Water contaminated with *Didymosphenia geminata* alters fish cell line viability

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Didymosphenia geminata (*D. geminata*) in Chilean rivers is a complex roblem. Its biology and effects on ecosystems is still being studied, at the moment not research has focused on its *D. geminata* effects at the cellular level. We developed an artificial river system to maintain *D. geminata* study material and evaluate effects of water contaminated with this diatom on the viability of two fish cell lines. Results indicate that CHSE-214 cells are sensitive to increasing *D. geminata* extract concentrations, reducing crop viability by 50% when exposed for 24 hours at a 0.01V/V dilution and reducing proliferative capacity by 70% on a 5 day temporal curve. SHK-1 cells showed lower sensitivity, presenting a decrease of 20% in viability at 24 hours, and a lower cell proliferation rate by day 5, but higher than of the CHSE-214 cells. Both lines were affected by exposure to *D. geminate* extracts, but CHSE-214 lines were more sensitive to polyphenols extracted from the microalgae. We conclude that certain cell types are sensitive to *D. geminata* in rivers, meaning that chronic effects on aquatic species contaminated with this diatom should be observed. Effects of this plague at a cellular level can be further studied to understand its full impact on river ecosystems.

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Water contaminated with *Didymosphenia geminata* alters fish cell line viability

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Abstract

Didymosphenia geminata (*D. geminata*) in Chilean rivers is a complex problem. Its biology and effects on ecosystems is still being studied, at the moment not research has focused on its *D. geminata* effects at the cellular level. We developed an artificial river system to maintain *D. geminata* study material and evaluate effects of water contaminated with this diatom on the viability of two fish cell lines. Results indicate that CHSE-214 cells are sensitive to increasing *D. geminata* extract concentrations, reducing crop viability by 50% when exposed for 24 hours at a 0.01V/V dilution and reducing proliferative capacity by 70% on a 5 day temporal curve. SHK-1 cells showed lower sensitivity, presenting a decrease of 20% in viability at 24 hours, and a lower cell proliferation rate by day 5, but higher than of the CHSE-214 cells. Both lines were affected by exposure to *D. geminata* extracts, but CHSE-214 lines were more sensitive to polyphenols extracted from the microalgae. We conclude that certain cell types are sensitive to *D. geminata* in rivers, meaning that chronic effects on aquatic species contaminated with this diatom should be observed. Effects of this plague at a cellular level can be further studied to understand its full impact on river ecosystems.

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Introduction

Didymosphenia geminata (*D. geminata*) is a large diatom species (~100 microns long) that has been molecularly identified in rivers of southern Chile [1]. The first report of *D. geminata* forming a bloom was in the Espolón river (around 44° S) in Patagonia in 2010 [2]. *D.geminata* has gained considerable attention as an invasive species in natural habitats [3, 4]. It shows an aggressive behavior in the southern hemisphere, probably because favorable climatic condition such as temperature and phosphorus concentrations in water which potentiates both growth and propagation [5, 6]. Biological invasions are a threat to freshwater environments and the ecosystem services they provide [7].

D. geminata produces sulfated polysaccharide stems and forms nuisance in river sediment that can grow up to 10 cm thick with 100% coverage in streams [8]. This microalgae belongs to the brown algae diatom, and is rich in antioxidants like polyphenols and diadinoxanthin [9]. The most significant impacts that this diatom presents to aquatic systems can be observed through physical changes: substantial increases in algal biomass, retention of fine sediment, hydrodynamic alteration, and consequently, effects on biogeochemical states and processes such as redox conditions, pH and nutrient cycling in benthic layers [6].

