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Short Communication

Screening of Chilean fish-killing microalgae using a gill cell-based assay

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ABSTRACT. Fish-killing algal species are responsible for important global economic losses to the finfish industry. Chile is the world's second-largest salmon exporter, and fish-killing algal blooms have widely impacted its production in the last decades. The lack of standardized analytical methods to quantify and characterize the so-called "ichthyotoxins" has hindered our understanding of the underlying ichthyotoxic modes of action. The novel application of a highly sensitive and reproducible fish RTgill-W1 cell line-based assay has allowed significant progress in the field. In this study, the ichthyotoxic potency of the main microalgae species, which has been reported in fish-killing events in the historical Chilean monitoring programs, was assessed. The dinoflagellate *Karenia selliformis* was the most ichthyotoxic species against the RTgill-W1 (cell viability down to 8%), representing the major threat for the local salmon industry. In comparison, the raphidophyte *Heterosigma akashiwo* and the dinoflagellate *Prorocentrum micans* were the least toxic (gill cell viability down to 81 and 89%, respectively). Importantly, ichthyotoxic flagellates were more toxic to fish gill cells upon rupture. These results have important implications for the mitigation and management of algal blooms by the salmon industry.
Keywords: harmful algal blooms; salmon farming; ichthyotoxic; gill damage; RTgill-W1; fish-kills; Chilean fjords

The 2016 "Godzilla Red Tide" event caused the most catastrophic episode in the history of the Chilean aquaculture due to the convergence of two potent toxic microalgae, the dictyochophyte *Pseudochattonella verruculosa* and the dinoflagellate *Alexandrium catenella*. This massive event produced the largest fish farm mortality ever recorded worldwide (export loss of USD 800 million), which when combined with PSP shellfish toxicity (~15% reduction in harvest compared to 2015), resulted in major social unrest and rioting in the south of Chile (Trainer *et al*., 2020). Historical fish-killing harmful algal bloom (HAB) events have, however, been reported along the southern Chilean fjords since the development of the salmon farming industry in 1982 (Table 1). Fish mortalities have been often associated with the rapid growth of harmful microalgal species, but this noxious effect can also be associated with harmless species because high biomass of any phytoplankton can generate oxygen depletion in the water column at the end of bloom due to bacterial degradation, as well as gas-bubble trauma due to extreme oxygen saturation from algal photosynthesis (Lutz, 1995). However, some microalgal species can kill fish at low cell concentrations via a high impact on the gill tissues. Two main mechanisms for fish gill damage by microalgae have been proposed: 1) damage due to the presence of penetrating or cutting micro-structures, and 2) due to the production of the so-called "ichthyotoxins" (Hallegraeff *et al*., 2017). Research on the latter has been an enormous scientific challenge because the production of these metabolites often differs from the well-studied paralytic, diarrheic, amnesic and neurotoxic phycotoxins.

The novel application of a standardized and highly sensitive, repeatable and reproducible rainbow trout *Oncorhynchus mykiss* RTgill-W1 cell line assay (Fischer *et al*., 2019) has become a popular screening tool for assessing toxicity of algal blooms in several im-

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Table 1. Summary of the main historical fish-killing algal blooms recorded in the south of Chile. The most damaging events are ranked according to their economic impact (Harmful effect: X -unknown, I -ichthyotoxic, M -mechanical gill damage).

