Polyphenols Extracts From Didymosphenia Geminata (Lyngbye) Schmidt Altered the Motility and Viability of Daphnia Magna

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Original Research

Keywords: Didymosphenia geminata (Lyngbye), D. geminata in rivers, significant ecological, scientific and societal concerns

Posted Date: February 5th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-186618/v1

Version of Record: A version of this preprint was published at Aquatic Ecology on August 7th, 2021. See the published version at https://doi.org/10.1007/s10452-021-09890-1.

Abstract

The invasive diatom Didymosphenia geminata (Lyngbye) Schmidt, *D. geminata* has invaded the austral zone of Chile, causing significant ecological, scientific and societal concerns. We aimed to evaluate the viability and motility *Daphnia magna* (*D. magna*), as a biosensor for effects of *D. geminata*. Toxicity assays were performed in dilutions of river water alone (V/V dilution) and in river water contaminated with *D. geminata* (V/V dilution) or polyphenols extracted from *D. geminata* under controlled conditions and different time (acute 30 min and 7 hrs). Our results indicated that *D. magna* was sensitive to increasing concentrations of *D. geminata* extracts. We observed a 50% (IC_{50}) viability reduction after 24 h of exposure to a 0.023 V/V dilution and the same value when using polyphenols from *D. geminata*; additionally, this treatment further reduced the motility capacity by 50% after 72 h. The *D. magna* organisms were acutely responsive, showing a 50% reduction in frequency at 15 min. We conclude that *D. magna* is sensitive to polyphenols produced by *D. geminata* in rivers, suggesting potential chronic toxic consequences on several aquatic species following exposure to these diatom substances.

Introduction

Biological invasions of non-native species are a significant threat to biodiversity (Firn et al. 2015; Gurevitch and Padilla 2004) and socio-ecological systems (Epanchin-Niell et al. 2010; Taylor & Bothwell 2014). On Austral native freshwater ecosystems, the recent proliferation of *Didymosphenia geminata* (Lyngbye) Schmidt (*D. geminata*) has become a significant concern because of the effect on the ecosystem services they provide (Reid and Torres 2014; Strayer 2010; Taylor and Bothwell 2014). *D. geminata* blooms, beat oligotrophic aquatic systems, for several hundred kilometres (Montecino et al. 2014; Pinto Torres et al. 2016). It was altering, the physicochemical conditions, of the river and the benthic fauna distribution, in particular macroinvertebrate community (Brand and Grech 2020). It was producing negative ecological and economic consequences (Alpert et al. 2000; Beville et al. 2012; Taylor and Bothwell 2014). Considerable attention has been paid to this *D. geminata* problem, as it has invaded more than 187 rivers in many other countries (Blanco and Ector 2009; Gretz et al. 2007). This benthic diatom has been declaring an invasive species in the Southern Hemisphere countries such as New Zealand (Kilroy and Unwin 2011), Argentina (Bothwell et al. 2014) and Chile (Reid and Torres 2014) since 2010 (Segura 2011). Under favourable environmental conditions, it is capable of producing blooms, with large amounts of extracellular stalks, in a short period and colonises far from its geographical range (Bishop and Spaulding 2017; Cullis et al. 2012). In the Southern, *D. geminata* has shown more aggressive performance, with a considerable impact due to the extensive formation of biomass (Kilroy et al. 2009), which is likely caused by the favourable climate and physicochemical water conditions (Kunza et al. 2018), allowing it to bloom over the river rocks or be present but not invading in the planktonic phase (Montecino et al. 2016).

The most commonly, described *D. geminata* impacts are physicochemical changes in the watercourses in oligotrophic locations, including substantial increases in algal biomass, the retention of fine sediment, and benthic hydrodynamic alterations, which consequently affect biogeochemical states and processes

such as redox conditions, pH and nutrient cycling in the benthic layer (Reid et al. 2012). Other impacts are described in the periphyton biomass and benthic communities, showing higher tolerant invertebrate groups densities such as Oligochaeta Chironomidae, Cladocera and Nematoda, Orthocladiinae and also non-insect taxa (Brand and Grech 2020; Kilroy et al. 2009)

The toxic impacts of this microalgae start with the alteration of microenvironments and microalgal communities according to the seasonal variations of *D. geminata* (Chester and Norris 2006; Figueroa et al. 2018) and the possibility of these microalgae spreading to new bodies of water (Montecino et al. 2014; Reid et al. 2012) trough different vectors (Leone et al. 2014) therefore more observational and experimental work suggest *D. geminata* is causing direct and indirect effects through the whole food web (Olivares et al. 2015; Parodi et al. 2015), during periphyton blooms (Chester and Norris 2006; Suren et al. 2003) with *D. geminata* (Kilroy et al. 2009) as well as after its removal (Larned and Kilroy 2014)

D. geminata toxicological potential to affect other organisms remains unknown. Moreover, this pennate microalgae is rich in antioxidants such as polyphenols and pigments as diadinoxanthin (Lohr and Wilhelm 1999), which was also reported as *D. geminata* polyphenol toxicity on two salmonids species cell lines (Olivares-Ferretti et al. 2019) and *Salmo salar* spermatozoa activation times (Olivares et al. 2015). A large-scale ecological change in freshwater ecosystems, perturbations to macroscopic organisms are often documented (Ricciardi and MacIsaac 2000a; Ricciardi and MacIsaac 2000b), suggesting the possibility that *D. geminata* polyphenol toxicity affected the behaviour of benthic bioindicator organisms, like *Daphnia magna* (*D. magna*) and used this model for explored the toxicity (Kim et al. 2003), as an excellent tool for assessment of this problem (Jellyman et al. 2011). *D. magna*, due to its short life cycle, morphological characteristics and reproductive capacity, makes them righteous organisms in the study of toxins in aquatic environments (Baun et al. 2008; Gerhardt 2007). Our study aimed to elucidate the toxicity levels and effects of *D. geminata* polyphenols on *D. magna* viability and motility using the frequency of movement as a parameter for sublethal effects.