Few studies have focused attention to environmental impacts, although it is mentioned indirectly in nutrient recycling mechanism or flow channel attenuation studies [10, 11]. Other studies indicate that *D. geminata* alters microenvironments and producing changes in invertebrate assemblages, associated with *D. geminata* altering fish community diets [12]. In addition, it disturbs aquatic invertebrate communities and drinking water filters system [13-15], although it is unknown whether the contamination effects are directly caused by *D. geminata*. In this line, recent studies have described toxic effects of microalgae on contaminated river communities of microcrustaceous [16] and effects on *Salmo salar* spermatozoa activation time [17]. Despite extensive research on *D. geminata* in recent years [18] with effects on ecosystems, advances on toxicological effects are still lacking. To date, studies on this diatom have focused on oligotrophic specimens, with no toxicity studies on vertebrates in rivers, for which this study aims to elucidate the effects of *D. geminata* on cell viability on salmonids.

In vitro cell cultures have proven to be a suitable tool to assess toxicity of different chemicals in fish [19]. For example, a study that assessed viability and cell proliferation in two fish cell lines showed significant differences in EC_{50} values for phenolic compounds (phenol and 2.4-dinitrophenol) [20]. The present study evaluated the sensibility against *D. geminata* of the fish cell lines SHK-1 and CHSE-214, as a model for various physiological effects in aquaculture (Atlantic salmon and Chinook salmon) [21, 22]. Water contaminated with the microalgae from an artificial river system, and a polyphenol extract derived from the microalgae were used to assess cell viability and proliferation.

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Methods

D. geminata sampling

Samples of *D. geminata* were collected from the Futaleufu and Biobío rivers during autumn and winter of 2015. We collected didymo, defined as snot rock with *D. geminata* samples and other biota present in the rock mucus, through the Chilean National Fishery Services (SERNAPESCA). The samples were transported to the laboratory in closed dark plastic boxes, at 10 ° C. River water and substrate (river rocks) colonized by the microalgae were also collected.

Maintenance protocol for *D. geminata* samples.

The didymo, a mixture of *D. geminata* and other biotic components of the snot rock, was immediately transported to the lab (for 8 hours) and maintained in a closed water recirculation systems called artificial rivers, in agreement with SERNAPESCA biosafety protocol. We got the authorization from permit order No. 3500 to obtain and preserve fresh *D. geminata* samples for laboratory studies, and for polyphenol extraction procedures, as previously described by our group (Parodi et al., 2015). We arranged our artificial river system by adding 50% original river water, plus 50% distilled water, in a total volume of 14 L, leaving a 15 cm water column over the rocks. Artificial rivers were maintained with an expanded polystyrene insulating cover and maintained at a controlled temperature of 12°C with a refrigerating gel system. Flow rates (1200 L/h) were controlled using a Plaset-Italy Model 71009 engine, whose two outputs were connected to a 1 inch diameter PVC pipe, capturing water from one end of the artificial river and leading into the other end (start), achieving steady flow and aeration. Macroscopic and microscopic changes in *D. geminata* artificial river systems were recorded daily.

Polyphenol extraction and HPLC

A *D. geminata* fresh sample obtained from artificial rivers was exposed to 20 ml hydroalcoholic mixture in 20% ethanol. Macerate and rupture was performed by using Misonix XL2000 Series ultrasound equipment, with 10 pulses of 30 minutes with intervals of 1 minute until decomposition occurred in all complex samples. Samples were then incubated for 20 min at 30°C under agitation, and filtered through a double gauze and a Wartman No. 2 (125 mm) as described by Jofre-Fernandez et al. (2013). Finally, polyphenol was measured in the extracts.

Folin and Ciocalteu reagents were used following protocols described by Lowry et al. (1951), and optical density was measured at 517 nm. Samples were frozen and passed through HPLC for their profile identification, following the protocols described by Lohr (Lohr M., 1999), and were then modified using extracts for the measurement of retention time and absorbance

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at 440 nm with the AC18 column (Macherey-Nagel, Duren, Germany). Organic compound presence was identified in the yellow sample fraction.

