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Quantifying harmful algal bloom thresholds for farmed salmon in southern Chile

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ABSTRACT

Harmful algal blooms (HABs) have affected salmon farms in Chile since the early 1970's, causing massive losses in fish. Two large HABs occurred in 2002 and 2009, during which *Alexandrium catenella* blooms killed tons of salmon over an extended geographic area in southern Chile. At the beginning of 2016, high and persistent densities of *Pseudochattonella cf. verruculosa* and *A. catenella* were detected in the estuarine and marine ecosystems of southern Chile. Mortality for this latter event reached 27 million salmon and trout (i.e. 39,000 tons). Unfortunately, the threshold concentrations of algae that could be harmful to the health of farmed salmon in southern Chile have not yet been quantified. Here, to protect fish farms from HABs, critical concentration levels, i.e. thresholds at which the behavior of farmed *Salmo salar* is affected by harmful algae were quantified using generalized linear mixed models (GLMM). An extensive database from southern Chile covering the period from 1989 to 2016 was analyzed. The database included salmon behavior, cell abundance of microalgae and oceanographic factors. For both species analyzed, the higher the cell abundance, the greater the probability of detecting anomalous behavior. A threshold of 397 cells/mL was estimated for *A. catenella*, although it can increase up to ca. > 975 cells/mL at a Secchi depth > 6 m and up to 874 cells/mL during flood tide. A threshold value < 1 cell/mL for *Pseudochattonella cf. verruculosa* was found to be associated with anomalous salmon behavior, which significantly increased at a water temperature of 11 °C. Evidence for a relationship between fish behavior and mortality is provided.

1. Introduction

The severity, frequency and geographic coverage of harmful algal blooms (HABs) has increased globally in recent decades; this tendency may continue due to climate change pressures and their effects on marine planktonic systems (Sellner et al., 2003; Anderson, 2009; Wells et al., 2015). The eradication of harmful algae from aquatic ecosystems is highly unlikely, as they expand into new habitats and adapt to a changing climate (Anderson et al., 2015). There is general scientific consensus that the types of resources affected by HABs and the associated economic losses are also increasing (Anderson et al., 2012).

Blooms of the dinoflagellate *Alexandrium catenella* have occurred in Chile since 1972 (Guzmán et al., 1975). The first recorded bloom was detected in the Magallanes region (53°S), following which there has been a continuous northward expansion of *A. catenella* up to 42°S, which has resulted in several toxic outbreaks in 1981, 1989 and 1991 (Lembeye, 1981; Uribe, 1988; Guzmán et al., 2002). Several outbreaks of *A. catenella* have occurred in southern Chile. An intense bloom was detected in the Aysén Region in 2002, which expanded northward to the Los Lagos Region (42°10′S). Since then there have been recurring outbreaks of *A. catenella* (Molinet et al., 2003). Exceptionally intense blooms affected the coastal zone of southern Chile in the austral summers of 2009 and 2016. Abundance of *A. catenella* reached ca. 5000 cells/mL in 2009, with resulting economic losses in the Chilean salmon farm industry of over $10 M USD (Mardones et al., 2010). Similar levels of *A. catenella* abundance (ca. 5000 cells/mL) occurred in 2016. For the first time the outbreak extended to more oceanic waters associated with the 2015–2016 El Niño (Guzmán et al., 2016; Hernández et al., 2016; Léon-Muñoz et al., 2018). The first *P. cf. verruculosa* bloom was detected in 2004, and blooms recurred in 2005, 2009 and 2011 (Mardones et al., 2012; Eckford-Soper and Daugbjerg, 2016). During the bloom that took place in early 2016 in northern Patagonia (Reloncaví Fjord and Sound, northern Chiloé Island), density of *P. cf. verruculosa* reached levels of 7700 cells/mL (Clément et al., 2016) and up to almost 20,000 cells/mL (Villanueva et al., 2016; Léon-Muñoz et al., 2018) according to the area. The *A. catenella* and *P. cf. verruculosa* blooms of early 2016 were

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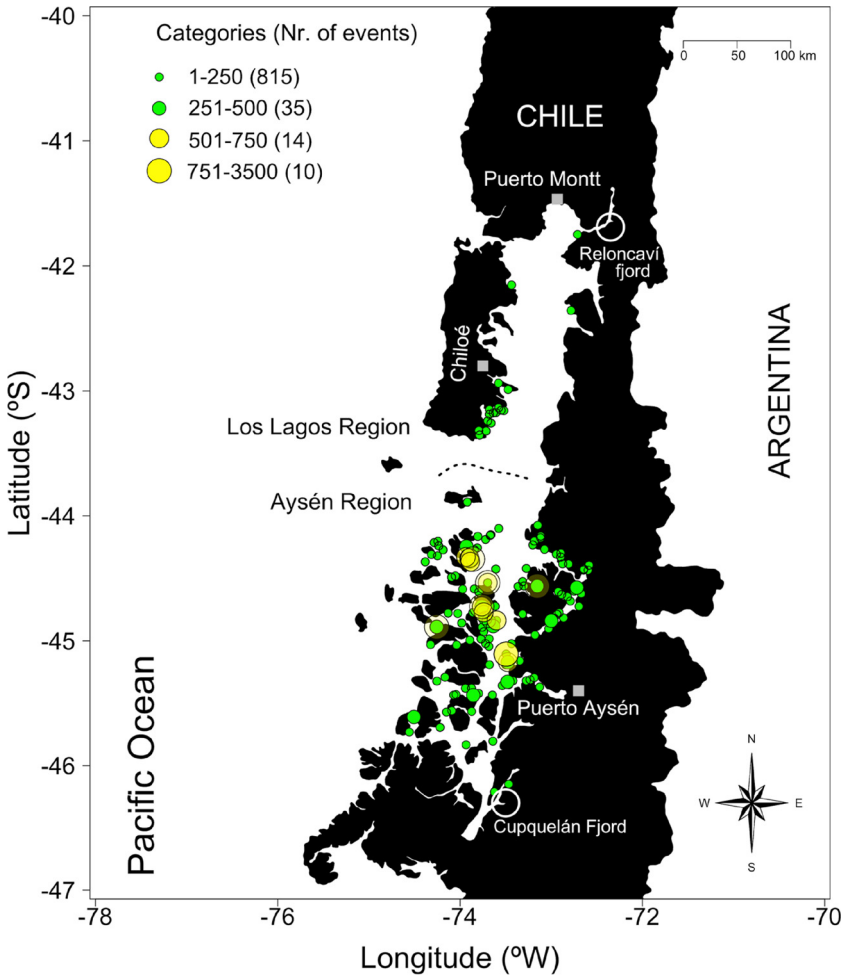

Fig. 1. Spatial distribution of *Alexandrium catenella* blooms in the Los Lagos and Aysén Regions between 1996 and 2016. All intensive blooms (> 500 cells/mL) were located in the Aysén Region. Four bloom categories expressed as cells/mL and the number of events belonging to each category (in parentheses) are presented.

economically devastating for the Chilean salmon farming industry, killing ca. 27,000,000 farmed salmon and trout (39,000 tons).

