Chapter

Development of Ex Vivo Model to Study the Effect of Rock Snot, *Didymosphenia geminata* (Lyngb.), on Cells and Gametes of Freshwater Fish

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Abstract

Rock snot, a species of diatom that produces nuisance growths in international freshwater rivers and streams, in Chile, Canada, New Zealand and other country, with consistently cold-water temperatures and low-nutrient levels, is produced by *Didymosphenia geminata* (Lyngb.) M. Schmidt (*D. geminata*) and is a complex problem in rivers. Its biology problem and its effects on ecosystems are still being investigated, yet no research has focused on the effects of *D. geminata* at the cellular level. We proposed to review and described an artificial river system to preserve *D. geminata* for obtained future study material to evaluate the effects of water contaminated by this diatom on cell models. Our preliminary results indicate the *D. geminata* altered several cell lines and cell function, and review of the literature suggested alteration in the biotic system on river with this plague. We proposed the described literature for exploring the possibility of building a model for maintaining *D. geminata* from Chilean rivers and used the biological material obtained from artificial river, like extract and contaminated water for studying the chronic effects on cells.

Keywords: didymo, cells, biotechnology

1. Introduction: threat to freshwater environment

Didymosphenia geminata (Lyngb.) M. Schmidt (*D. geminata*) has attracted considerable attention as an invasive nuisance species in natural habitats, in different country, like Chile, Argentina, Canada, Poland and New Zealand [1, 2]. The species also show nuisance effect in the southern hemisphere [3, 4]. Biological invasions are a threat to freshwater environments and the ecosystem services they provide [5, 6]. *D. geminata* is a large diatom species (~100 μm long) that produces sulphated polysaccharide stems and forms nuisance strands that can grow up to 10 cm thick with 100% coverage in streams [7]. This microalga, belonging to the brown algae diatom family, is rich in specific molecules such as antioxidants, which include polyphenols and diadinoxanthin [8]. What is the nuisance effect of this plague? The most significant impacts that this diatom presents to aquatic systems

can be observed through its physical changes, substantial increases in algal biomass, stagnation of fine deposits, hydrodynamic change and their secondary effects on biogeochemical states and processes, such as redox conditions, pH and nutrient cycling in the benthic layers [9]. Recently, *D. geminata* has been molecularly identified in the rivers of southern Chile [10] and was compared with different samples; in fact, Argentinian and Chilean *D. geminata* are the same species, but the authors did not describe their origin [11]. This observation is important because the movements of *D. geminata* can improve our understanding of the species' dispersion; in fact, an essential work in New Zealand proved the invasion of *D. geminata* and gave us clues about the source of these diatoms [12].

Few studies have focused on environmental impacts, although these were mentioned indirectly in nutrient recycling mechanism and flow channel attenuation studies [13–15]. Other studies have indicated that *D. geminata* alters microenvironments and reduces fish populations [16] by altering fish community diets [17]. Also, it disturbs aquatic invertebrate communities and the filters of drinking water systems [18, 19], although it is unknown whether the contamination effect is direct. Recently, the toxic effects of microalgae on contaminated river communities [14] and the effects on *Salmo salar* spermatozoon activation time have been described [20]. Despite extensive research on *D. geminata* in recent years [21] with results on ecosystem effects, further advances on the toxicological effects are still needed.

2. Method

We used a collection of different databases, which included ScienDirect and Scopus, and we do it with a selection of the only index articles published. Based on the previous report from our group laboratory, we made a collection of the 135 different articles and review; quantitative, qualitative and mixed method evaluations, with and without comparative groups, were eligible for inclusion in the review. The metadata and abstracts from the literature search were transferred to software [EndNote X9 (Thomson Reuters, CA, USA)] and duplicated are eliminate. Two reviewers observed each title and abstract and selected studies that potentially met the eligibility criteria, or these keywords are used for exploring the internal database building whit Endnote. These papers were obtained in full, and two reviewers screened the full papers for inclusion, with any disagreements resolved by consensus with reference to the full papers and a third reviewer if necessary.