Cell culture and cytotoxicity studies

The cells lines are buy to European Collection of Authenticated Cell Cultures (ECACC), the CHSE-214 cells (ECACC N° 91041114) is a fish cells line derived from *Oncorhync hustshawytscha* embryo. CHSE-214 cells were cultured with MEM (Gibco), supplemented with 10% fetal bovine serum (Life Technologies), L-glutamine 2 mM (Corning), and antibiotic/antimycotic solution (penicillin 10,000 IU/ml, streptomycin 10,000 µg/mL and amphotericin 25 µg/mL). SHK-1 cells (ECACC N° 97111106) correspond to a fish prefrontal kidney cell line derived from leucocytes from *Salmo salar*. SHK-1 cells were maintained in an incubator at 17°C. Cells were cultured in a Leibowitz L-15 medium (Gibco) and supplemented with 10% fetal bovine serum (Life Technologies), L-glutamine 2 mM (Corning), antibiotic/antimycotic solution (penicillin 10,000 IU/ml, streptomycin 10,000 µg/mL and amphotericin 25 µg/mL) and β-mercaptoethanol 40 µm. The subculture procedures were carried out in a biosafety cabinet and cells were seeded in 24 wells plates for a parallel experiment. For cell maintenance and subculture, the medium was changed every 3 days.

Cellular subculture

Cells were washed with PBS and detached with a trypsin/EDTA 0,25% (HyClone) solution for two minutes. Cells were then placed in complete medium to inhibit the trypsin. Cells were then collected in sterile 15 ml tubes and centrifuged at 300 RCF for 10 minutes. Cells on the bottom were resuspended in 1 ml base medium and counted in a haemocytometer as described elsewhere. Seeding was performed at a density of 1,2 x 10^5 cells/ml in 16 mm diameter culture plates with 250 µl medium. After 24 h, cells were exposed to the polyphenol extract. To assess toxicity, cell cultures were exposed to polyphenol extract over time, to obtain temporal curves. Supravital stains and morphology (cytochemistry) was measured to explore the response mechanisms to the extract. *D. geminata* was exposed to decreasing concentrations of the polyphenol extract (0.1%, 0.01% and 0.001% respectively) for 24 h, and the proliferation rate was measured counting cells per area in a method modified by our group (Parodi et al., 2002).

Viability analysis by MTT and staining with Phalloidin 488

Cells grown in culture plates were exposed to fresh water, water contaminated with *D. geminata*, or water with polyphenol extract, for different times, to observe the viability and to generate a temporal curve of the effects. The doses of water or water contaminated with *D. geminata* were made in a dilution V/V on cultured medium. Before the incubation cells were washed and incubated with working MTT solution. MTT solution was 5 mg/ml (12 mM) and 0.5 mg/ml (1.2 mM). Cells were then incubated for 2 hours at 17°C to allow the formation formazan crystal, then supernatants were removed and 150 ul DMSO was added

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per well. Cultures were left at room temperature until the formazan crystals dissolved. The volume was recorded and 300 µl distilled water was added. Absorbance at DO 570 nm was measured (Spectroquant ® Pharo 300, Merck) and compared against the reagent blank (150 µl DMSO plus 300 µl water). For the blanks, reactive mixtures were taken to zero and measured to ensure a good calibration. Results were normalized against the control and expressed as a viability percentage. Cells were stained with Phalloidin 488, washed 3 times with PBS, and fixed with 4% PFA at pH 7.4 for 10 minutes at room temperature. Samples were then washed twice with PBS at room temperature and incubated with Phalloidin 488 50 nM for 20 min following manufacturer dilution at 17°C, in a humidity chamber. Finally, 2 final washes with PBS were performed and samples were mounted and stained with Dako for fluorescent microscopy. All solutions were maintained at 17°C. Cells were observed with a Nikon Eclipse E200 microscope, with a CMOS 519MU 5.0 M camera and analyzed with the ImageJ software.

Data Analysis

Unless indicated otherwise, results, including image analysis, are presented as the average ± SEM. Statistical comparisons were performed using the Student´s ¨t¨ test or ANOVA. A probability level (P) less than 0.05 is considered statistically significant.