Chilean salmon farms are located mainly in the Los Lagos and Aysén Regions. This ecosystem (northern and central Patagonia) is one of the most extensive fjord systems in the world. It is characterized by complex morphology that includes channels, gulfs, estuaries and bays (Pantoja et al., 2011) (Figs. 1 and 2). From an oceanographic perspective it is a marine ecosystem influenced by high salinity deep oceanic water and by low salinity surface freshwater from multiple sources (rivers, precipitation, snow/glacier melting) (Iriarte et al., 2010). These contrasting oceanographic conditions produce a high degree of variability characterized by marked horizontal and vertical gradients in salinity, density, light availability and inorganic nutrient ratios (Silva and Palma, 2006; Iriarte et al., 2014; Jacob et al., 2014). Major changes in atmospheric and oceanographic conditions during the summer of 2016 caused a weakening of vertical stratification in western Patagonia which allowed the advection of more saline and nutrient-rich waters, causing major blooms of *P. cf. verruculosa* and the worst mass mortality of farmed salmon species ever recorded in this area (Léon-Muñoz et al., 2018).

Studies have been conducted to determine the factors associated with the growth and toxin production of Chilean strains of *A. catenella* to understand better the mechanisms that control harmful algal blooms in southern Chilean marine and estuarine ecosystems. Laboratory studies have shown that the growth of *A. catenella* strains is related to changes in water temperature, photoperiod, nutrient concentrations and salinity (Aguilera-Belmonte et al., 2013; Avila et al., 2015). Field studies analyzed the dynamics of outbreaks in relation to oceanographic conditions (Díaz et al., 2014) and have provided knowledge of genetic, morphological and toxicological diversity among *A. catenella* strains in southern Chile (Varela et al., 2012). Also, specific ichthyotoxic mechanisms have been proposed to explain the massive mortality of farmed salmon due to *A. catenella* blooms (Mardones et al., 2015).

There is no specific quantitative information for *A. catenella* or other ichthyotoxic microalgae in terms of the minimal cell abundance levels necessary to produce harmful effects on farmed salmon. Historically, Chilean salmon farmers have used reference levels estimated for the same (or similar) species of microalgae from different ecosystems worldwide. The Chilean salmon farm industry (Technological Salmon Institute; INTESAL) has proposed 500 cells/mL and 10 cells/mL as ichthyotoxic reference levels for *Alexandrium catenella* and *Pseudochattonella cf. verruculosa*, respectively. The Chilean National Fisheries and Aquaculture Service (SERNAPESCA) recently proposed, based on the work of Mardones and Clément (2016), harmful reference levels greater than 300 cells/mL and greater than 50 cells/mL for *A. catenella* and *P. cf. verruculosa,* respectively (SERNAPESCA, 2017). Consequently, the main objective of this study is to quantify, using historical data, the cell abundance thresholds of *A. catenella* and *P. cf. verruculosa* to maintain the well-being of farmed salmon. In other words, critical thresholds (CT) of cell abundance at which the behavior of farmed salmon is affected were quantified.

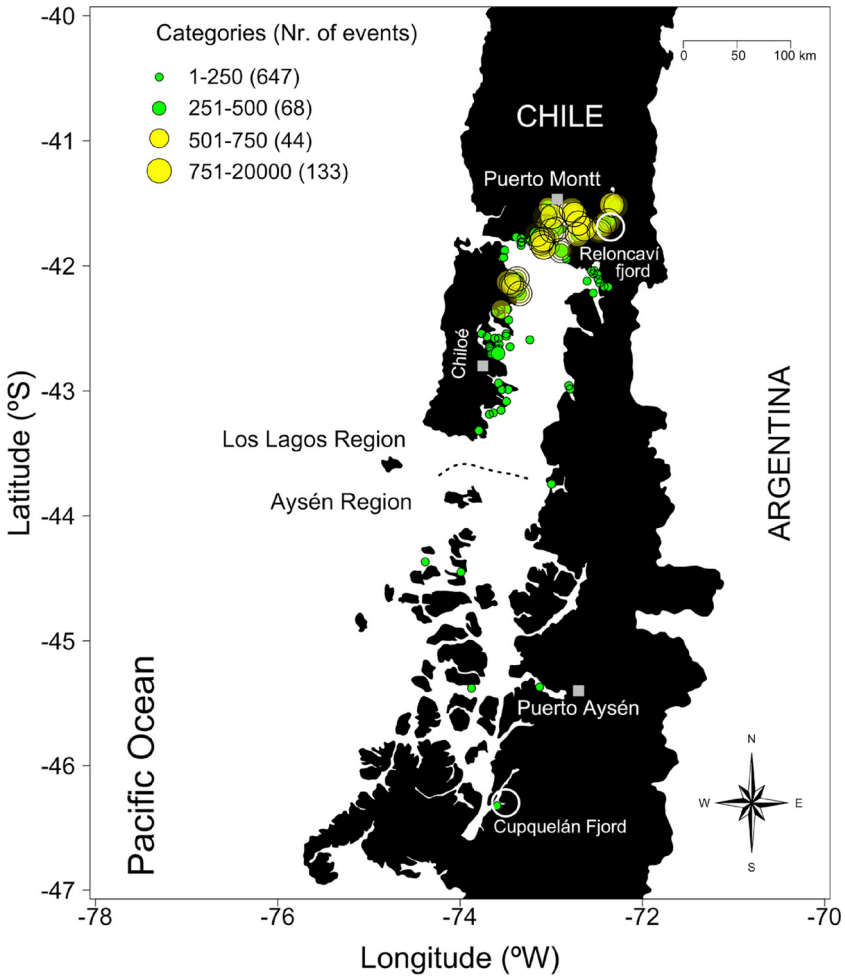

Fig. 2. Spatial distribution of *Pseudochattonella cf. verruculosa* blooms in the Los Lagos and Aysén Regions between 2004 and 2016. Almost all *P. cf. verruculosa* blooms were located in the Los Lagos Region between Chiloé Island and Reloncaví Fjord. Four bloom categories expressed as cells/mL and the number of events belonging to each category (in parentheses) are presented.

2. Materials and methods

2.1. Study area

Microalga cell abundance and oceanographic/meteorological factors were sampled in the coastal zone of southern Chile. This study is focused only on the Los Lagos and Aysén Regions as noted in Section 2.4, so that only harmful algal blooms between Reloncaví Fjord (approx. 41.5°S) and Cupquelán Fjord (approx. 46.2°S) were used for the estimation of critical thresholds (Figs. 1, 2, 3).

2.2. Harmful algal bloom database

This database summarizes the sampling effort conducted by the Chilean salmon farming industry to identify and estimate cell abundance (cells/mL) of several microalga species of different functional groups (e.g. diatoms, dinoflagellates, radiophyceae) between January 1989 and March 2016 (N = 339,205). Sampling was carried out as part of the activities of the Phytoplankton Monitoring Program of INTESAL, which includes the collection of discrete quantitative samples in the coastal zone of the Los Lagos, Aysén and Magallanes regions where salmon farming takes place. Oceanographic and meteorological factors were also quantified. Phytoplankton sampling is mostly conducted during spring, summer and fall on a weekly basis at more than 350 salmon farms. When blooms are detected, sampling frequency increases to a daily scale. Cell counts were conducted using standard inverted microscopy.

