Mat thickness associated with *Didymosphenia geminata* and *Cymbella* sp. in the southern rivers of Chile

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ABSTRACT

Didymosphenia geminata is a diatom that can alter aquatic systems. According to the international literature, chemical and hydraulic factors have a greater influence on the proliferation of *D. geminata*, but the study of other microalgae that could be associated with her has been poorly addressed. The objective of this study is to evaluate the relationship between the structure of mat thickness *D. geminata* and another taxon that produces mucilage, *Cymbella sp.*, while also considering physical and chemical factors. To do this, two samples were taken, one in the spring of 2013 and the other in the autumn of 2014, from eight rivers in central-southern Chile-South America, where the benthic community was characterized and the thickness of the mat was measured. The results indicate that in the presence of both taxa the thickness of the mucilage layer is doubled. However, antecedents suggest that *D. geminata* is the main producer of mucilage, being seconded by *Cymbella sp.* The present study contributes evidence about the relationship between mat thickness *D. geminata* and other microalgae contribution, and aquatic condition for this development.

Key words: mat thickness, microalgae, stalk, and mucilage tubes.

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INTRODUCTION

Since the beginning of the 2000s, the massive proliferation of mucilage in rivers by *Didymosphenia geminata* (Lyngbye) A. Schmidt, has aroused the interest of scientists and governmental entities, due to its ability to severely alter the physical and biological conditions of aquatic systems (Spaulding & Elwell, 2007).

D. geminata has been described as a freshwater diatom that produces stalks in large quantities (Blanco & Ector, 2009), generating massive growths and forming thick benthic algal mats that can significantly alter river ecosystems. Numerous reports from around the world have noted its massive proliferation, being observed in regions of North America, Europe and Asia, as well as in New Zealand, Chile, and Argentina, (Kawecka & Sanecki, 2003; Kumar et al., 2009; Kilroy & Bothwell, 2012; Reid et al., 2012; Sastre et al., 2013; Montecino et al., 2016; Ladrera et al., 2018). The dispersión of *D. geminata* is attributed mainly to the anthropic vector (Cullis et al., 2012; Reid et al., 2012), however, there is also evidence of biological vectors such as *Neovison vison* (Leone et al., 2014).

The presence and massive growths of *D. geminate* have been related to chemical, physical and biological variables. For example, the presence of nitrogen-fixing cyanobacteria would favor its colonization (Novis et al., 2016), low levels of dissolved reactive phosphorus would favor its blooms (Bothwell et al., 2014; Bray et al., 2017), while the increase of the river's bed shear stress favors its removal (Cullis et al., 2013), as well as sediment transport, both conditioned by increased flow (Miller et al., 2009).

Although other diatoms can generate stalks or tubes of mucilage (Bahls, 2007), the possible role of these diatoms as mats producers or in a possible synergistic relationship with *D. geminata* has not been studied, with most efforts focusing on the particular monitoring of *D. geminata* as the main cause of the massive increase of mats.

This problem has already been observed by other researchers, who have begun to warn of other possible microalgae producing such blooms (Bahls, 2007; Suzawa et al., 2011; Furey et al., 2014; Khan-Bureau et al., 2014, 2016). However, there are few publications to date that document the relationship between the abundance of other microalgae and the mat thickness, that focus exclusively on qualitative analysis at the microscopic scale.

Previous works have already identified species of the genus Cymbella as potentially harmful algae. In the United States, sites with gray mucilage associated with the species *Cymbella Janischii* (A. Schmidt) De Toni, have been registered (Bahls, 2007; Khan-Bureau et al., 2014), and in Japan, a country that has not been invaded by *D. geminata*, algal blooms have also been found associated with *C. Janischii* (Suzawa et al., 2011). In several places in Chile, Jaramillo et al. (2015) recorded 7 species of Cymbella together with the presence of *D. geminata*, through molecular analysis, without detecting the presence of *C. janischii*. The 7 species are *Cymbella affinis, Cymbella cistula, Cymbella mexicana, Cymbella proximal, Cymbella aspera, Cymbella lanceolata y Cymbella tumida* (Brébisson) van Heurck. In none of these studies was the mat thickness estimated to be related it to the abundance of species of the genus Cymbella.

In light of this background, the objective of the present study is to evaluate the relationship of *D. geminata* and *Cymbella sp.* with mat thickness in rivers of the south-central zone of Chile. For this, three specific objectives are proposed: (I) quantify the mat thickness in different rivers, (II) relate mat thickness with the presence and abundance of *D. geminata* and Cymbella sp, and (III) to evaluate the relationship between physical and chemical conditions, mat thickness, *D. geminata* and *Cymbella sp.* presence and their abundance.