Results

Organic compound profile

Samples were taken from various Chilean contaminated rivers, confirmed by SERNAPESCA. *D. geminata* samples were macerated and hydroalcoholic extraction was performed for HPLC analysis. Samples showed an organic content of 440 nm, similar to that described for other brown algae, particularly diatoms with yellow pigment, which indicates the presence of organic polyphenol as previously described. Figure 1 show a representative profile of five different collection points. The table insert displays retention times and peak values at 440 nm.

D. geminata contaminated water affects cell cultures

SHK-1 and CHSE-214 cells were used to assess the effects of *D. geminata* contamination or its polyphenol extract on cell viability. Figure 2A show SHK-1 cells exposed to fresh water (control) compared with cells exposed to water contaminated with *D. geminata*, showing microscopic alterations in culture morphology. Phalloidin 488 stained cells show a decrease in the fluorescent signal in cells exposed to contaminated water (bottom panel). Figure 2B show CHSE-214 cell counts in presences of *D. geminata* contaminated water or uncontaminated river water dilutions V/V. Figure 2C shows the reduction of culture viability when exposed to increasing concentrations of polyphenols, obtained from *D. geminata*

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samples. Figure 3A shows an image of SHK-1 cells exposed to *D. geminata* contaminated water with a reduced signal. The lower panel show a fluorescent image of cells stained with Phalloidin 488, where a signal reduction is observed. Figure 3B shows an MTT viability curve of cells exposed to different dilutions of *D. geminata* in contaminated water and uncontaminated river water, and a reduction of the viability was observed when cells were exposed to contaminated water. Figure 3C show the effect of increasing polyphenol concentrations on culture viability; the increase of polyphenol concentration reduces the viability of the cell lines. Overall, our results suggest that high contaminant concentrations affect the fish cell viability when exposed for 24 hrs. This may be important when describing chronic effects on aquatic vertebrate species in contaminated rivers, or when algae bloom is observed.

Effect of *D. geminata* contaminated water on proliferation

The effects on cellular proliferation were evaluated in cultures exposed to a single dose of contaminated water (0.001 V/V). Cells showed less development during incubation with contaminated water. Both cell lines showed reduction in development, as seen in Figure 4A, which can be an interesting chronic effect to observe in previous figures. Figure 4B show the acute effect of a single dose of a 0.01 V/V dilution of *D. geminata* contaminated water. Cultures were exposed for 24 h and cells from both cell lines were subsequently counted after several days. Our result showed a different reduction effect in the cell lines, which suggests a different type of mechanism that causes cell death in each cell line, or a differential resistance of each cell lines against the exposure to *D. geminata*.

Discussion

Our data suggests that rivers contaminated with *D. geminata* can generate toxicity, especially in embryonic cells (CHSE-214). We used cell observation to follow the artificial river contamination with *D. geminata*, like a previous report of our group [23]. The *D. geminata* samples showed the presence of biocompounds described for brown microalgae and special for isolated *D. geminata* cells, which were present in our extracts. Figure 1 show that in HPLC profiles, biocompounds present in the *D. geminata* extract are identified as pigments, measured at 440 nm. This profile has not been previously described for *D. geminata*, but it is accepted that this kind of pigment is abundant in this type of diatom and matches with that described at 440 nm, presenting peaks at 10 and 15 minutes for fucoxanthin and diadinoxanthin, respectively (Lohr M.,1999). Our data proposes an HPLC signature for the microalgae *D. geminata* present in contaminated rivers, showing a peak at 3 minutes that has not been described for brown algae from the diatom family. This HPLC signature or "fingerprint" could be used in natural rivers to search for contamination and specially during

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algae bloom. We expect to evaluate this procedure in natural rivers through future project that can confirm the presences of this biocompound, helping to explain toxicity mechanisms, and generating a valuable tool for further studies in rivers.