The cell abundance of *A. catenella* and *P. cf. verruculosa* were analyzed in this study; maps showing HABs for the former and latter species are presented in Figs. 1 and 2, respectively. Oceanographic factors considered in this study include salinity (psu), water temperature (°C) and oxygen (mg/L) measured on a quantitative scale. Qualitative factors such as Secchi depth readings (4 levels) and tides (4 levels) were also analyzed. Individual fish behavior of Atlantic salmon (*Salmo salar*), Pacific salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) were measured on a qualitative scale of three categories: “normal”, “irregular swimming” and “loss of appetite”. Only two categories were used in the estimation of harmful thresholds as explained in the Supplementary material section (Tables 1 and 2). A subset of the full database was analyzed representing the days when *P. cf. verruculosa* or *Alexandrium catenella* cells were detected. At the same time, the behavior of Atlantic salmon was quantified. For example, when *P. cf. verruculosa* cells were detected, the subset was ca. 53% of the total observations. This means that 53% of observations when *P. cf. verruculosa* cells were detected were of Atlantic salmon behavior, while 28% and 19% of the remaining observations were of Pacific salmon and rainbow trout behavior, respectively.

2.3. Fish mortality database

Mortality rates of the farmed salmonids (i.e. *Salmo salar*, *Oncorhynchus kisutch* and *Oncorhynchus mykiss*) in the Los Lagos, Aysén

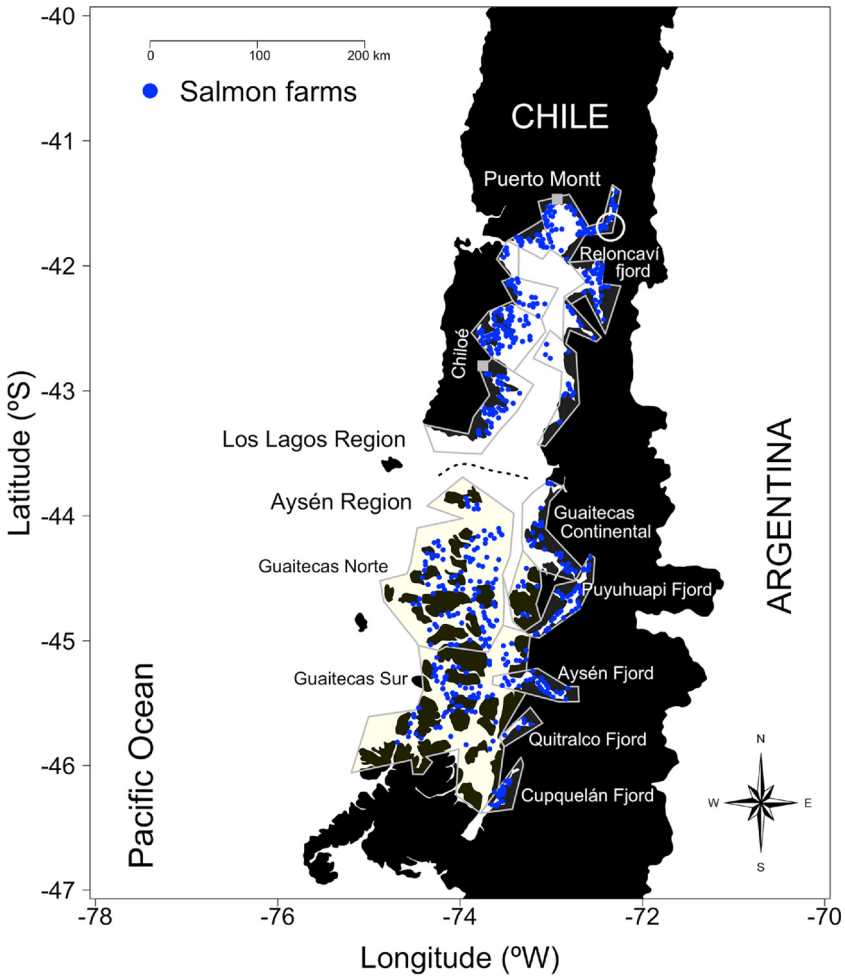

Fig. 3. Phytoplankton subdivisions in the Los Lagos Region and Aysén Region used by the Technological Salmon Institute (INTESAL) for management purposes. Locations of salmon farms are marked with blue dots. The two subdivisions most exposed to the open ocean, Guaitecas Norte and Guaitecas Sur, are highlighted on the map.

and Magallanes Regions due to harmful algal blooms and other causes were recorded weekly per farm from December 2010 to April 2016 (N = 50,491). In addition, the database includes the number of farmed fish, their average biomass and stocking density per week and per farm for each year.

2.4. Treatment of database and preliminary analysis

Blooms in the Magallanes Region were excluded from this study because only three blooms of *A. catenella* and none of *P. cf. verruculosa* were recorded in the database. Only information from Los Lagos Region was used in the calculation of critical thresholds (CT) of *P. cf. verruculosa* because cell abundance of this species was detected in Aysén Region for only 3 of 247 observations. In the case of *A. catenella*, analyses were focused on cell abundance in Aysén Region, which represented more than 87% of the total observations.

Cell densities of *P. cf. verruculosa* were analyzed only for the year 2016 (N = 494) (which includes more than 80% of the historical records) to avoid data with possible misidentification of this species, as occurred in the past (Mardones et al., 2012). Morphological and phylogenetic analyses of the 2016 blooms show that the ichthyotoxic species *P. cf. verruculosa* was involved in the massive mortality of farmed salmon in southern Chile (Paredes et al., 2016).

To avoid subjective and erroneous misclassification of fish behavior by salmon farmers, the categories “irregular swimming” and “loss of appetite” were considered together as a single qualitative category called “abnormal behavior”. In consequence, only two categories were used for the estimation of critical thresholds, “normal” and “abnormal” behavior (see Supplementary material), making no distinction of fish responses towards *A. catenella* or *P. cf. verruculosa*. A summary of the data used for the calculation of critical thresholds using *A. catenella* and *P. cf. verruculosa* counts for each farmed salmon behavior category in the Aysén Region (Table 1) and Los Lagos Region (Table 2) is presented in the Supplementary material section. Not all data contained cell counts when oceanographic factors were recorded, and vice-versa. Because of this, the data sets used to model the probability of abnormal fish behavior (and critical thresholds) in response to different *A. catenella* and *P. cf. verruculosa* cell abundance levels were selected according to the concurrence of cell abundance and oceanographic factors. Box-plots for both ichthyotoxic microalgae species are presented as preliminary evidence to describe the behavioral response of salmon to cell abundance levels, and to show how salmon behavior is associated with mortality on a weekly scale. In addition, the salmon behavior-mortality relationship was tested with the non-parametric Mann-Whitney U test (Sokal and Rohlf, 1997).

2.5. Statistical models

Generalized linear mixed-effect models (GLMM; Zuur et al., 2009; Bolker, 2015; Korner-Niegervelt et al., 2015) were used to quantify threshold abundance levels for *A. catenella* and *P. cf. verruculosa*. A logit-link function for binomial distribution was selected because the

Table 1
Critical thresholds (CTs) calculated using cell abundance (CA, cells/mL) of *Alexandrium catenella* from Chile and of *Alexandrium fundyense* from Canada. Significant covariates like Secchi depth readings (SDR) at 1–2 m, 2–4 m, 4–6m, > 6 m and tidal phases (low tide, flood tide, high tide, ebb tide) were considered for the estimation of CTs. INTESAL corresponds to the Technological Salmon Institute (Chile) and LCI to the 95% lower confidence interval. NA means not available. *Only valid for farms located in Guaitecas Norte and Guaitecas Sur areas. LC50 is the concentration that killed 50% of individuals within 24 h. Cell abundances (CA, cells/mL) at SDR of 4–6 m and SDR > 6 m are approximate values obtained using the closest observations of fitted curves.