MATERIALS AND METHODS

Study area

The study was carried out in eight rivers located in four basins in the south-central zone of Chile (Fig. 1) that have *D. geminata* presence records (Montecino et al., 2016). These rivers present circumneutral pH, cold and oligotrophic water and substrates composed mainly of boulders.

Within these rivers, 25 short river reaches of 50 m were identified and georeferenced. In the event that two or more reaches were in the same river, these were distanced by at least 2 km. We selected in each river reach between 1 and 6 sampling points that represented all hydromorphological diversity of the river reach. All sampling points were sampled and represent the unit of analysis of the present study (Fig. 1).

Sampling was carried out in two occasions, late spring (sampling 19 river reaches, 74 sampling points, December 2013) and early autumn (sampling 17 river reaches, 49 sampling points, March 2014). During the late spring season, the water temperature varied between 9 ° C and 18 ° C, with an average conductivity of 45.7 µS/cm. Meanwhile, the fall season had lower temperatures between 4 ° C and 12 ° C, and higher average conductivity (52.6 µS/cm).

Sampling methods

The periphyton community of each sampling point was analyzed and the mat thickness measured in centimeters with a graduated ruler. 1000 mm^3 of mat sample was taken using a blunt syringe, and if the layer had a thickness of less than 1 mm, an area of 400 mm^2 was swept with a brush, assuming a volume of 400 mm^3. The samples were fixed with 1000 mm^3 solution of potassium iodide with iodine in water (lugol) and stored in sealed containers.

Biological samples were analyzed quantitatively in the laboratory. For this, a sub-sample of 1000 mm^3 was obtained and was deposited in a Sedgewick Rafter chamber and analyzed using a Zeiss Axisostar II Plus microscope, with inverted objective microscopy set at 40x. Identification was performed up to the genus taxonomic level and considered *D. geminata* as the most likely representative of its genus in Chilean rivers (Jaramillo et al., 2015). The results were expressed as cell density (cel/mm^3).

In each river reach, the following physical and chemical parameters were measured using a HANNA Model HI 9828 multi-parameter probe: water temperature, pH, electrical conductivity, dissolved oxygen and oxygen saturation. In addition, samples were taken to estimate the levels of calcium (Ca), total phosphorus (P), iron (Fe), nitrate (NO_3), nitrite (NO_2) and silicate (SiO_2) through the analysis protocols of "Standard Methods for Examination of Water & Wastewater" (Apha, 2005). At each sampling point, the depth was measured using a graduated bar and the superficial, middle and background velocities were measured using a Globalwater FP 101 digital flowmeter model.

Analysis of *D. geminata* and *Cymbella sp.*

A two-way ANOVA with permutations was performed to determine differences in mat thickness between point with presence or absence of *D. geminata* and *Cymbella sp.* Then, a posteriori non-parametric Tukey test was performed. Sampling points were divided into four categories: *D. geminata* presence only (A/D); *Cymbella sp.* presence only (C/A); the presence of both (C/D) and, the absence of both (A/A).

A Smooth Surface model was fitted to evaluate the relationship between the mat thickness (independent variable) and cell densities of *D. geminata* and *Cymbella sp.* (predictor variables). The model was adjusted using REML.

Analysis of physical and chemical variables

Three analyses were performed to establish a relationship between physical and chemical conditions, mat thickness and absence or presence of *D. geminata* and *Cymbella sp*. First, through one-way ANOVAs with permutations, the values of physical and chemical parameters were compared according to categories A/D, C/A, C/D and A/A. Then, a non-parametric tukey test was performed. The physical and chemical parameters with significant differences were correlated with the total richness of taxa of each sampling point. Secondly, a Random Forest classification model was adjusted to determine the discriminating ability of physical and chemical parameters on the presence or absence of *D. geminata* and *Cymbella sp.* Finally, a Random Forest regression model was performed to determine the ability of the presence or absence of *D. geminate* and *Cymbella sp.*, and the physical and chemical conditions, to predict mat thickness.

Regarding the statistics analysis used, ANOVAs of one and two ways were applied from the methodology used by Anderson & Legendre (1999). A non-parametric tukey test was carried out using the "mctp" program by "narpcomp" package (Konietschke et al., 2015). The Random Forest analysis (Breiman, 2001) was performed using the "caret" package. The Smooth Surface model was adjusted with the "ordisurf" program from "vegan" package. All the analyses were performed using R project software (R Core Team, 2017).

RESULTS

Mat thickness

The average mat thickness was 0.46 cm, with a maximum of 3 cm and a minimum of 0 cm. In 74% of samples, a visible mat was detected (> 0.1 mm), and only 10% of the sampling points had a mat greater than 1 cm.