3. *Didymosphenia geminata* (Lyngb.) M. Schmidt maintenance and hydrological problems

The patterns of silica, phosphorus and electrical conductivity observed in the presence of *Cymbella* sp. and *D. geminata* have previously only been documented for *D. geminata* and phosphorus [22] which coincide with the results presented by Gretz et al. [23] in which a range of phosphorus levels in *D. geminata* has frequently been detected in the USA. The richness of microalgae does not decrease with increasing phosphorus and conductivity, so the patterns of *D. geminata* and *Cymbella* sp. are not frequently recorded for these parameters. Regarding the temperature, the results could be interpreted as an extension of the thermal niche of *Cymbella* sp. facilitated by the presence of *D. geminata* and the existence at higher temperatures than those recorded in the absence of *D. geminata*. As a hypothesis, the next process is proposed to explain the following:

1. How *D. geminata* colonises a habitat?

2. Why *D. geminata* produces a thicker mat at higher temperatures [24]?

3. How a mat of greater thickness generates a microhabitat that facilitates colonisation by *Cymbella* sp., thus increasing its thermal niche?

The development of kind of model will help to understand these issues and develop studies about the biology and relationship of *D. geminata* with other diatoms, such as *Cymbella* sp. In this case, building an artificial model, such as river model, helps us to follow this process and to suggest a biological mechanism for *D. geminata* development about other diatoms and river species.

The conditions in which *D. geminata* is established are, and the subsequent development of rock snot is even more complex. The studies have focused mainly on the physicochemical conditions of the development of *D. geminata* [1, 24], and very few investigations have incorporated other physical-hydrological factors, such as turbidity, runoff speed, shear stress, substrate [19, 25] and the like. This view leads to many degrees of freedom for assessing the main factors that condition the establishment and proliferation of *D. geminata*. It is thus necessary to reduce the degrees of freedom to model these conditions and to work in more controlled conditions through an in vitro model, such as what we intend to develop in our project.

An in vitro model of an artificial hydraulic system is designed using their hydraulic similarity (a hydraulic physical model), which maintains the development of controlled fluvial and ecological processes. The hydraulic similarity must be carried out, considering the dimensionless numbers of the hydraulics that allow for the characterisation of the regime, such as the Froude number. However, the challenge not only lies in the design via the dimensionless numbers of the hydraulic system but must also be a system that maintains the eco-hydraulic conditions that are typical of the rivers in which the establishment of *D. geminata* has been evidenced, such as the runoff speed, turbidity, depth, and type of substrate. In other words, it will be necessary to replicate as faithfully as possible the hydraulic, thermal and ecological conditions of the rivers in which the samples of *D. geminata* will be collected, but the artificial hydraulic system design must be hydraulically similar; this is the challenge of the current project being discussed.

4. *Didymosphenia geminata* (Lyngb.) M. Schmidt maintenance: the first model

We assessed whether it was possible to keep *D. geminata* in a laboratory setting close to the conditions found in a river. Accordingly, aquarium systems that regulate water variables were implemented, which allowed us to observe the maintenance or development of *D. geminata*. The substrate with the adhered *D. geminata* was collected from the rivers and placed in the aquarium. The aquarium maintained light and flow conditions and a water column of approximately 15 cm. This *D. geminata* maintenance aquarium was observed continuously for 2 months, and then a second tank was prepared with distilled water, a flow pump and uncontaminated river substrate that was washed and sterilised by an autoclave. *D. geminata* was maintained as presented in a report by Olivares et al. [20]. Notably, after 4 weeks, *D. geminata* showed a population of microalgae that appeared to exhibit cyclical behaviour, ranging from a few to large numbers of *D. geminata* per field (subjective observations). Our previous data suggested that it is possible to keep and replant *D. geminata* in artificial river conditions; we propose to improve

this protocol to generate a new version for the river model [26]. This work allows us to suggest some ideas for the management of *D. geminata* in the laboratory, allowing open protocols for in vitro studies of *D. geminata* to obtain a better understanding of its biology. Observations made in this earlier study indicate that it is possible to keep *D. geminata* in the laboratory, which seems to contradict what is mentioned in the literature regarding both its maintenance [27] and preservation [28]. It may even be possible to move and transport samples from the collection sites for analysis [29] and thus achieve complex systems of general diatom cultures from biofilm communities [30].