Species	*A. catenella*	*A. catenella*	*A. catenella*	*A. fundyense*
Source	This study	Fuentes et al. (2008)	a) Mardones et al. (2015) b) Mardones and Clément (2016)	a) Chang et al. (2007) b) Martin et al. (2006) c) Burridge et al. (2010)
Type of study	Statistical analysis to detect abnormal fish behavior using INTESAL database	Observational studies of HABs associated with changes in fish behavior	a) Laboratory experiments for the calculation of LC50 b) Observational studies of HABs associated with increased fish mortalities	Laboratory experiments for the calculation of LC50
Location	Chile, Aysén Region	Chile, Aysén Region	Chile; Los Lagos, Aysén and Magallanes regions	Canada, Bay of Fundy
Fish/stage	*Salmo salar*/adults	NA	a) *O. mykiss*/gill cells b) *Salmo salar*/adults	*Salmo salar*/smolts
Critical thresholds:				
CA (cells/mL)	397 (LCI = 174)	> 356	a) 191 (K7 strain, Magallanes Region); 1996 (Ester2 strain, Aysén Region) b) 300	a) 614 b) 640 c) 614
CA at SDR of 1–2 m	NA	NA	NA	NA
CA at SDR of 2–4 m	568	NA	NA	NA
CA at SDR of 4–6 m	> 800	NA	NA	NA
CA at SDR > 6 m	> 975	NA	NA	NA
CA at low tide	NA	NA	NA	NA
CA at flood tide	874*	NA	NA	NA
CA at high tide	NA	NA	NA	NA
CA at ebb tide	NA	NA	NA	NA

response variable is dichotomous (normal or abnormal salmon behavior) (McCullagh and Nelder, 1989; Lane et al., 2009). The predictor variables include microalga cell abundance (continuous variable) and oceanographic factors (continuous or categorical variables) as fixed terms (Section 2.2). Factors with less than 20 samples per level were discarded in accordance with the recommendation of Bolker et al. (2008).

Cell densities were scaled, i.e. the mean was subtracted and the result divided by the standard deviation, as in Díaz et al. (2014). Following that, normality of cell abundance observations was induced by a Box-Cox transformation that maximized log-likelihood values (Cryer and Chan, 2008). Salmon farms and/or months were used as random variables (random terms) to account for non-independence of cell abundance within farms and/or months. Predicted cell abundance associated with 50% normal and 50% abnormal salmon behavior were defined as a critical threshold (CT) value. This means that cell abundance levels below this critical threshold (CT = 0.5) are statistically associated with salmon behavior that in general terms is closer to a completely abnormal state (category 0) than to a completely normal state (category 1). A probability of normal behavior equal to 0.2, for example, means that it is highly unlikely that salmon behave normally. Lower 95% confidence intervals (LCI) of cell abundance associated with critical thresholds (CT) were calculated (when possible) using $R = 2000$ random samples (Korner-Niegervelt et al., 2015). The use of mixed models allows analyzing the non-normal observations of the response variable, including a nested structure (random effects) in predictors (Bolker, 2015). The goodness-of-fit for models using cell abundance to predict probabilities of normal salmon behavior were assessed by comparing fitted values with original data with slightly jittered vertical observations to improve figure visibility (Korner-Niegervelt et al., 2015).

Critical thresholds were calculated only for models that always included cell abundance as an explanatory variable and in which all coefficients were statistically significant ($p < 0.05$). Models were not compared using selection criteria like the likelihood ratio test (Bolker et al., 2008), because this was not the objective of this study. All

Table 2
Critical thresholds (CTs) calculated using cell abundance (CA, cells/mL) of *Pseudochattonella cf. verruculosa* from Chile and New Zealand and of *Pseudochattonella farcimen* from Denmark. Water temperature at 11 °C was included as a significant covariate for the estimation of CTs. INTESAL corresponds to the Technological Salmon Institute (Chile). LC50 is the concentration that killed 50% of individuals within 24 h. NA means not available.

Species	*P. cf. verruculosa*	*P. cf. verruculosa*	*P. verruculosa*	*P. farcimen*
Source	This study	a) Mardones et al. (2012) b) Mardones and Clément (2016)	MacKenzie et al. (2011)	Andersen et al. (2015)
Type of study	Statistical analysis to detect abnormal fish behavior using INTESAL database	Observational studies of HABs associated with increased fish mortalities	Observational studies of HABs associated with increased fish mortalities	Field and laboratory experiments
Location	Chile/Los Lagos Region	Chile/Los Lagos Region	New Zealand/Queen Charlotte Sound	Denmark/Snaptun Harbor
Fish/stage	*Salmo salar*/adults	*Salmo salar*/adults	*O. tshawytscha*/adults	*O. mykiss*/500g
Critical thresholds:				
CA (cells/mL)	< 1	a) 22 b) 50	~10	500
CA at water temperature of 11 °C	2150	NA	NA	NA

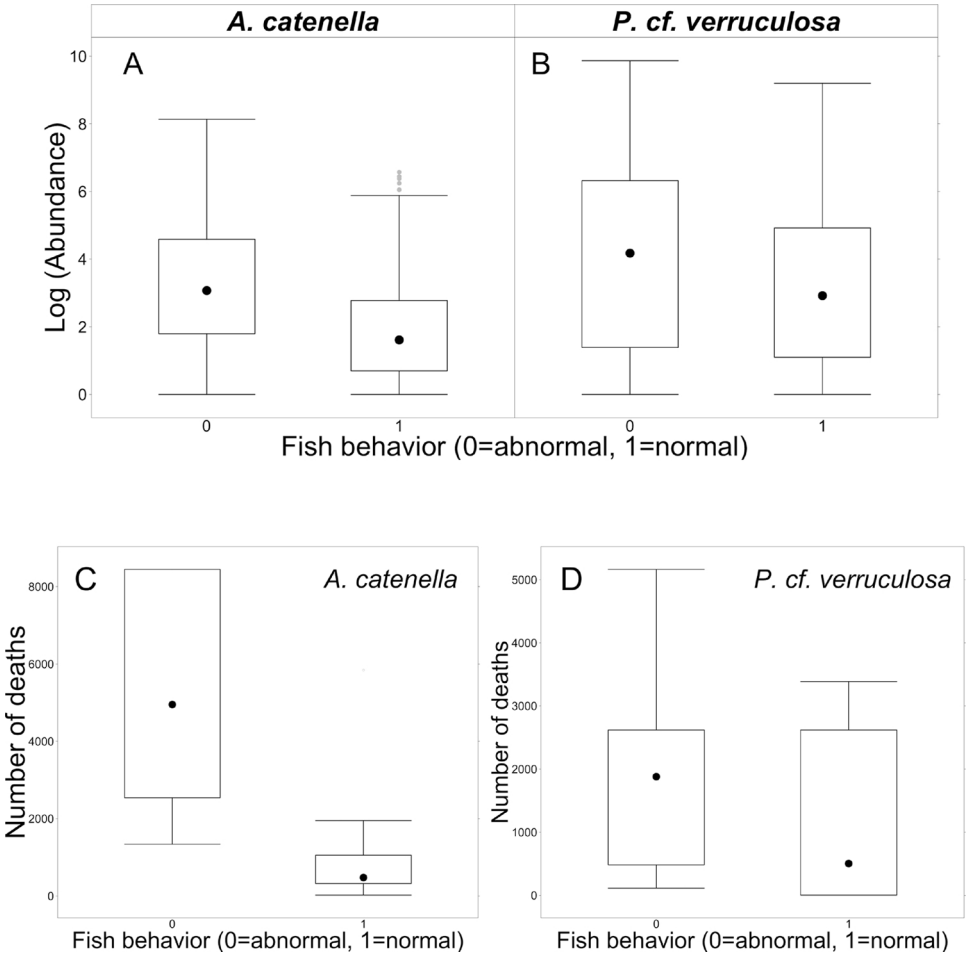

Fig. 4. Box-plots of a) *Alexandrium catenella* and b) *Pseudochattonella cf. verruculosa* cell abundance levels (cells/mL) on a logarithmic scale for abnormal (=0) and normal (=1) salmon behavior. Box-plots of the number of weekly dead salmon due to c) *A. catenella* and d) *Pseudochattonella cf. verruculosa* blooms for abnormal (=0) and normal (=1) fish behavior. Medians are represented by the dots. Outliers representing salmon mortalities greater than 10,000 fish for *A. catenella* and greater than 8,000 fish for *P.cf. verruculosa* were removed to improve figure clarity.