Mat Relationship with *D. geminata* and *Cymbella sp.*

Of the 36 samples, *D. geminata* was detected in 13 river reaches during spring, and 17 river reaches during autumn, and *Cymbella sp.* was detected in 14 river reaches during spring, and 18 river reaches during autumn. At the sampling point level, *D. geminata* was detected in 89 points, 25 in autumn and 64 in summer, and in the case of *Cymbella sp.*, was detected in 76 points, 28 in autumn and 48 in spring.

The two-way ANOVA analysis shows that mat thickness increases significantly in the presence of *Cymbella sp.* (p value = 0.017; p perm = 0.022) and *D. geminata* (p value < 0.01; p perm < 0.01), but there is no significant interaction between *Cymbella sp.* and *D. geminata* (p value interaction = 0.9, Fig. 2B). The posterior tukey test suggests that mat thickness at sampling points with the absence of both genera (A/A) differ significantly from sampling points in the presence of both genera (C/D) (p value = 0.01). Categories C/A and A/D do not differ significantly from the rest (A/A – C/A, p value = 0.96; A/A – A/D, p value = 0.16; C/A – A/D, p value = 0.6; A/D – C/D, p value = 0.64; C/A – C/D, p value = 0.19) (Fig. 2A).

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From category C/A, 75% of the sampling points did not exceed 3 mm of mat thickness, with a median of 0, that is to say, they do not present visible mucilage. On the other hand, 50% of the A/D sampling points have a visible mat, with a median of 3 mm thick. Thus, *Cymbella sp.*, in the absence of *D. geminata*, recorded thinner mats than *D. geminata* in the absence of *Cymbella sp.* Finally, in the presence of both genuses (C/D), 50% of the sampling points had a visible mat with a median of 5 mm of thickness (Fig. 2A).

The relationship between mat thickness and cell density of *D. geminata* presents an inverse pattern (Fig. 3B). The relationship between *Cymbella sp.* and the mat thickness could also be interpreted as inverse, being less clear than that expressed by *D. geminata* (Fig. 3A). The relationship of densities between *Cymbella sp.* and *D. geminata* presents an inverse form, i.e., a higher density of *Cymbella sp.*, a lower density of *D. geminata* and vice versa (Fig. 3C). In Fig. 3C, it is observed that higher values of mat thickness are associated with low cell densities of *D. geminata* and intermediate cell densities of *Cymbella sp.* A large production of mucilage at low cell densities of *D. geminata* is a result expected in the literature (Bothwell et al., 2014). The Smooth Surface of the mat thickness fitted to the relationship of *Cymbella sp.*- *D. geminata* explains 8.3% of the deviation, which is considered significant ($r^2 = 0.058$, p-value = 0.035).

Participation of physical and chemical variables

When evaluating the differences in physical and chemical conditions in the presence/absence of *Cymbella sp.* and *D. geminata*, variables such as pH, dissolved oxygen, calcium, iron, sample depth and background velocity did not change significantly. On the other hand, the variables that varied between categories were water temperature (p anova < 0.001, p perm = 0.002), electrical conductivity (p anova < 0.001, p perm = 0.001), total phosphorus (p ANOVA = 0.001, p perm = 0.001) and silicates (p ANOVA < 0.001, p perm = 0.001). However, when performing the subsequent tukey test, only the water temperature varied between the C/A, A/D and C/D categories, which implies that only the temperature marks differences between the two taxa in terms of habitat preferences (Fig 4).

Regarding phosphorus and silica, the categories of presences of both taxa (C/A, A/D or C/D) always recorded lower concentration of both parameters compared to A/A. In the case of the electrical conductivity, a preference of both taxa is registered by the average and lower range of the registered concentrations. Finally, in the case of temperature, the categories A/A and C/A are present at lower temperatures, while the A/D and C/D categories are present at higher temperatures (Fig. 4).

By correlating these four parameters with the total richness of taxa, it is observed that only silica is inversely and significantly related to the total richness, but not the temperature, phosphorus and electrical conductivity (Kendall test: Temp., τ=0.12, p value=0.06; Sílice, τ=-0.13, p value=0.05; CE, τ=0.11, p value=0.09; P, τ=-0.03, p value=0.61). This implies that the preference for lower concentrations of silica shown by *Cymbella sp.* and *D. geminata* is also reflected by the rest of the registered benthic community.

According to the first Random Forest model, *Cymbella sp.* presence is explained by physical and chemical variables with a 29% error, whereby variables with the greatest power of discrimination being electrical conductivity, phosphorus and iron. With respect to *D. geminata*, its presence is explained by physical and chemical variables with an 11.1% error, being temperature, phosphorus and silica variables with the highest power of discrimination (Table 1).