Previous studies have suggested effects of *D. geminata* on aquatic organisms and spermatozoa [17, 18] that correlate with our data. This study indicates that water contaminated with this diatom reduces the viability of CHSE-214 cell and affects SHK-1 cells as well, suggesting a chronic effects on aquatic organisms due to river water contamination with *D. geminata*, as shown in figures 2 and 3. Interestingly, CHSE-214 cells showed increased sensitivity to contamination, from which it can be interpret that embryonic cells are more sensitive to *D. geminata* (Figure 2) and the polyphenols described, unlike kidney cells such as SHK-1. The data presented indicates that cells may be more sensitive in early stages of development or in the gametes, as previously described [17]. Reports on spermatozoa showed no effect on viability, but did showed physiological effects which suggest that cellular effects are even more complex than those described so far. Results in Figure 3A show that not only does cell viability decrease, but cell division decreases as well when exposed to *D. geminata* contaminated water. Thus, a greater effect on CHSE-214 cells and significant effects on SHK-1 cells can be observed (Figure 4).

Changes in microinvertebrate compositions through the observation of *Salmo trutta* diet have been described [12], which are consistent with the changes in biota from rivers contaminated with *D. geminata*. Our results suggest a complex effect at the cellular level of polyphenols present in this microalgae. This is relevant since polyphenols, when concentrated and present for long periods of time (longer than 24 hrs), could lead to chronic effects, generating deleterious effects on superior aquatic species. *D. geminata* can be considered toxic in upwelling conditions (bloom), where it is most active, altering aquatic community viability (macroinvertebrates and fish), trough physical effects of river coverage and sediment trapped in estuaries [24] or conglomerate composition, mainly mucilage [25].

Conclusion

Our study aim to assess the harmful effects of *D. geminata* at a cellular level, as well as other effects of this diatom. We suggest that there is an effect on fish organs due to water contaminated with the diatom. This is of great interest considering if these same effects can occur in native freshwater species, which could be a bio-indicator of water quality, as well as for other species or biological models [26]. However, native fish species reproduction has decreased in Chile, presenting a low success rate [27, 28]. Furthermore, there are no records of biota or species affected by the microalgae in Chilean rivers, showing a lack in research on the effects of *D. geminata* on the organs of native river species. Our data can be used to support research in rivers contaminated with *D. geminata* and the direct effect on biota and native fish. Our results suggest toxic and complex effect but we do not described direct effect in the natural river.

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Compliance with Ethical Standards:

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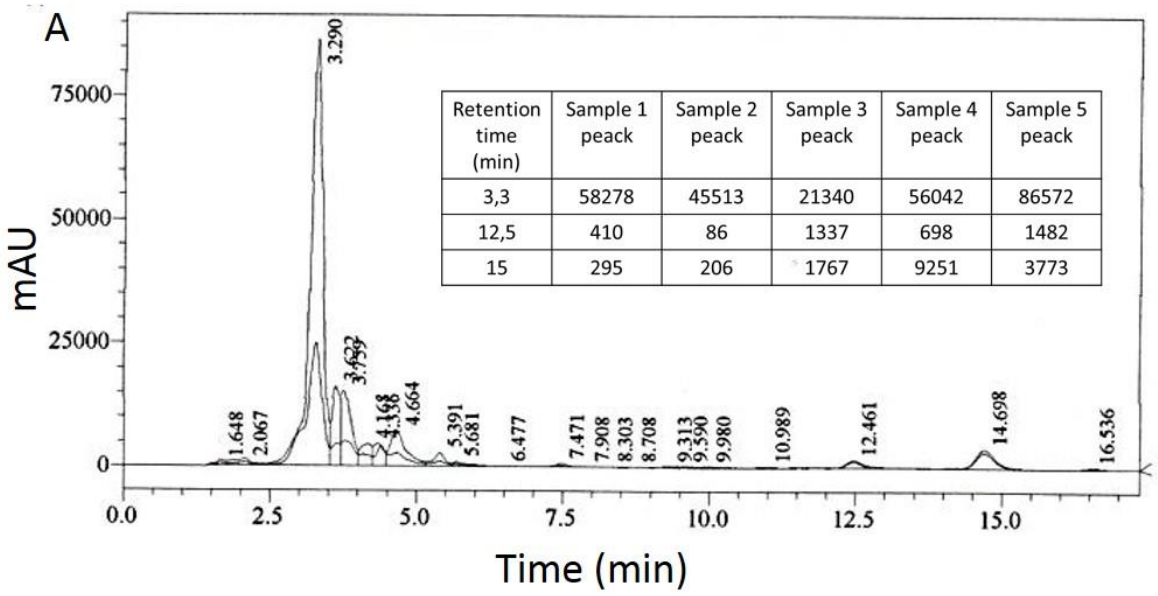
Ethical approval: Not animal are used in the present work. Not human are used in the present work.