analyses were conducted using the statistical and R programming software (R Development Core Team, 2017) and packages "lme4", "TSA", and "arm", available at the CRAN repository (www.r-project.org/).

3. Results

3.1. Preliminary analysis of Salmo salar behavior and cell abundance

Box-plots of *A. catenella* and *P. cf. verruculosa* cell abundance (on a logarithmic scale) calculated for abnormal (=0) and normal (=1) salmon behavior show that for both species abnormal behavior is more associated with high cell abundance than with normal behavior. For *A. catenella*, the median number of cells associated with salmon abnormal behavior was 2.83 cell/mL and 1.60 cell/mL for normal behavior (Fig. 4a). For *P. cf. verruculosa*, the median number of cells associated with salmon abnormal behavior was 4.09 cell/mL and 2.39 cell/mL for normal behavior (Fig. 4b). Differences in medians between the two categories (salmon normal and abnormal behavior) for both microalga species are remarkably higher on a nominal scale. Median values of cell abundance of *A. catenella* for abnormal and normal salmon behavior were 17 and 5 cell/mL, respectively, while median values of *P. cf. verruculosa* cell abundance were 60 cell/mL for abnormal behavior and 11 cell/mL for normal behavior.

3.2. Preliminary analysis of Salmo salar mortality and behavior

A non-parametric Mann-Whitney *U* test (Sokal and Rohlf, 1997) indicated that the median of dead salmon measured weekly per farm due to *A. catenella* blooms was significantly different ($W = 164$, $p < 0.001$) between the abnormal and normal salmon behavior categories. A boxplot shows that the median of dead fish associated with abnormal behavior (4951) was more than 10 times greater than the median of dead fish associated with normal behavior (476) (Fig. 4c). For, *P. cf. verruculosa*, a significant difference between medians was found in fish mortality between normal and abnormal salmon behavior categories (Mann-Whitney *U* test $W = 263$, $p < 0.05$). The medians of weekly dead fish obtained for abnormal and normal salmon behavior were 1880 and 503, respectively (Fig. 4d). Unfortunately, it was not possible to use a GLMM model to quantify the effect of cell abundance levels on salmon mortality due the low number of observations in which the number of weekly dead fish per farm and algal abundance (cells/mL) were recorded in the same week ($N = 28$ for *A. catenella*, $N = 41$ for *P. cf. verruculosa*).

3.3. Effect of Alexandrium catenella abundance on Salmo salar behavior

The probability of salmon behavior being completely abnormal increased as cell abundance increased (values on the y axis tend to 0). A critical threshold (CT) of 397 cells/mL was estimated using a GLMM model, in which cell abundance is the only explanatory variable and

