density than high-density microalgal cells is unclear. Bacteria produce toxins only at high cell densities, with coordinated production resulting in more potent effects (Doekes et al., 2019 and references therein). The factors promoting or inhibiting toxin production in marine microalgae have not been well studied, but in contrast to bacteria, microalgal toxins seem to be synthesized as by-products of core catalytic pathways (Verma et al., 2019).

The results obtained in this study for *P. verruculosa* differed from those of field studies, in which the highest toxicity reportedly occurred at a low cell density (~5 cells mL^{-1}) (Mardones et al., 2012). Instead, fish cell viability decreased by a maximum of ~40% in cultures exposed for 1 h to 2×10^5 *P. verruculosa* cells mL^{-1}. Consistent with this result,

Mardones et al. (2019) found that a 1-h exposure of RTgill-W1 cells to a Chilean strain of *P. verruculosa* at a density of 10^5 cells mL^{-1} decreased the viability of the fish cells by up to 45%. Our study also showed that fresh extracts of *P. verruculosa* and *H. akashiwo* were highly toxic, a finding consistent with that reported by Mardones et al. (2019). In fact, unlike the study of Andersen et al. (2015), in which *Pseudochattonella* cells themselves were more toxic than either cell lysates or culture supernatants, we found that the viability of CHSE-214 cells exposed to intact cells of *P. verruculosa* was reduced by only 22% whereas stronger effects occurred in fish cells exposed to cell lysates and supernatants. Conversely, direct exposure to intact *H. akashiwo* cells resulted in a greater toxicity. These results suggest that the lytic compounds of *P. verruculosa* are released whereas those of *H. akashiwo* are attached to the cell.

The ichthyotoxicity pathways of *H. akashiwo* and *P. verruculosa* are also not well understood, but in members of the Raphidophyceae and Dictyochophyceae the production of free fatty acids (FFA), reactive oxygen species (ROS) (Marshall et al., 2003), polyunsaturated fatty acids (PUFA) (Andersen et al., 2015), and brevetoxins (Dorantes-Aranda et al., 2015) has been invoked. ROS generation might be aimed at reducing the growth of other species competing for the same niche and/or as a defense mechanism to ward off possible predators, or to signal unfavorable environmental conditions (Diaz and Plummer, 2018). Other studies have also implicated ROS, alone or in combination with PUFA, in the toxicity of some species of microalgae (Marshall et al., 2003; Skjelbred et al., 2011; Mardones et al., 2015). Whether *H. akashiwo* and *P. verruculosa* release PUFA or generate extracellular ROS has not been experimentally demonstrated, nor has the involvement of these compounds in ichthyotoxicity, although the production of PUFA by *Pseudochattonella* has been reported (Dittami and Edvardsen, 2012). Marshall et al. (2003) found that *Chattonella marina* cells are ruptured when they are sucked into the gill lamellae of breathing fish, with the subsequent release of their contents, including high levels of ROS and FFA. The resulting lipid peroxidation in the gills of the fish reduces both respiratory and osmoregulatory capacity, allowing the transfer of FFA and oxygen into the bloodstream to cause the death of the fish. Similar results were reported for shellfish larvae, in which direct contact of the velum with *H. akashiwo*, *C. antiqua*, and *C. marina* resulted in mucosal trapping and the subsequent death of the larvae, with higher mortalities induced by *C. marina* and *C. antiqua* than by *H. akashiwo* (Basti et al., 2016). In a recent study, however, the activation by these raphidophytes of detoxification enzymes (GST) rather than antioxidant enzymes (SOD) involved in ROS effects was demonstrated (Basti et al., 2021). Thus, the mechanisms of oxidative stress imposed by ichthyotoxic species are likely complex.

The present study also showed the significant effect of salinity on the ichthyotoxic potential of *H. akashiwo* and *P. verruculosa*, with a salinity of 30 psu inducing a greater toxicity of the fish cell cultures than a salinity of 20 psu in all exposure treatments. However, salinity had less effect on the ichthyotoxicity of *H. akashiwo* than on that of *P. verruculosa*. Moreover, the ichthyotoxicity of intact *H. akashiwo* cells was higher at low cell densities and lower at high cell densities but under all conditions higher than that of *P. verruculosa*. In the latter, ichthyotoxicity was dose dependent, increasing with increasing cell density.

5. Conclusions

This study examined the combined effect of temperature and salinity on the growth of fish-killing Chilean strains of *H. akashiwo* and *P. verruculosa* and revealed differences in the optimal growth condition of these two microalgal species. While the overall growth rate of *P. verruculosa* was higher than that of *H. akashiwo*, the latter species was able to grow under all of the tested temperature and salinity conditions. By contrast, optimal growth of *P. verruculosa* was obtained only at the highest salinity (30 psu), independent of the temperature, which suggests that salinity is an important determinant of the ichthyotoxic potential of this species. The high growth rate of *P. verruculosa* at high salinity and temperature would explain the exceptional bloom of this species, and the high-level of ichthyotoxicity, that occurred in Southern Chile in 2016. However, the ability of *H. akashiwo* to reach high cell densities over a wide range of salinities and temperatures points to the potential for future HABs of this species in areas not previously affected. Both *H. akashiwo* and *P. verruculosa* have been associated with the mass mortality of fish, especially salmon. *H. akashiwo* is ichthyotoxic at low cell densities and via direct cell contact while the ichthyotoxicity of *P. verruculosa* requires high cell densities and is mediated by the release of intracellular lytic components. Given the anomalous meteorological and oceanographic conditions associated with blooms of Chilean strains of *H. akashiwo* and *P. verruculosa*, the role of other factors in the proliferation of these marine microalgae should be explored. For example, high nitrate levels were present during the 2016 bloom while other studies suggest a role for ROS and PUFA. Furthermore, whether other growth conditions, such as the stationary growth phase, increase or decrease the ichthyotoxic potential of either species remains to be determined.

CRediT authorship contribution statement

Alondra Sandoval-Sanhueza: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Alejandra Aguilera-Belmonte:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing. **Leila Basti:** Validation, Writing – original draft, Writing – review & editing. **Rosa I. Figueroa:** Methodology, Software, Validation, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Carlos Molinet:** Software, Formal analysis, Investigation, Writing – review & editing, Visualization. **Gonzalo Álvarez:** Methodology, Validation, Investigation, Writing – review & editing. **Sandra Oyanedel:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – review & editing, Supervision. **Pilar Riobó:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Supervision. **Guido Mancilla-Gutiérrez:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. **Patricio A. Díaz:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

We have not financial interests/personal relationships which may be considered as potential competing interests.

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