According to the second Random Forest model, physical, chemical and biological variables (presence of *D. geminata* and *Cymbella sp.*) can explain up to 42% of the variance of mat thickness, but the variables with the greatest power of discrimination are pH, silica and river depth. The presence of both microalgae has a low influence on the models (Table 1).

DISCUSSION

***D. geminata*, *Cymbella sp.* and their relationship with mucilage**

Results show how points presents different mat thickness according to *D. geminata* and *Cymbella sp.* presence or absence. In the presence of both, mat thickness is doubled. But, only in *Cymbella sp.* presence, is more likely that mats do not be visible (< 0.5 mm). Contrarily, only in *D. geminata* presence, sampling points have a visible mat in most of the cases (median of 3 mm). Thus, by itself, *Cymbella sp.* does not have the ability to produce massive growth that have *D. geminata*. This imply that mat thickness duplication in presence of both due occur through an interaction between both species.

Greater mat thickness at a lower cell density of *D. geminata* is consistent with the documented paradox between *D. geminata* and mucilage production, a process associated with low cell abundance (Bothwell et al., 2014). In the case of *Cymbella sp.*, a greater mat thickness is present at intermediate densities, suggesting the existence of a cellular optimal for the production of mucilage. However, there are no studies investigating the production of mucilage associated with this genus. These results suggest that *D. geminata* could develop mucilage in early stages of colonization of a habitat, but not *Cymbella sp.*, which would require a certain cell density to produce more mucilage.

We suggest that the inverse relationship of cell densities between *D. geminata* and *Cymbella sp.* would be a consequence of their relationship through the mucilage: the lower cellular density of *D. geminata* would produce mucilage (increase mat thickness), which increases the colonization possibility of *Cymbella sp.*

(and other diatoms (Ladrera et al., 2018)), and then would begin mucilage production of this taxon. Eventually, the presence of two taxa that produce mucilage would doubles the mat thickness. Previous studies have documented the ability of *D. geminata* to generate suitable habitat for small diatoms and other microorganisms (Domozych et al., 2010; Ladrera et al., 2018) by reducing cutting effort through increased mat thickness (Cullis et al., 2013), which favors the colonization of cosmopolitan diatoms (Kilroy et al., 2009) and is consistent with the suggested process.

There is currently no literature that investigates the production of mucilage by the genus *Cymbella sp.*, so it is hasty to suggest that the greater mat thickness at intermediate densities it is the result of an optimum production of mucilage. As a hypothesis, we suggest that this result could respond to bidirectional regulation of mucilage production: at low cell density of *Cymbella sp.* there would be an Allee effect (Stephens & Sutherland, 1999) and at high densities there would be greater intraspecific competition (Begon et al., 2005), making it difficult in both cases to produce mucilage. Although asexual microorganisms are less susceptible to the Allee effect (Sarnelle & Knapp, 2004), it does affect ecosystem engineering species, such as mucilage-producing microalgae (Stephens & Sutherland, 1999). In this way, according to the detected pattern of *Cymbella sp.* this study suggests an interesting hypothesis to be developed in future studies.

Role of physical and chemical variables

The patterns of silica, phosphorus and electrical conductivity observed in the presence of *Cymbella sp.* and *D. geminata* has previously only been documented for *D. geminata* and phosphorus (Bray et al., 2017), which coincide with the results presented by Spaulding & Elwell (2007) where a range of phosphorus levels in *D. geminata* is frequently detected in the USA. The richness of microalgae does not decrease with increasing phosphorus and conductivity, so the pattern recorded by *D. geminata* and *Cymbella sp.* for these parameters is not frequent.

Regarding temperature, the results could be interpreted as an extension of the thermal niche of *Cymbella sp.* facilitated by *D. geminate* presence, living in higher temperatures than those recorded in *D. geminate* absence. As a hypothesis, the next process proposed is to explain the results: 1.- *D. geminata* colonizes a habitat, 2.- at higher temperatures *D. geminata* produces more mucilage (Bothwell et al., 2014), 3.- mucilage thick this mat and generates a microhabitat that facilitates the *Cymbella sp.* colonization, increasing its thermal niche. This concurs with what was discussed in the previous section of the discussion, including with temperature as a catalyst for the facilitation process.

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In the Random Forest model of mat thickness, pH, river depth and silica had a greater influence than that explained by the presence of both microalgae. However, silica explains both the mat thickness and *D. geminate* presence, allowing us to assume that silica explains the mat thickness because it determines the presence of *D. geminata*, which produces mucilage. On the other hand, none of the physical and chemical variables that explain the presence of *Cymbella sp.* explain the mat thickness. However, *Cymbella sp.* by itself may have more importance than *D. geminata* in the Random Forest model. We suggest that *Cymbella sp.* explains an increase in mat thickness due to its interaction with *D. geminata* which doesn´t depend strongly on physical and chemical factors, while *D. geminata* is seconded by its relationship with silica. The relation between river depth and mat thickness is due to an increase in sunlight at lower depths, which in general favours the production of microalgae mucilage.