Ranking	Species	Date	Location	Region	Impact	Harmful effect	Reference
	Prorocentrum micans	1983	Seno Reloncaví	Los Lagos	Wild fish mortality	X	Lembeye & Campodonico (1984)
6	*Heterosigma akshiwo*	September, 1988	Seno Reloncaví, Hornopirén	Los Lagos	Salmon Mortality (>5,000 t)	I	Clément & Lembeye (1993)
	Leptocilyndrus inimum	November, 1989	Chiloé	Los Lagos	Salmon mortality	M	Clément (1994)
	Chaetoceros convolutus	July, 1991	Hornopirén	Los Lagos	Salmon mortality	M	Fuica *et al.* (2007)
	Leptocilyndrus minimum	October and December, 1993	Chiloé Island and Calbuco	Los Lagos	Salmon mortality	M	Clément (1994)
	Leptocilyndrus minimum	January and December, 1994	Chiloé Island	Los Lagos	Salmon mortality	M	Fuica *et al.* (2007)
	Chaetoceros convolutus	January-April, 1995	Seno Reloncaví-Chiloé Island	Los Lagos	Salmon mortality	M	Fuica *et al.* (2007)
	Dictyocha speculum	March, 1995	Chiloé Island	Los Lagos	Salmon mortality	M	Fuica *et al.* (2007)
	Chaetoceros convolutus	May, 1997	Seno Reloncaví	Los Lagos	Salmon mortality	M	Fuica *et al.* (2007)
	Leptocilyndrus minimum	July and December, 1998	Seno Reloncaví-Chiloé Island	Los Lagos	Salmon mortality	M	Fuica *et al.* (2007)
	Karenia selliformis	March, 1999	Chiloé Island	Los Lagos	Salmon mortality	I	Clément *et al.* (2001); Guillou *et al.* (2002)
	Chaetoceros convolutus	May, 1999	Seno Reloncaví	Los Lagos	Salmon mortality	M	Fuica *et al.* (2007)
	Heterosigma akashiwo	March, 2000	Seno Reloncaví	Los Lagos	Salmon mortality	I	Fuica *et al.* (2007)
	Heterosigma akashiwo	March, 2002	Reñihue	Los Lagos	Salmon mortality	I	Mardones *et al.* (2012)
5	*Alexandrium catenella*	October-April, 2002	Chiloé Island-Moraleda Channel	Los Lagos-Aysén	Three human fatalities/50 intoxicated/ 1,800 t salmon mortality	I	Molinet *et al.* (2003)
	Leptocilyndrus danicus	February, 2004	Lemuy Channel	Los Lagos	Salmon mortality	M	Fuica *et al.* (2007)
	Pseudochattonella spp.	2004	Seno Reloncaví-Chiloé Island	Los Lagos	Salmon mortality	I	Mardones *et al.* (2012)
4	*Alexandrium catenella*	2006	Chiloé Island-Aysén	Los Lagos-Aysén	One human fatality/seven intoxicated/salmon mortality	I	Fuentes *et al.* (2008)
	Pseudochattonella spp.	2009	Seno Reloncaví-Chiloé Island	Los Lagos	Salmon mortality	I	Mardones *et al.* (2012)
3	*Alexandrium catenella*	February-April, 2009	43 to 47°S	Los Lagos-Aysén	Two human fatalities/20 intoxicated/salmon mortality (>USD 10M)	I	Mardones *et al.* (2010, 2015)
	Chaetoceros criophylus	June, 2014	Seno Reloncaví	Los Lagos	Salmon mortality	M	INTESAL*
	Thalassiosira pseudonana	December, 2014	Aysén fjord	Aysén	Salmon mortality	X	INTESAL*
	Thalassiosira pseudonana	March, 2016	Seno Reloncaví	Los Lagos	Salmon mortality	X	INTESAL*
2	*Pseudochattonella verruculosa*	January-March, 2016	Seno Reloncaví-Chiloé Island	Los Lagos	Salmon mortality (>US$800M; 40,000 t)	I	Clément *et al.* (2016); Mardones *et al.* (2019)
1	*Alexandrium catenella*	March-May, 2016	offshore bloom; 39 to 45°S	Aysén, Los Lagos, Los Ríos	Massive mortality of marine species	I	IFOP (2016)
	Karenia spp.	January-February, 2017	46°S	Aysén-Magallanes	Salmon mortality (121 t)	I	SERNAPESCA (2017)*
	Karenia selliformis	February, 2018	Quellón, Chiloé	Los Lagos	Salmon mortality (1,600 t)	I	SERNAPESCA (2018)*
	Pseudochattonella verruculosa; Vicicitus globosus	March, 2019	Pilpilehue, Chiloé	Los Lagos	Salmon mortality (150 t)	I	SERNAPESCA (2019)*