The maintenance of *D. geminata* in the aquarium was successful following the protocol described in Section 2 and achieved a viability of 90%. In this protocol, the critically essential parameters appear to be the following: the use of polluted river substrate, a constant water flow, low temperatures and a water column of 15 cm above the substrate. Moreover, we observed a set of simple organisms, indicating a complex ecological system associated with *D. geminata*, but it is not possible to induce controlled contamination of a new complex substrate with more details in the flow; in addition, the river conditions need to be prepared and studied [20, 26].

Studies from other groups have addressed these questions by making direct observations in rivers. Observing their characteristics to model the growth of *D. geminata* [31] and evaluating the effect of phosphorus as a nutrient function or process of *D. geminata* [27, 29], the type of substrate required [19] or the effect of nutrients on the development of this diatom [32], such studies require observations in the field, which make it difficult to control environmental and seasonal variables, as well as requiring that the seasonality of the hydrologic regime be considered at the time of collection. In contrast, this project suggests a *D. geminata* maintenance system that would allow *D. geminata* studies in the laboratory and provide the basis for generating systems that could test the conditions of *D. geminata* development or control. For example, the effect of shear stress in rivers could be mimicked in scaled models using hydraulic flow to simulate runoff characteristics.

However, not all of the river model is complete, and in our experiment and those of other groups, the culture of *D. geminata* has not yet been reported. In previous work, we cited where a complex culture protocol for single *D. geminata* had explored and replicated a closed in vitro model similar to a river for the study of *D. geminata* that is not present in the existing literature. The feasibility of keeping *D. geminata* in river model suggests the possibility of replicating and maintaining it in the laboratory but does not indicate that it is a culture medium because the conditions that mimic the observed outcrop have not been determined. However, it presents a new tool for learning more about *D. geminata* and its growth conditions.

5. Cellular effects

Recent studies have suggested additional toxic impacts of *D. geminata* on invertebrates [7] and benthic river communities [18] or of the treatments used for its removal [33]. This information on the effects of the microalgae is just recently being explored. Previous data suggested that *D. geminata* can mainly affect the activation time of *Salmo salar* spermatozoa, secondary at present of the polyphenols, and a possible mechanism is the alteration of the calcium in the cells. This is an evidence of alterations in cell physiology, possibly due to the presence of polyphenols; more importantly, however, our findings indicate that the effects of this plague are more complex and should be closely watched. *D. geminata* should be considered as a toxicological agent that affects cell function.

Evidence suggests an effect of water contaminated with *D. geminata* on the motility time of *Salmo salar* spermatozoa. A decrease is observed in their activation time and the percentage of motile cells, without affecting the viability of the spermatozoa or motile spermatozoon kinetics. These effects could be mediated by the polyphenol content present in *D. geminata* and could be released into rivers contaminated by this microalga. *D. geminata* has become a pollution problem in the rivers of southern Chile. It was initially detected in rivers in the "Los Lagos" Chilean area, but since then there have been at least three more regions that reportedly have this plague, and projections are that it will continue to spread if barriers are not raised. No evidence exists that it can be maintained in a cultivated system, despite its rapid growth [15].

In contrast, it is possible to find research indicating that under various conditions, it is not feasible to keep *D. geminata* in closed systems [34]. An older study suggests a prolonged protocol for keeping *D. geminata* but not that made in culture [35]. Other models have described the effects of *D. geminata* on the microflora of rivers, reporting changes mainly in aquatic species, but with no reports of similar results in more complex forms [7]. Our findings indicate that the viability of *Salmo salar* spermatozoa is not affected when exposed to water contaminated with *D. geminata*. However, the reaction time and the mass of motile spermatozoa are changed. A decrease of 50% in the activation time was shown, which alters the functions of the spermatozoa. The Powermilt compound has been used as a spermatozoon activator as described by Olivares et al. [20]. This compound produced an excellent response in our samples, with an average activation time of 4 min. To understand the sperm activation mechanism, under *D. geminata* presence, experimental observations were made about whether it was able to inhibit the Powermilt's effects on the activation times. Upon dilution with water contaminated with *D. geminata*, we observed a decreased effect of the Powermilt stock on activation; the sperm activation it was fully inhibited when using 100% *D. geminata*. This data suggests that the release of molecules into river water containing *D. geminata* would reduce the activation time of *Salmo salar* spermatozoa without affecting their kinetics. Another study with metals in the water showed effects on the kinetic parameters [36], but our results showed that the contaminated water altered the activation processes, except for the already activated spermatozoa. This outcome suggests a complex mechanism that reduces the number of cells that can be activated but not the quality of this activation, which involves a different contamination mechanism, such as metal in the water.