Since there are no limnological data linked to mat thickness in Chile, there is no information to compare or establish the ability of *Cymbella sp* to produce mucilage, which highlights the novelty of the present study.

CONCLUSION

The presence of *D. geminata* and *Cymbella sp.* increases the mat thickness, in this can suggest that *D. geminata* would produce mucilage to a greater extent, and its presence could favour the production of mucilage by *Cymbella sp.* The pH, silica and river depth are more relevant when it comes to predicting mat thickness than both microalgae, despite this being a biological process. The findings of this study suggest that it is necessary to evaluate together the microalgae community when we try to find the relationship between mucosal development and a species. Further research must continue to investigate the processes that determine the biological production of mucilage in rivers.

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Figure 1

Study area and sampling scheme

(A) A total of 25 river reaches were sampled during two seasons (spring and autumn). A/A, reaches with absence of both *D. geminata* and *Cymbella sp.*; A/D, reaches with the presence of *D. geminata* only; C/A, reaches with the presence of *Cymbella sp.* only; and C/D, reaches with the presence of both species. (B) The study was carried out in the regions of Bío-Bío, Araucanía and Los Ríos, in the south-central zone of Chile. (C) In each river reach between 1 and 6 sampling points were registered, with a total of 123 sampling points during both campaigns.

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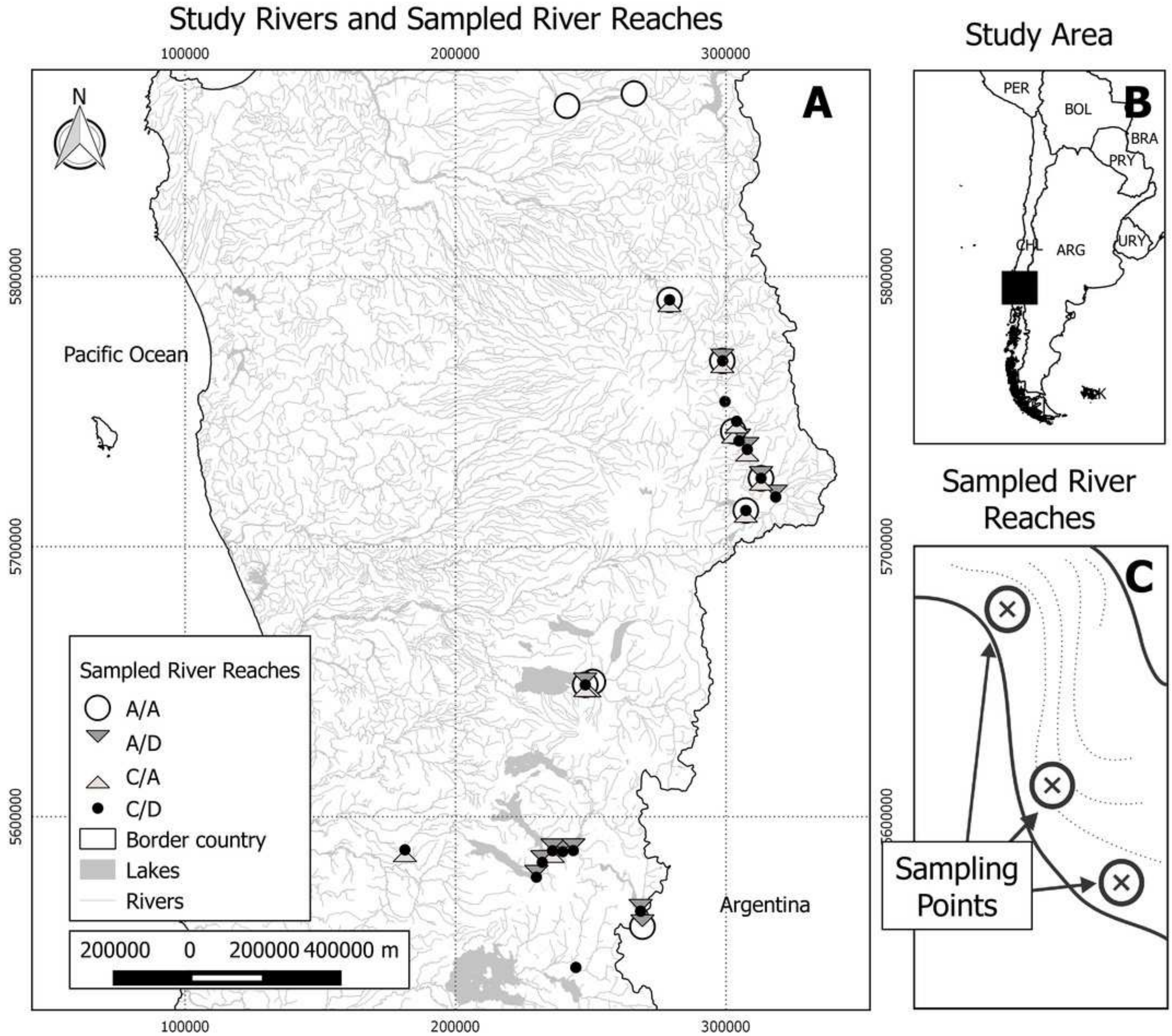
Study Rivers and Sampled River Reaches
100000
200000
300000
5800000
5700000
5600000
A
N
Pacific Ocean
Argentina
Sampled River Reaches
A/A
A/D
C/A
C/D
Border country
Lakes
Rivers
200000 0 200000 400000 m
Study Area
B
PER
BOL
BRA
PRY
CHL
ARG
URY
Sampled River Reaches
C
Sampling Points