*Data from the Instituto Tecnológico del Salmon (INTESAL, Chile) were obtained from monthly reports kindly provided by INTESAL's data managers and data from the Servicio Nacional de Pesca (SERNAPESCA, Chile) were obtained from annual salmon farming health reports (www.sernapesca.cl).

pacted coasts (Tanneberger *et al.*, 2013; Dorantes-Aranda *et al.*, 2015; Natsch *et al.*, 2018). Through this cell line assay, ichthyotoxic compounds produced by Chilean strains of *A. catenella* and *P. verruculosa* have been initially explored (Mardones *et al.*, 2015, 2018, 2019). Synergism between the long-chain (≥C20) polyunsaturated fatty acids (PUFA) docosahexaenoic acid (DHA, 22:6ω3; 16-20% of total fatty acids) and

reactive oxygen species (ROS -as superoxide anion) has been suggested as a primary ichthyotoxic mechanism in *A. catenella*, but cannot be explained by paralytic shellfish toxins (Mardones *et al*., 2015, 2018). Furthermore, the role of mucocysts has been pointed for a more in-depth research insight, which could provide a greater understanding as to the harmful mode of action of *P. verruculosa* (Mardones *et al*., 2019).

Ichthyotoxic microalgae is a major concern for the Chilean Government, and thus highly expensive monitoring programs are conducted along the southern Patagonian coast. Despite such monitoring efforts, many of the fish-killing microalgae species that occur in the Chilean fjords are practically unstudied concerning their potential toxicities. As a result, it is frequent that Chilean monitoring programs alert fish farmers about the presence of a "putative" ichthyotoxic microalgae species only based on their ichthyotoxic characteristics reported in other coastal areas.

This study aims to assess the ichthyotoxic potency of the main microalgae species that has been reported in fish-killing events in the historical Chilean monitoring programs, in order to rank their potential impact on the local aquaculture.

Nine potentially ichthyotoxic microalgae strains and one non-toxic species (*Teleaulax* sp. as a control treatment) were obtained from the Harmful Algal Collection of the Centro de Estudios de Algas Nocivas in Chile (CREAN/IFOP) (Table 2). Algae were grown in F/2 medium using seawater at 30 of salinity and kept at 17°C under 16:8 h light:dark cycle at 100 µmol photons m^{-2} s^{-1} of light intensity (cool white fluorescent lamp). The gill cell line RTgill-W1, originally from rainbow trout (Bols *et al*., 1994), was obtained from the American Type Culture Collection (CRL-2523, ATCC). Cells were cultured at 17ºC in the dark in Leibovitz's L-15 medium (L1518 Sigma), supplemented with 10% (v/v) fetal bovine serum (FBS, 12003C, Sigma), and an antibiotic-antimycotic solution (A5955, Sigma) containing amphotericin B (25 mg mL^{-1}), streptomycin (10 mg mL^{-1}) and penicillin (10,000 units mL^{-1}) in 25 cm^2 culture-treated flasks (3100-025, Iwaki). 0.25% trypsin-0.02% EDTA in Hank's balanced salt solution (59428C, Sigma) was used to detach cells that grew as an adherent monolayer at the bottom of the flask. Subcultures were normally established twice per week at a ratio of 1:2 with L-15 medium renewal.