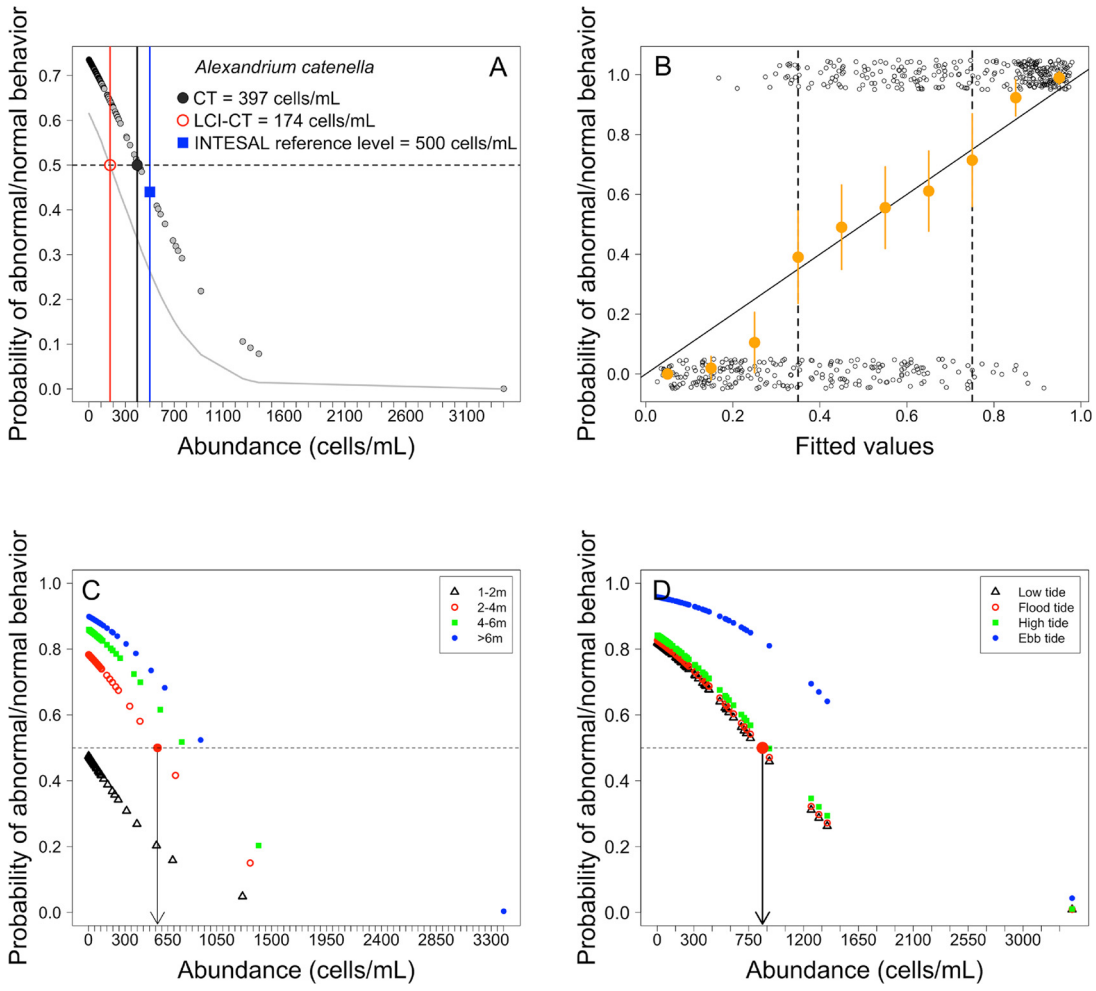

Fig. 5. a) Predicted probability of normal salmon behavior (gray dots) and 95% lower confidence intervals (LCI, gray line). Probabilities of 0 and 1 represent completely abnormal and normal salmon behavior, respectively. The 50% abnormal-50% normal behavior reference line used to calculate a critical threshold is indicated with a dotted line. The critical threshold (CT, black circle) calculated for *A. catenella* cell abundance levels is 397 cells/mL. The lower confidence interval for CT (LCI-CT, red dot) is 174 cells/mL. Vertical lines show the cell abundance levels for LCI-CT (red line), CT (black line) and historical INTESAL (Technological Salmon Institute) reference value (blue line). Predicted values were obtained from a GLMM that includes abundance as a fixed factor and farms as a random factor; b) goodness-of-fit plot obtained with a GLMM model with cell abundance as the explanatory variable and farms as a random term. Open circles represent abnormal salmon behavior (=0) or normal salmon behavior (=1) jittered in the vertical direction. Orange circles represent the means calculated within intervals of 0.1 along the x axis, and vertical bars are 95% confidence intervals. The diagonal black line represents a perfect match between observations and fitted values. A close agreement between fitted values and observations is visible between 0.35 and 0.75, which are marked with vertical dotted lines; **c)** predicted probability of normal salmon behavior according to cell abundance and Secchi depths of 1–2 m (open black triangles, reference level), 2–4 m (open red circles), 4–6 m (closed green squares) and $\geq$ 6 m (closed blue circles). Critical thresholds vary between ca. 570 cells/mL (marked with an arrow) and ca. > 975 cells/mL for Secchi depths of 2–4 m and $\geq$ 6 m, respectively. The dotted line indicates the 50% abnormal-50% normal behavior reference line used to calculate critical thresholds; d) predicted probability of normal salmon behavior for Guaitecas Norte and Guaitecas Sur according to the tidal phase: low tide (open black triangles, reference level), flood tide (open red circles), high tide (closed green squares) and ebb tide (closed blue circles). The critical threshold estimated for flood tide is ca. 874 cells/mL (closed red circle, marked with an arrow). The 50% abnormal-50% normal behavior reference line used to calculate critical thresholds is shown by a dotted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

farms is a random factor. The intercept and slope parameters of the logistic function are statistically significant ($p < 0.01$). The lower confidence interval associated with this critical threshold (LCI-CT) is 174 cells/mL (Fig. 5a). Using the historical reference value proposed by INTESAL, the probability of observing abnormal salmon behavior is below 0.5, which is closer to completely abnormal (category 0) than completely normal behavior (category 1). In other words, INTESAL's historical reference value slightly overestimates the cell abundance level necessary to affect salmon behavior according to this one-fixed-term model (Fig. 5a). The plot of fitted values against observations shows that the means were very close to the fitted values between the range of 0.35 and 0.75 overlapping 95% confidence intervals after taking the averages of zeros and ones using size classes of 0.1 (Fig. 5b). This means that the predictions of the model are reliable within the 0.35–0.75 range, which includes the critical threshold (CT = 0.5) used to predict abnormal salmon behavior.

3.4. Effects of Alexandrium catenella abundance and water transparency (Secchi depth readings) on Salmo salar behavior

Cell abundance was a fixed factor in the model, Secchi depth was also a fixed factor with four levels: 1–2 m, 2–4 m, 4–6 m, > 6 m, and farm was a random factor. The GLMM model shows that cell abundance was statistically significant ($p < 0.01$), as were Secchi depths of 2–4 m ($p < 0.05$), 4–6 m ($p < 0.01$) and > 6 m ($p < 0.01$) compared to the reference Secchi depth of 1–2m. The deeper the Secchi reading (and in consequence visibility), the higher the probability of observing normal salmon behavior (category 1) (Fig. 5c). Coefficients associated with

Secchi depths of 2–4m, 4–6 m and > 6 m were 1.38, 1.90 and 2.28, respectively. Critical thresholds varied between ca. 570 and ca. > 975 cells/mL for Secchi depths of 2–4 m and > 6 m, respectively (Fig. 5c). The curve for the Secchi depth reference level (1–2 m) is located below the 50% abnormal-50% normal behavior, which means that it is not useful for calculating a critical threshold.

3.5. Effect of Alexandrium catenella abundance and tides on Salmo salar behavior

The tides were considered a factor with four levels: low tide, flood tide, high tide and ebb tide. As in earlier GLMM models, cell abundance and farm were considered fixed and random terms, respectively. Coefficients for tidal phases were non-significant ($p > 0.05$); a significant coefficient ($p < 0.01$) was obtained only for cell abundance. Applying the same model focusing only on the areas in Aysén Region that are more exposed to the open ocean, which includes salmon farms located in Guaitecas Norte and Guaitecas Sur (Fig. 3), significant coefficients for cell abundance ($p < 0.01$) and flood tide ($p < 0.05$) were obtained. The critical threshold estimated for flood tide is ca. 874 cells/mL (Fig. 5d). Other tidal phases were not significant. It is interesting to note that the critical threshold for ebb tide was more than twice the value obtained for low, flood and high tides (Fig. 5d).

3.6. Effect of Pseudochattonella cf. verruculosa abundance on Salmo salar behavior

As expected, as cell abundance increased, the probability of observing completely abnormal behavior also increased and vice-versa (Fig. 6a). These probabilities were estimated using a GLMM with cell abundance as the explanatory variable and farms and months as random factors. Intercept and slope parameters of the logistic function were statistically significant ($p < 0.01$). It was not possible to estimate a CT associated with the 50% abnormal-50% normal salmon behavior reference line because the maximum predicted probabilities were below 0.5 (Fig. 6a,b). A cell abundance threshold of 1 cell/mL was estimated using the maximum predicted probability (0.41121). This means that the expected critical threshold associated with the 50%–50% reference line falls below 1 cell/mL (CT < 1 cell/mL) (Fig. 6a,b). In addition, for the latter threshold (1 cell/mL) an extremely low probability of normal salmon behavior of 0.165 was obtained, as shown by its lower confidence interval (Fig. 6a). A very similar probability of salmon behavior was obtained using the historical reference value proposed by INTESAL (10 cells/mL) (0.41121), in agreement with predictions obtained in the current study associated with 1 cell/mL (0.41065) (Fig. 6b).

Fig. 6c shows the plot of fitted values against observations. Unfortunately, after taking the averages of zeros and ones using size classes of 0.1, the mean obtained for size class 0.4–0.5 is zero because no observations of normal salmon behavior were reported for that class (Fig. 6c). The fitted value for size class 0.30–0.40 is close to the diagonal black line that represents perfect agreement between observations and fitted values. This means that conservatively a lower threshold should be used as the critical value.

3.7. Effect of Pseudochattonella cf. verruculosa abundance and water temperature on Salmo salar behavior

Cell abundance and water temperature coefficients were significant ($p < 0.01$) in a GLMM model with farms as a random term. A critical threshold (CT) of 2150 cells/mL was obtained using the lowest observed water temperature (11 °C) (Fig. 6d). It was not possible to estimate a critical threshold using mean and maximum water temperatures (14 °C and 19 °C, respectively) because the maximum predicted probabilities estimated for both curves were less than 0.5 (Fig. 6d).

4. Discussion

Critical thresholds were estimated for *A. catenella* and *P. cf. verruculosa* in the Aysén and Los Lagos Regions of southern Chile, respectively. A critical threshold (CT) of 397 cells/mL for *A. catenella* was obtained, with a lower confidence interval of 174 cells/mL. For *P. cf. verruculosa* a CT < 1 cell/mL was estimated. Critical thresholds are not fixed and consequently can change according to changes in oceanographic factors.