Figure 2

Graphs of the relationship between mat thickness and presence/absence of *Cymbella sp.* and *D. geminata*

A) Boxplot thickness in the presence (P) or absence (A) of *Cymbella sp.* and *D. geminata*. Letters above (a, b and c) represent the homogeneous groups of the tukey test. B) Graph of the interaction between presence and absence of *Cymbella sp.* and *D. geminata* against the average mat thickness.

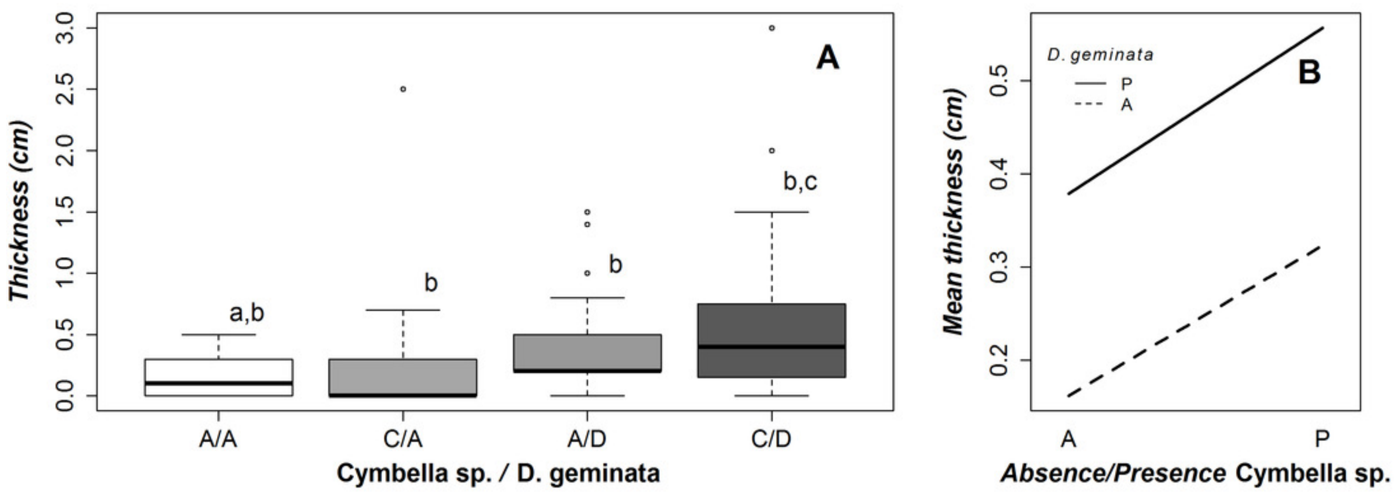

Figure 3

Graphs of the relationship between mat thickness and cell density of *Cymbella sp.* and *D. geminata*.

A) Mat thickness as a function of cell density of *Cymbella sp.* B) Mat thickness as a function of cell density of *D. geminata* C) Smooth Surface model of mat thickness in cell density of *Cymbella sp.* versus cell density of *D. geminata* plot. Smooth Surface is significant (p value = 0.03) and accounts for 8.3% of the variance.