Ethical approval: This article does not contain any studies with human participants performed by any of the authors. This article does not contain any studies with animals performed by any of the authors.

Figure Legends



Retention time (min)	Sample 1 peack	Sample 2 peack	Sample 3 peack	Sample 4 peack	Sample 5 peack
3,3	58278	45513	21340	56042	86572
12,5	410	86	1337	698	1482
15	295	206	1767	9251	3773

Figure 1. Biomolecules from *D. geminata* extract. Image A shows an example of chromatography from HPLC system from 5 different rivers. The insert shows a table with values of peak and retention time, comparing the 5 different rivers contaminated with *D. geminata*

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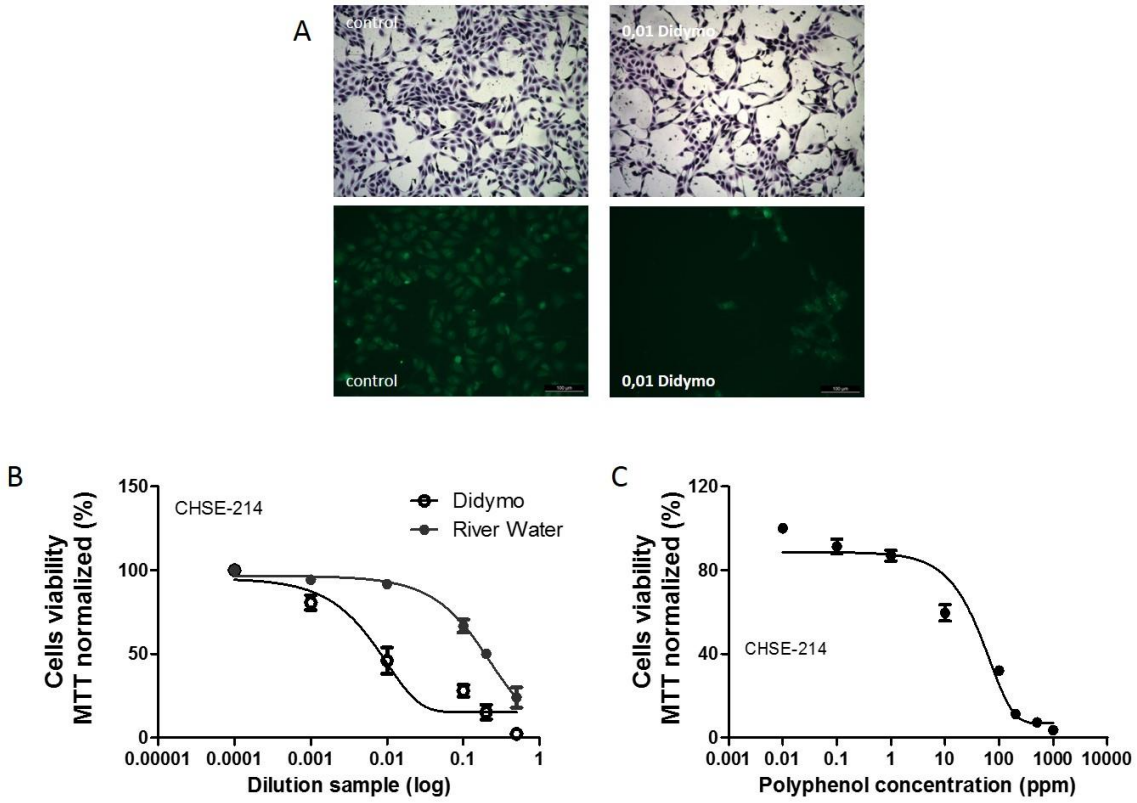