The organic content of *D. geminata* has proven to be rich in antioxidant polyphenols that are common in brown algae, such as diadinoxanthin. This type of molecule has cellular effects that range from beneficial to toxic, depending on the concentration. In the samples maintained or recovered from rivers, there was a ratio of approximately 200 ppm of polyphenols per 10 g of *D. geminata* that could be extracted. To explore whether it was possible to associate this organic content to the functional effects of *D. geminata*, the spermatozoa were activated with increasing concentrations of the extracts, thus showing a dose-response effect when inhibiting the time activation of the samples with an IC_{50} of 15 ppm of polyphenols. We conclude that the presence of polyphenols mediates the effect observed in the waters contaminated with *D. geminata*, but we cannot reject other mechanisms for altering cellular function.

Furthermore, in vitro cell cultures have proven to be a suitable tool for assessing the toxicity of different chemicals in fish [37]. A polyphenol study in two fish cell lines showed significant differences in the EC_{50} values for phenolic compounds (phenol and 2,4-dinitrophenol) in CHSE cells when assessing cell viability and proliferation [38].

In other study, we evaluated the cell sensibility of SHK-1 and CHSE-214 to *D. geminata*, two cell lines that will be used to assess various physiological fish farming activities (to evaluate SHK-1 and CHSE-214 cell sensitivities against *D. geminata*), which will also be used to determine multiple physiological aquacultural effects on Atlantic salmon and Chinook salmon [39, 40].

In general, the current studies on this diatom have focused on oligotrophic specimens, with no toxicity studies on species living above biota in rivers, which this project aims to elucidate for the effects of *D. geminata* on cell viability, using in vitro model of the river.

This aim is of great interest for analysing whether these same effects occur in native freshwater species, as this could be a bioindicator of water quality, as well as for other species or biological models [41]. In this regard, the reproduction of native