The cytotoxicity assay with the 10 microalgae strains was carried out using conventional multi-well microplates, according to Dorantes-Aranda *et al*. (2011). Cultures with confluent gill cells were trypsinized (59428C, Sigma) for detachment, counted using a hemocytometer and adjusted to a concentration of 2×10^5 cells mL^{-1} in L-15 medium. Subsequently, gill cells were seeded in quadruplicate in 96-well flat-bottom microplates (3860-096, Iwaki, Japan), using a volume of 100 µL per well. After 48 h at 17ºC in the dark for gill cell attachment, L-15 medium was discarded, the cells rinsed with PBS and exposed to 100 µL of lysed cells and supernatant medium from the 10 strain cultures at 30 of salinity at 17ºC in the dark. Supernatant and lysed cell treatments were prepared from all cultures in the exponential growth phase at 2,000 cells mL^{-1}. The lysed cell suspension was prepared by sonication of diluted samples for 10 min at an amplitude of 10 µm peak to peak at 17ºC and filtered using a syringe with a nylon filter (0.22 µm). After two hours exposure, the viability of the gill cells was determined using L-15/ex medium (Schirmer *et al*., 1997), a modified version of the L-15 medium, containing 5% of the indicator dye alamarBlue (DAL1025, Invitrogen) (Pagé *et al*., 1993). The medium containing the indicator dye was added to all cell-seeded wells and incubated for one hour in the dark (Dayeh *et al*., 2005). Using a microplate reader (FLUOstar OMEGA, BMG Labtech), the fluorescence signal of alamarBlue was detected using excitation and emission filters of 540 and 570 nm, respectively. The viability of the gill cells was expressed as a response percentage of the treatments relative to the controls (% of control). Analysis of variance (ANOVA) from simple linear regression models on gill cell viability against intra- and extracellular compounds was performed, to explore the ichthyotoxic of the 10 microalgae strains. Normality and homogeneity of variances were assessed by the Kolmogorov-Smirnov method and Levene's test. A *post-hoc* analysis using a Tukey HSD test was performed to determine differences among treatments. The null hypothesis (no difference in responses) was rejected in all statistical analyses if the respective *P*-value was <0.05. These analyses were performed using the R software 3.0.1 (Ihaka & Gentleman, 1996). Algal biovolume was calculated according to Hillebrand *et al*. (1999).

Historical monitoring data in Chile shows that after the startup of the salmon industry in 1982, the first harmful algal blooms (HABs) producing fish-kills were related to the occurrence of flagellates of the class Dinophyceae and Raphydophyceae. During the 90's, salmon mortalities were mainly linked to harmful diatoms of the genus *Leptocylindrus* and *Chaetoceros*, whereas since 2000, there has been an increase in the intensity, duration and geographic coverage of ichthyotoxic flagellates (see the type of harmful effect in Table 1). This study tested the ichthyotoxic potency of the most recurrent phytoflagellates producing fish-killing events in the last 36 years in southern Chile (Fig. 1, Table 2). After two hours exposure of microalgae

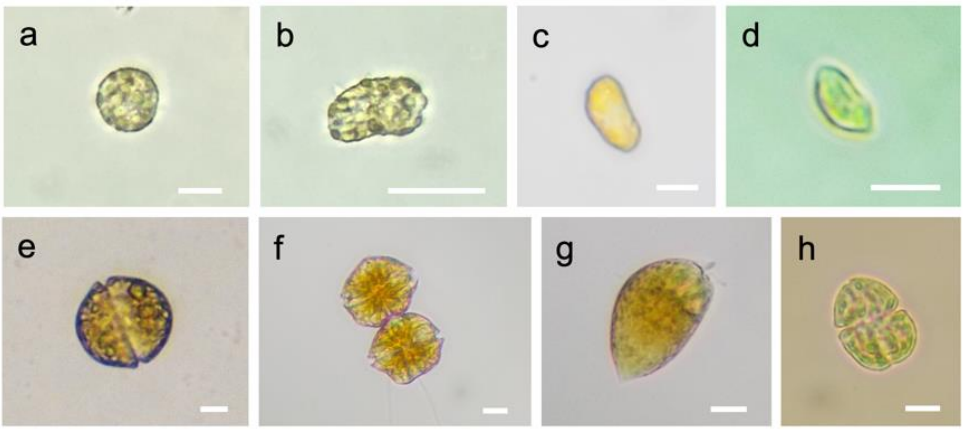

Figure 1. Microalgae species used in the study. a) *Vicicitus globosus*, b) *Pseudochattonella verruculosa*, c) *Heterosigma akashiwo*, d) *Teleaulax* sp., e) *Alexandrium ostenfeldii*, f) *Alexandrium catenella*, g) *Prorocentrum micans*, and h) *Karenia selliformis.* Scale bar: 10 μm.