Figure 2. Effect on CHSE-214 cell line. Image A shows an example of microphotographs in the control, and exposed to water contaminated with *D. geminata* (Didymo) 0.01 V/V for 24 hrs. Upper panel shows panoptic staining. Lower panel shows alexa 488 phalodin staining. Image B shows cells viability, by MTT in a curve concentration of river water (Control) and water contaminated with *D. geminata* (Didymo)Image. C shows cells viability, by MTT in a curve concentration with extract polyphenols from *D. geminata*. The microphotographs are representative of the 5 independent observations. Each point represents (mean ± SEM), measurements of at least 5 independent experiments.

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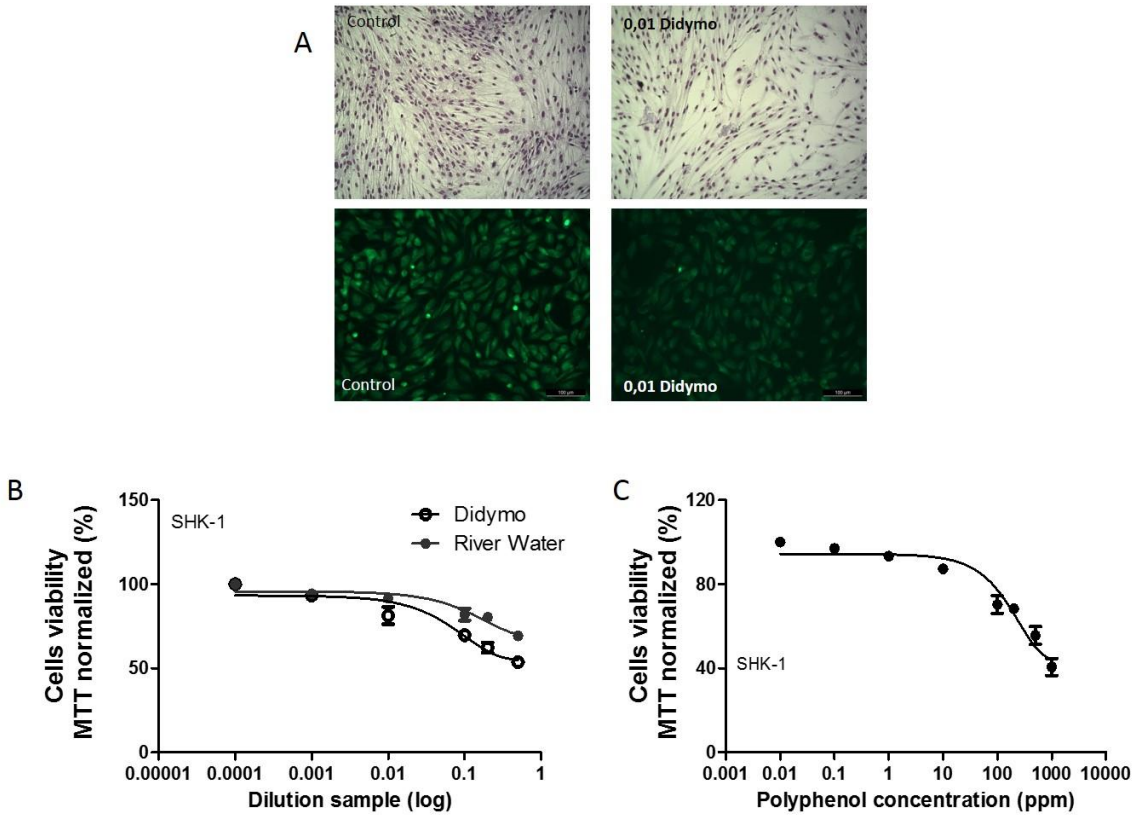

Figure 3. Effect on SHK-1 cell line. Image A shows an example of microphotographs in the control, and exposed to water contaminated with *D. geminata* (Didymo) 0.01 V/V for 24 hrs. Upper panel shows panoptic staining. Lower panel shows alexa 488 phalodin staining. Image B shows cells viability, by MTT in a curve concentration of river water (Control) and water contaminated with *D. geminata* (Didymo).Image. C shows cells viability, by MTT in a curve concentration with extracts polyphenols from *D. geminata*. The microphotographs are representative of the 5 independent observations. Each point represents (mean ± SEM), measurements of at least 5 independent experiments.