When Secchi disc readings were available, a CT of 568 cells/mL was obtained for *A. catenella* when visibility increased at 2–4m, and > 975 cells/mL when visibility increased at a depth > 6 m (Fig. 5c). As visibility increased, CT also increased. On the other hand, when water transparency decreased and in consequence visibility decreased at 1–2m, the general behavior of salmon was closer to the abnormal state (probability closer to 0 than to 1) independent of the cell abundance level (Fig. 5c). In the latter situation it is not necessary to calculate the CT, given that the very low visibility at 1–2 m in itself should be interpreted as a CT. Results obtained in the current investigation suggest that the co-occurrence of *A. catenella* and other harmful species (i.e. *Dinophysis acuminata*, *Protoceratium reticulatum*, *Pseudonitzschia cf. australis*, *Pseudonitzschia cf. pseudodelicatissima*) as occurred in southern Chile during summer 2016 (Buschmann et al., 2016) can decrease the water column transparency and Secchi disc readings, and their synergistic action can increase the sensitivity of farmed salmon. Therefore, changes in salmon behavior could be detected at lower *A. catenella* abundance levels when co-occurrence with other microalga species (not necessarily ichthyotoxic) occurs. This hypothesis should be tested when abundance of *A. catenella* and other species at different Secchi depth readings becomes available.

Tidal phases also play a role in estimating critical thresholds of blooms detected in salmon farms more exposed to the open ocean. For Guaitecas Norte and Guaitecas Sur (Fig. 3), the critical threshold estimated when *A. catenella* cells were sampled during ebb tide (CT > 1800 cells/mL) was twice as high as when sampled during flood tide (CT = 874 cells/mL) (Fig. 5d). A critical threshold should not be used under this scenario because it would be misleading. The extremely high critical threshold reflects the movement of phytoplankton cells out of salmon farms during ebb tide (MacKenzie et al., 2011; Blauw et al., 2012), which decreases the availability but not the abundance of cells, giving an erroneous signal to farm managers that an extremely high number of cells is necessary to achieve the 50% reference level in the estimation of a critical threshold.

It was not possible to estimate a critical threshold for *P. cf. verruculosa* associated with a 50% reference level. This means that cell abundance levels on salmon farms during the detected bloom were associated with abnormal salmon behavior probabilistically. The estimated probability was less than 0.5 independent of the cell abundance level (Fig. 6a,b). Consequently, a critical threshold below 1 cell/mL (CT < 1 cell/mL) is suggested as an abundance level already associated with abnormal salmon behavior. No fitted values between class 0.4 and 0.5 were available after fitting a GLMM with cell abundance as an explanatory variable with farms and months as random terms (Fig. 6c). According to this result, the model cannot be used to calculate a critical threshold using the 50% reference level. In consequence, as suggested above, a CT < 1 cell/mL for *P. cf. verruculosa* should be considered by salmon farm managers as an early warning signal of deteriorating wellbeing. In addition, temperature can change the critical threshold, as Fig. 6d shows. A critical threshold of 2150 cells/mL was estimated for a water temperature of 11 °C. For temperatures of 14 °C and 19 °C predicted curves fall below the 50% reference line (Fig. 6d). Therefore, a farm manager should only use CT = 2150 cells/mL as a signal of impending abnormal behavior when water temperature is around the annual minimum of 11 °C. When it exceeds 11 °C the probability of observing normal salmon behavior decreases. For example, an increase of 3 °C from 11 °C to 14 °C is associated with a decrease in probability of