Table 2. Species of marine microalgae used for exposure experiments to test their toxicity on fish gill cells RTgill-W1. The nontoxic species *Teleaulax* sp. was used as a negative control. *(Original code: ARC498).

Class	Species	Strain code	Origen	Isolator (Year)
Cryptophyceae	*Teleaulax* sp.	TL_01	Reloncaví Fjord/Los Lagos	J.I. Mardones (2019)
Dictyochophyceae	*Pseudochattonella verruculosa*	PV_01*	Huar Island/Los Lagos	A. Martin (2016)
	Pseudochattonella verruculosa	PV_06	Pilpilehue/Los Lagos	J.I. Mardones (2019)
	Vicicitus globosus	VG_01	Hualaihué/Los Lagos	J.I. Mardones (2019)
Dinophyceae	*Alexandrium catenella*	AC_01	Asasao/Aysén	B. Olivares (2019)
	Alexandrium ostenfeldii	AO_01	Calbuco/Los Lagos	J.I. Mardones (2018)
	Karenia selliformis	KS_01	Guaitecas/Aysén	J.I. Mardones (2018)
	Karenia selliformis	KS_02	Guaitecas/Aysén	J.I. Mardones (2018)
	Prorocentrum micans	PM_01	Reloncaví Sound/Los Lagos	J.I. Mardones (2018)
Raphidophyceae	*Heterosigma akashiwo*	HA_01	Hornopirén/Los Lagos	J.I. Mardones (2019)

Strains were obtained from the Harmful Algal Collection of the Centro de Estudios de Algas Nocivas in Chile (CREAN/IFOP).

cultures against fish gill cells, all algae cultures except the control (*Teleaulax* sp.) and one *Karenia* strain (KS_01), showed significant differences in the gill cell viability response between the supernatant and lysed cell algae treatments (Figs. 2a-b; ANOVA, $P < 0.05$), and no correlation with algal biovolume (Fig. 2c; $R^2 < 0.001$). This result agrees with several studies that have shown that ruptured microalgae cells exhibit consistently higher toxicity than supernatant against RTgill-W1 cells (Dorantes-Aranda *et al*., 2011, 2015; Mooney *et al*., 2011; Mardones *et al*., 2015). While specific lytic compounds produced by some phytoflagellates are released into the surrounding aquatic environment, the most active portion remains cell-bound. Based on these findings, mitigation approaches that are currently used in salmon farms during bloom events, where the main target is to destroy the harmful algae cells (by rupturing), are likely to make effects worse.

As observed in the lysed-cells treatment (the most toxic fraction), the most cytotoxic species at 2,000 cells mL^{-1} -in decreasing order- were *Karenia selliformis*, *A. catenella*, *Vicicitus globosus*, *Alexandrium ostenfeldii*, *P. verruculosa*, *Heterosigma akashiwo*, and *Prorocentrum micans* (Fig. 2b). Except for *K. selliformis* and *A. catenella*, the cytotoxic potency observed in all surveyed species is low, considering that 2,000 cells mL^{-1} could be accepted as an intense HAB event in natural oceanic conditions. Assuming that ichthyotoxins are equivalent to allelopathic compounds, our results could be a consequence of *in vitro* monoclonal cultures, where the microalgae do not require the release of toxic compounds under no competence nor predation pressure. This outcome might partially explain the observed mismatch in ichthyotoxic potency between natural algae blooms (higher toxicity) and laboratory studies on target species (lower toxicity).