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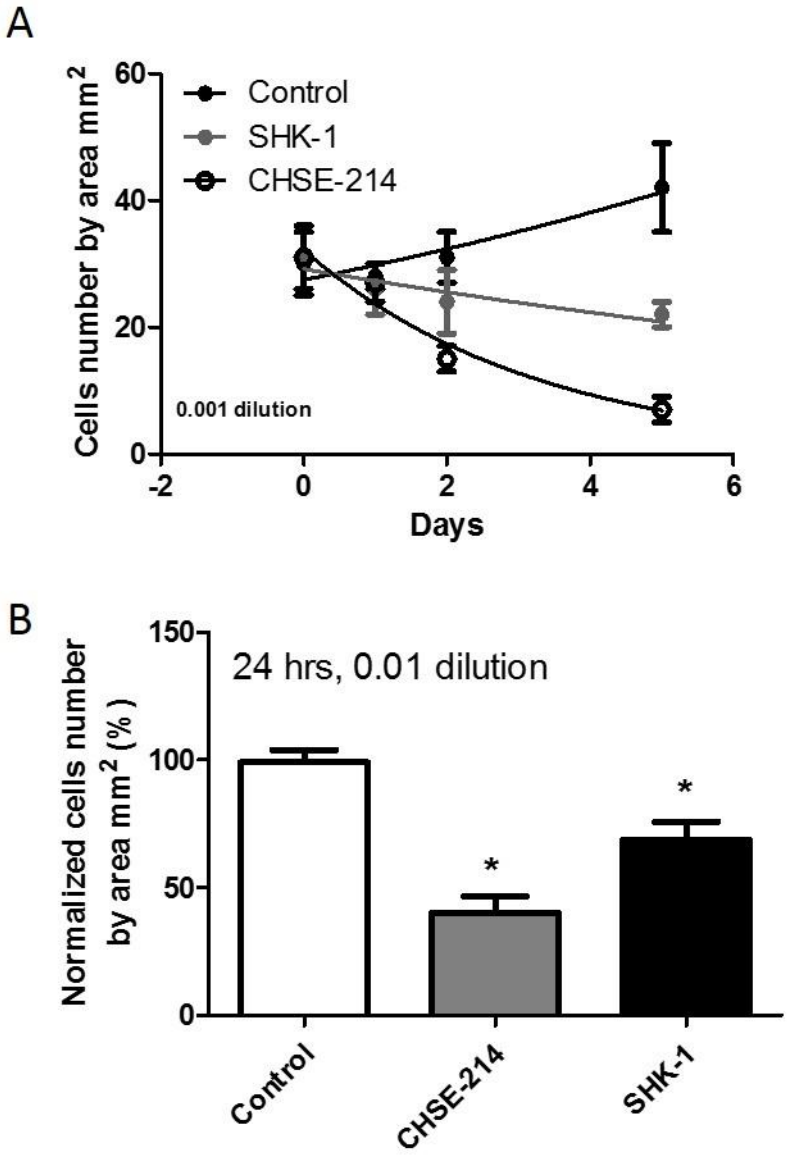

Figure 4. Effect on cellular proliferation and viability. Image A shows a quantification of the number of cells after 5 days of culture in absence or presence of water contaminated with *D. geminata* (Didymo) 0.001 V/V dilution in both cell lines. Image B is representative of the quantification at 24 hours of treatment with water contaminated with *D. geminata* (Didymo) 0.01 V/V dilution, compared to both cell lines. Each bar or point represents (mean ± SEM), the measurements of at least 5 independent cultures and experiments. The asterisk indicates $p < 0.05$ (ANOVA).

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