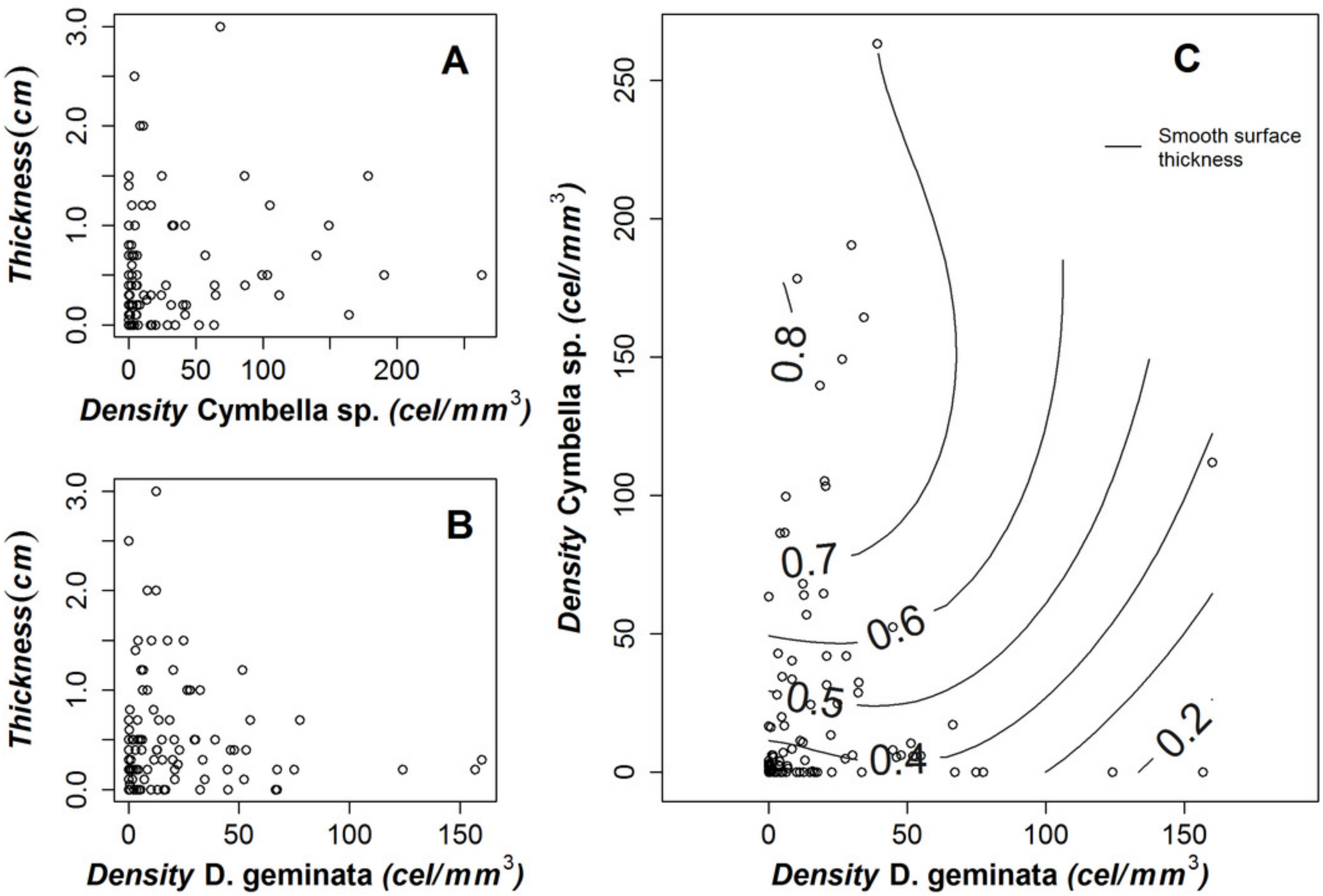

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Figure 4

Boxplot of physical and chemical conditions per river reach sampled according to the presence/absence of *D. geminata* and *Cymbella sp.*

Letters on each group indicate homogeneous groups thrown by Tukey test. A) Water temperature. Sites in the presence of *D. geminata* differ from sites without any of the microalgae studied. B) Water conductivity. Sites in the absence of both microalgae differ from the rest of the categories. C) Total phosphorus. Sites in the absence of both microalgae differ from the rest of the categories. D) Total silica. Sites in the absence of both microalgae differ from the rest of the categories. The rest of the chemical variables measured did not yield significant results.