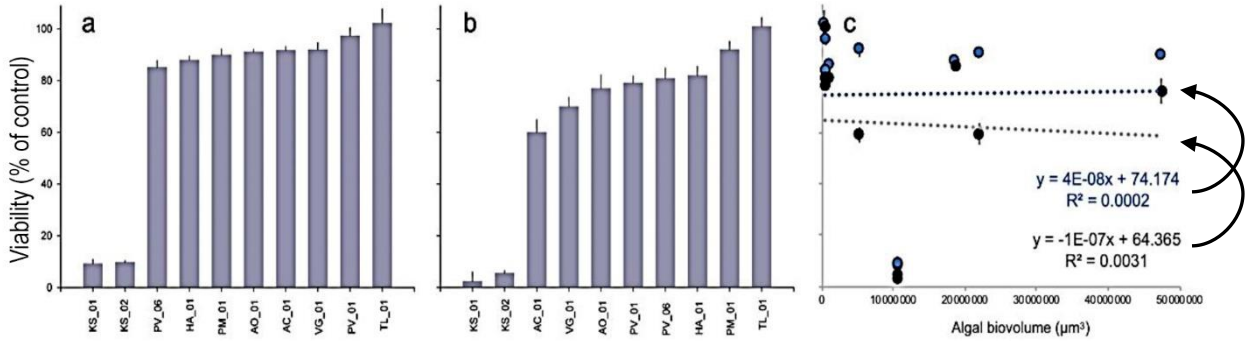

Figure 2. Loss of viability of RTgill-W1 cells after 2 h exposure to a range of algal cultures, arranged from most ichthyotoxic (left) to harmless (right). a) supernatant, b) lysed cells, and c) effect of supernatant (blue circles) and lysed cell (black circles) treatments on gill cell viability estimated from total algal biovolume. The trend line and equation of linear adjustment are presented for all data. The nontoxic species *Teleaulax* sp. was used as a negative control. VG_01: *Vicicitus globosus*, PV_01 and PV_06: *Pseudochattonella verruculosa*, HA_01: *Heterosigma akashiwo*, TL_01: *Teleaulax* sp., AO_01: *Alexandrium ostenfeldii*, AC_01: *Alexandrium catenella*, PM_01: *Prorocentrum micans*, KS_01 and KS_02: *Karenia selliformis.*

Coupling historical fish-killing events (Table 1) and results in this study regarding the ichthyotoxic potency of algae species, two main considerations should be taken into account by the Chilean salmon industry. First, *Karenia selliformis* has been observed to bloom in offshore areas (2-10 NM), barely affecting inshore salmon farming activities (<2 cells mL^{-1}). Exceptional outbreaks of this toxic dinoflagellate, in inner channels, might, however, be even more damaging than the *Pseudochattonella* bloom recorded in 2016 (Mardones *et al*., 2019); thus, early warning systems should be activated when the presence of this dinoflagellate species. Second, according to the present historical analysis, there is an apparent increment in the occurrence of phytoflagellates and their ichthyotoxic potency during the last decade in southern Chile (see the ranking in Table 1). This fact might be the result of enhanced monitoring efforts (especially from the salmon farming industry) and improved expertise in taxonomic identification of cryptic toxic flagellates species, but also due to the observed resilience of some toxic flagellate to projected climate variability in the eastern Pacific coast (Mardones *et al*., 2016). Expected climate change or exceptional warmer environmental conditions might increase the occurrence of ichthyotoxic microalgae, as reported by Trainer *et al*. (2020).

In conclusion, this study has shown that the application of an *in vitro* fish gill assay is an effective method to carry out sensitive screening tests for the toxicity of marine microalgae. An important output to consider by salmon farmers is that ichthyotoxic flagellates are more toxic to fish gill cells upon rupture and that *Karenia* spp. are more toxic than previously thought. These results have important implications for the way the salmon industry should manage and mitigate algal bloom impacts.

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