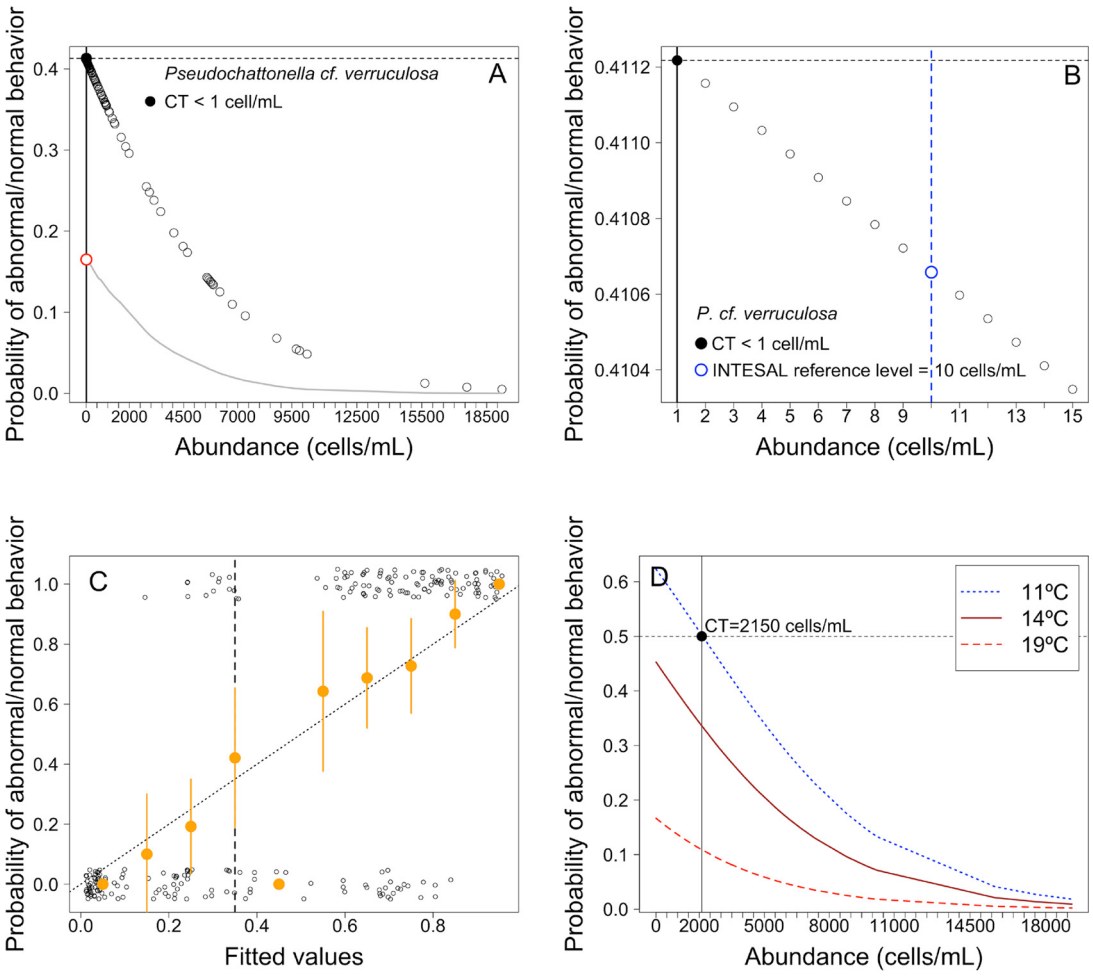

Fig. 6. a) Predicted probability of normal salmon behavior (open circles) and 95% lower confidence intervals (LCI, gray line) for a threshold of 0.41121. The red open circle indicates the estimated value (0.165) for 95% LCI for the latter threshold. The critical threshold (CT, black closed circle) calculated for *P. cf. verruculosa* cell abundance levels equals < 1 cell/mL. The vertical line shows the cell abundance levels for CT (black line). Probabilities of 0 and 1 represent completely abnormal and normal behavior, respectively; b) augmented area of relationship between predicted probability of normal salmon behavior and cell abundance between 1 and 15 cells/mL. The CT obtained in this study (< 1 cell/mL, black closed circle) and the historical INTESAL reference value (10 cells/mL, blue open circle) are associated with very similar probabilities of normal salmon behavior (0.41121 and 0.41065, respectively); c) goodness-of-fit plot obtained with a GLMM model with cell abundance as the explanatory variable and farms and months as random terms. Black open circles represent abnormal salmon behavior (=0) or normal salmon behavior (=1) jittered in the vertical direction. Orange closed circles represent the means calculated in intervals of 0.1 along the x-axis, and vertical bars are 95% confidence intervals. The diagonal black line represents a perfect match between observations and fitted values. A close agreement between fitted values and observations is visible for fitted values equal to and below 0.35 (marked with a vertical dotted line); d) predicted probability of normal salmon behavior for minimum (11 °C, blue dotted line), mean (14 °C, brown continuous line) and maximum (19 °C, red dashed line) water temperatures recorded when *Salmo salar* behavior and *P. cf. verruculosa* cells were quantified. The critical threshold (CT, black closed circle) estimated for a temperature of 11 °C is 2150 cells/mL. The vertical line shows the cell abundance levels for CT (black line). Probabilities of 0 and 1 represent completely abnormal and normal behavior, respectively. Critical thresholds for other temperature curves were not possible to calculate because maximum values did not reach the 50% abnormal-50% normal reference line (dotted line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observing normal behavior from 0.5 to 0.34. Furthermore, an increase of 5 °C from 14 °C to 19 °C was associated with an even more marked decrease in probability, from 0.34 to 0.11. This means that a CT = 2150 cells/mL is not valid when water temperature is around 19 °C, because in this case the probability of observing normal salmon behavior is close to zero (Fig. 6d). These results are in agreement with surface water temperature (15–16 °C) found prior to the massive bloom of this species during summer 2016 in Patagonia (Léon-Muñoz et al., 2018), because at this range of temperature the model predicts normal salmon behavior below 0.34, which is far from the 50% reference level (Fig. 6d).

4.1. Critical thresholds for the genera Alexandrium and Pseudochattonella

Abundance levels of *A. catenella* between 250 and 300 cells/mL have been reported as the cause of mortality of farmed salmon in southern Chile (Mardones et al., 2015). Toxicity varies according to the strain, the least toxic strain having an LC50 of 1996 cells/mL when gill cells are exposed to cultured *A. catenella* (Mardones et al., 2015). When the concentration of *A. catenella* exceeded 356 cells/mL unusual behavior was observed in salmon on farms in the Aysén Region, which was followed by gill damage and massive mortality (Fuentes et al., 2008).

No effect was observed when salmon smolts were exposed under laboratory conditions to *A. fundyense* cells obtained in the Bay of Fundy (eastern Canada) at a concentration of 250 cells/mL, however, a lethal concentration of 614 cells/mL was determined that killed 50% of smolts within 24 h (LC50) (Chang et al., 2007). For the same species and ecosystem, concentrations of 2000 cells/mL killed all salmon smolts, and an LC50 of 640 cells/mL was estimated (Martin et al., 2006). Concentrations of 800 cells/mL of *Alexandrium tamarense* were

associated with elevated *Salmo salar* mortality rates on Nova Scotia salmon farms in the year 2000 (Cembella et al., 2001). Burridge et al. (2010) reported mortality rates above 50% of *Salmo salar* smolts exposed to cultured *A. fundyense* at concentrations of 2000 cells/L, including rapid behavioral responses of fish that included loss of equilibrium and erratic swimming, followed by death. In addition, a 24 h LC50 was estimated as 614 cells/mL, and a non-observable effect level based on lethality was estimated at 100 cells/mL (Burridge et al., 2010). As expected, the estimated CT for *Alexandrium catenella* in this study was lower than the LC50 calculated under experimental conditions, and close to the abundance levels reported in southern Chile, which were capable of changing the behavior of farmed salmon.

Threshold levels have traditionally been calculated based on experimental conditions (bioassays) that measure the effects of different toxicity levels on fish, embryo cells, gill cells and hepatocytes (Skjelbred et al., 2011). These analyses have been difficult with *P. cf. verruculosa* (Eckford-Soper and Daugbjerg, 2016). For example, no acute toxic effects of *Pseudochattonella* cells were observed on salmon smolt mortality and swimming behavior under experimental conditions (24 h of exposure to 30,000 cells/mL), however, fish gills showed pathological lesions (e.g. hypertrophy, edema, necrotic cells) (Skjelbred et al., 2011). Similar results were obtained when rainbow trout smolts were exposed to cultures of *P. farcimen* for 3 days (Andersen et al., 2015). It appears that adult fish (> 2 kg) are more sensitive to *Pseudochattonella* cells than younger fish (Backe-Hansen et al., 2001). Observations at Danish farms indicate that rainbow trout begin to show effects at abundance levels of ca. 500 cells/mL (Andersen et al., 2015). Only 10 cells/mL of *P. cf. verruculosa* were sufficient to cause mortality of *Oncorhynchus tshawytscha* in New Zealand farms (MacKenzie et al., 2011), and a slightly higher level (22 cells/mL) was sufficient to produce between 2% and 5% salmon mortality in farms in southern Chile (Mardones et al., 2012). The latter results indicate that the critical threshold estimated in this study (CT ≤ 1 cell/mL) is a reasonable abundance level capable of modifying salmon behavior in Chilean farms. Critical thresholds for *A. catenella*, *P. cf. verruculosa* and related species that were calculated using cell abundance (cells/mL) in different aquatic ecosystems are compared in Tables 1 and 2.

4.2. Interpretation of critical thresholds

Critical thresholds (CTs) for both microalga species that were estimated in this study are lower than historical values used by INTESAL for management purposes. CTs are more conservative because they focus on estimating levels that change behavior, rather than lethal concentrations. These values should be interpreted probabilistically, meaning that microalga cell densities above critical thresholds are generally more closely associated with abnormal than with normal salmon behavior. The hypothesis underlying this study is that changes in salmon behavior (e.g. loss of appetite, erratic swimming) and consequent changes in general wellbeing are related to the abundance of harmful microalgae (e.g. Treasurer et al., 2003). Other factors not considered in this study, like stocking density, water currents and chemical treatments can alter the behavior of farmed salmon (Turnbull et al., 2005; Oppedal et al., 2011), which is why the proposed critical thresholds should be used as an auxiliary tool for managing salmon farms. For example, stocking density can influence farmed Atlantic salmon welfare, which is measured including fish behavior and feeding response. A stocking density above 22 kg/m^3 is associated with a reduced welfare score (Turnbull et al., 2005). Farmed salmon behavior also can change from a circular polarized group structure to a fixed group position where fish swim against the current when tidal current velocity changes from low/moderate to high velocities (Johansson et al., 2014). It has been detected that when farmed salmon are exposed to delousing chemicals, fish move to the surface and concentrate in the 1-m water column upper layer (Oppedal 2011). For that reason, CTs estimated in this study do not reflect the specific conditions that promote change in behavior of salmon at particular farms. Cell abundance levels above these thresholds should be viewed as impending changes in salmon behavior. CTs should trigger management actions to avoid or mitigate as much as possible the exposure of salmon to harmful algae to prevent or reduce high mortality levels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.hal.2018.05.004.

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