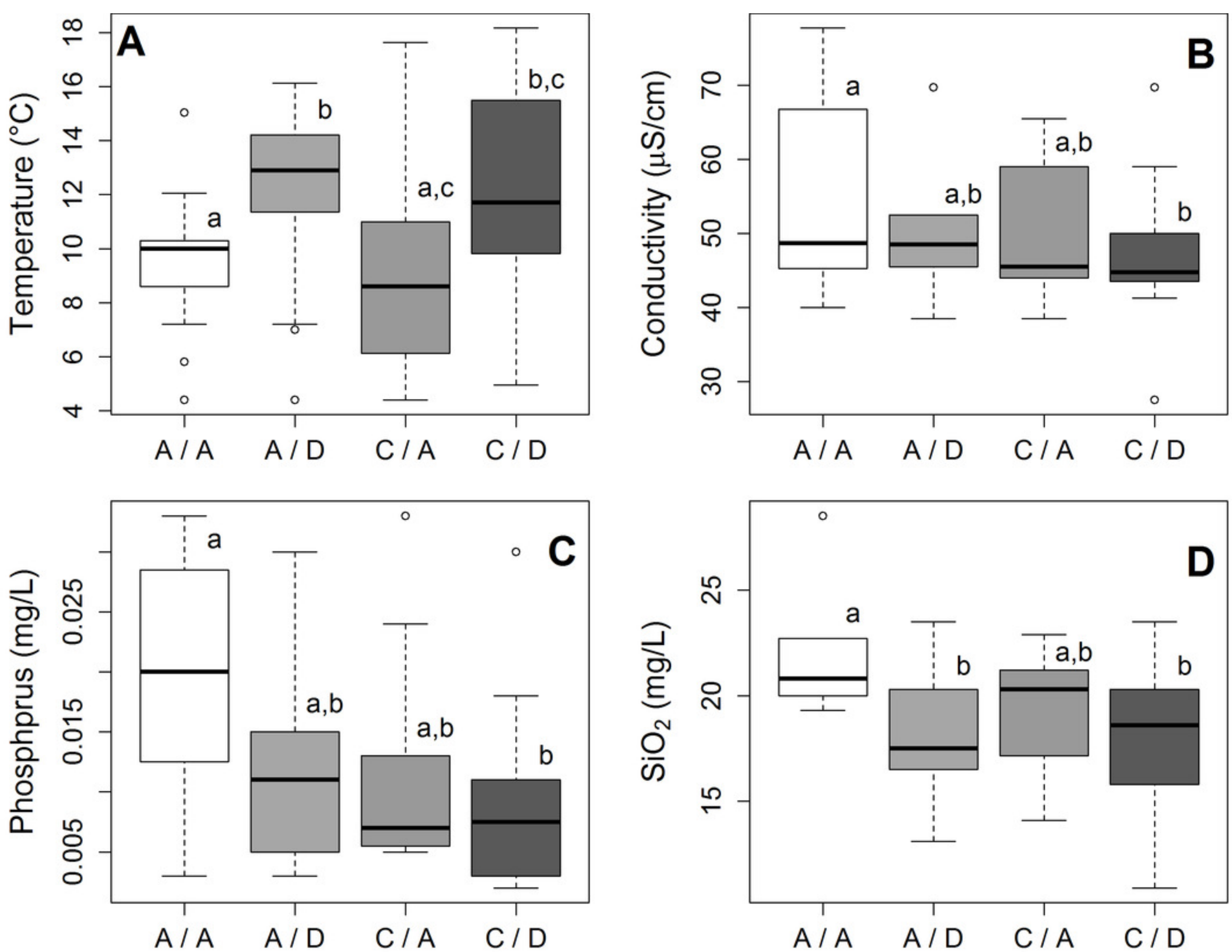

Table 1 (on next page)

Results of Random Forest analysis to determine discriminatory capacity of physical, chemical and biological variables on mat thickness, and in the presence of *Cymbella sp.* and *D. geminata*.

The percentage of the variable importance represents the participation of each variable in each model generated by Random Forest to fit the final model.

Table 1. Results of Random Forest analysis to determine discriminatory capacity of physical, chemical and biological variables on mat thickness, in the presence of *Cymbella sp.*, and in the presence of *D. geminata*. The percentage of the variable importance represents the participation of each variable in each model generated by Random Forest to fit the final model.

Macroscopic mat thickness		Presence *Cymbella sp.*		Presence *D. geminata*	
MS residuals	0.17	Error model	21.32%	Error model	5.56%
Var. Explained	42.34%	Error class *Cymbella sp.*	29.41%	Error class *D. geminata*	9.87%
Percentage of the variable importance					
Temp	31.83%	Temp	0.00%	Temp	**100.00%**
pH	**100.00%**	pH	31.21%	pH	24.70%
CE	19.89%	CE	**100.00%**	CE	44.82%
OD	35.16%	OD	21.76%	OD	44.82%
Ca	54.05%	Ca	8.79%	Ca	15.04%
P	14.91%	P	**93.37%**	P	**64.54%**
Fe	3.19%	Fe	**92.96%**	Fe	18.03%
SiO2	**92.70%**	SiO2	57.29%	SiO2	**95.72%**
Depth	**80.76%**	Depth	47.83%	Depth	0.00%
Vel fond	0.00%	Vel fond	17.57%	Vel fond	14.94%
Didy	26.14%	Didy	Not participate	Didy	Not participate
Cym	33.42%	Cym	Not participate	Cym	Not participate

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