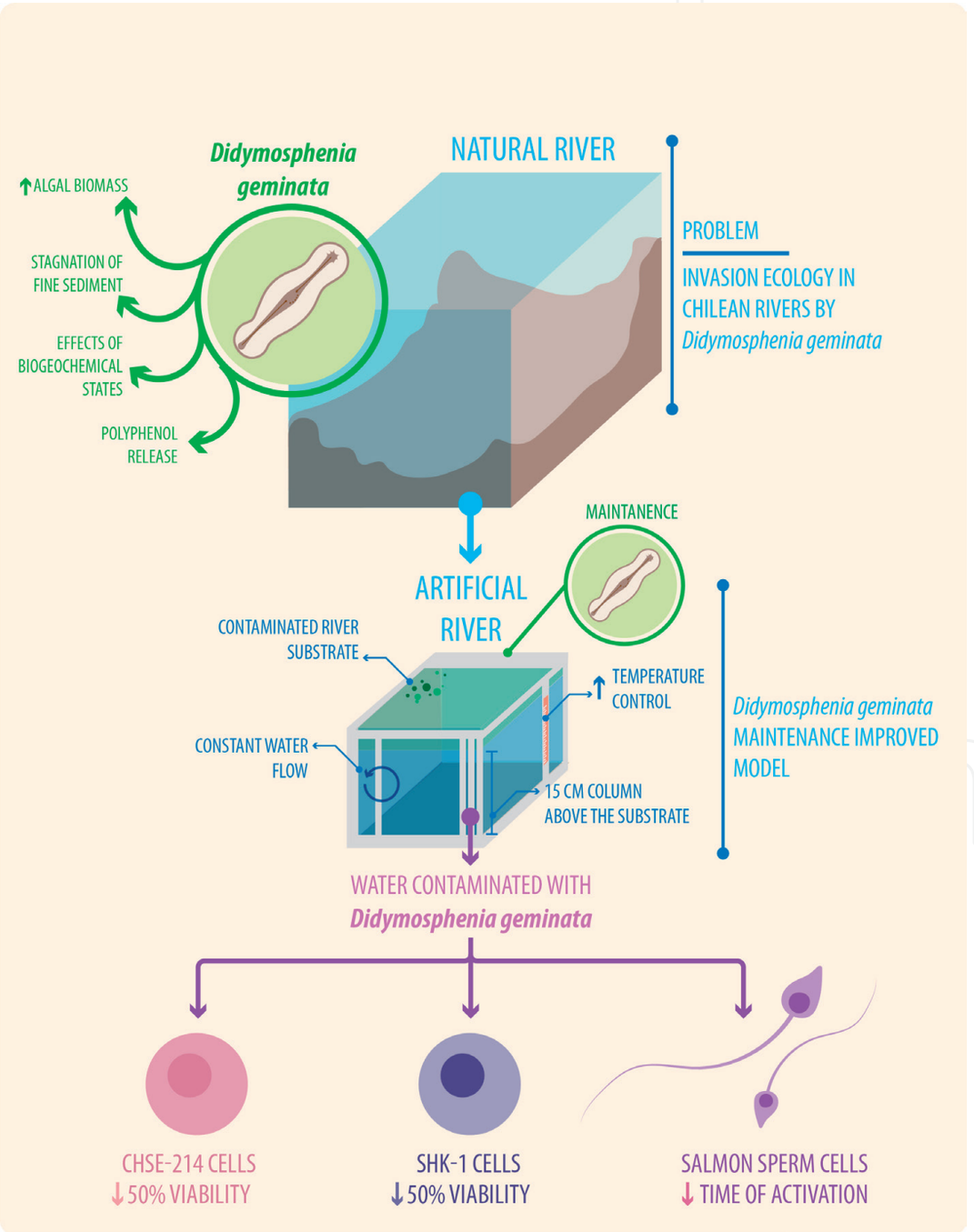

Figure 1.
A model summarizing the chapter. We present the idea of developing an in vitro *system for maintaining* D. geminata *and obtaining biological material for cell studies.*

fish species was reduced and exhibited a low success rate [42, 43]. Furthermore, there are no records where species are mentioned as being affected by the microalgae in rivers, revealing a deficiency in the research on the effects of *D. geminata* on the organs of native river species. Internationally, changes have been indicated in microinvertebrate compositions through *Salmo trutta* diet observations [17] and are consistent with the changes in the biota from rivers contaminated with *D. geminata*. Previous results suggest complex cellular-level effects of the polyphenols present in the microalgae. These effects could be considered relevant, since the compound, when concentrated and present for long periods of time, could lead to the chronic impacts (longer than 24 h), thereby generating deleterious effects on superior aquatic species. *D. geminata* can be considered toxic in upwelling conditions (blooms), where it is most active, thus altering the aquatic community's viability (macroinvertebrates and fish) because of the physical effects of river coverage and sediment trapped in estuaries [9] or the composite mass, which is mainly mucilage [44]. What we presumed is a possible development of a system of river model by maintaining *D. geminata* in a laboratory to obtain biological material to describe its cellular effects better and understand the impact of contamination by *D. geminata* in rivers. We summarise our idea in **Figure 1**, a model of the general purpose of developing a system to study *D. geminata*, and apply the study to the biological effects of rivers contaminated by this diatom. We present the models to explain the future of our work.

6. Concluding remarks

It is possible and necessary to improve a system of maintenance and study of the development of the *D. geminata* in river model. At the same time, this model helps in obtaining biological material to research at the cellular level of the effect of this contamination in rivers. Our Chapter, present a review to supports the idea at is possible the development of a system of river model and are necessary for explored and explain several mechanism of the *D. geminata* on river and different biological model, included cell model. We build the first model, but more improvement is necessary to have a more robust river model where *D. geminata* is maintained. Allowing the handling of biological samples for cell studies and this helps to us to report from this model, the controlled biological material from river model improved with *D. geminata* and without *D. geminata.* The maintenance of *D. geminata* and the effects of the organic material obtained in cellular models in scientific publications and future description of *D. geminata* biology.

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