Methods

Samples collection

Rock and water samples, control without *D. geminata* and with 12 bolons and with *D. geminata* were collected from each of three rivers from central and south Chile during the autumn and winter of 2019 as follows: Bio-Bio River, W318358, N5718384, W304806a and Point Lamin, W307366, N5713357N5713357; Espolón River at Futaleufú Point, W2667125, N52413723; Futaleufú River at Yelcho Point, W269107, N5213938. The samples were provided by the Chilean National Fishery Services (SERNAPESCA). After collection, the samples were immediately transported to the LaBCeMA laboratory, Universidad Mayor Temuco. A mixture of water and rocks, with *D. geminata* from all- each river was stored in coolers and maintained at 4 °C until arrival at the laboratory.

D. geminata **laboratory maintenance**

The samples were distributed in an implemented “artificial river” recirculation system (Parodi et al. 2015), Control water, without *D. geminata* samples and water contaminated to generate a closed system of water contaminated with *D. geminata* for six months of collection. According to the SERNAPESCA biosafety protocol for laboratory assays (Authorisation No. 3500). We followed the methodology standardised by Parodi et al. (2015). Briefly, the artificial river system was prepared by mixing 50% original river water from each of the collection points the different collection points, with 50% distilled water for a total volume of 14 L, leaving a 15 cm water column above the rocks. All artificial river from each location was maintained under a controlled temperature of 12 °C using an expanded polystyrene insulating cover and a refrigerating gel system. The flow rates (1200 L/h) were controlled using a Plaset-Italy Model 71009 recirculation engine, which maintained a steady flow and aeration. Macroscopic and microscopic changes in the artificial river systems were recorded daily for six months each year. The viability of the *D. geminata* population was observed with neutral red staining, and enough material was cultured to generate a 1 g polyphenol extract from 10 ml of wet *D. geminata* for use in the subsequent procedures and experiments.

Polyphenol Extraction And Liquid Chromatography (hplc) Peak Detection

In previous report we used the follow protocol (Olivares-Ferretti et al. 2019) A total composed wet *D. geminata* sample (10 ml) was obtained from two to five rocks collected (n = 12) and maintained in a single artificial river; no cultures of the sample were used, only fresh material was used, and it was exposed to liquid nitrogen. Samples from each point (10 ml) were macerated, and the cell frustules were ruptured by sonication (Misonix XL2000 Series) in 30-minute pulses with one-minute intervals until the biomass (even complex samples) was wholly homogenised. A total of 10 ml distilled water was then added to the macerated samples and the samples were collected in 15-ml tubes. The tubes were then incubated at 30 °C under agitation for 20 minutes, filtered through double gauze and a Whatman No. 2 (125 mm) filter, and collected in a 20-ml glass flask (following Jofre-Fernandez et al. 2013). Finally, polyphenol detection was performed using a total of 1 g of extract. Samples were diluted with Folin-Ciocalteu reagent following the protocol described by Lowry et al. (1951), and the polyphenol absorbance was measured at 517 nm. A total of 12 samples per year were frozen to avoid degradation until analysis by the HPLC service at the Universidad Austral de Chile to identify their profiles (Lohr and Wilhelm 1999). The presence of organic compounds was detected in the yellow fraction of the samples. A total of 5 different extracts from different collection points were used for the retention time measurements. A description of the antioxidant profiles of the samples and the absorbance at 440 nm with the AC18 column were determined (Macherey-Nagel, Duren, Germany).

Daphnia magna **laboratory maintenance**

D. magna specimens were used according to the NCh 2083 standard: With constant temperature 16 °C, with a light/dark cycle (12 h/12 h). The culture water for *D. magna* was prepared with 25 ml saline

solutions (calcium chloride: 11.76 gr/L; magnesium sulphate: 4.93 gr/L; sodium bicarbonate: 2.59 gr/L; potassium chloride: 0.23 gr/L) and filled up to 1000 ml with distilled filtered water. Finally, the water was aerated for 24 hours before use. The growing culture density was from 10 to 15 *D. magna* organisms per 200 ml of water. *D. magna* were fed with 5 ml of the microalgae *Chlorella vulgaris* and *Selenastrum capricornutum* (*Raphidocelis subcapitata*) 30 ml/L per 500 ml of culture (25–35 *D. magna*) every 2 days. The approximate life cycle of *D. magna* is 3 to 4 weeks. The time to mature from juveniles to adults is approximately 15 days.

D. magna **viability**

D. magna specimens from at least the third generation were obtained by acyclic parthenogenesis under specific growing conditions describe previous on laboratory maintained. Then, neonates of *D. magna* organisms used in the test can be collected by filtration through a sieve (opening size of 560-µm for *D. magna)* or separated manually within 24 hours of birth. Then, ten specimens for conditioning were split into an individual glass with 50 ml of artificial river water with oxygen. The specimens were exposed to the condition for 24 to 72 hours, and the mobile forms for each condition was recounted for an indication of viability after this time incubation. The control needed to show viability more significant than 80% to be considered correct for studying the viability of the different conditions, nine independent experiment was made for described the viability of *D.magna* on different experiment condition.

D. magna **motility**

The motility experiment is made using fresh *D. magna*, take for the cultured and deposit in a volume of 1 ml of artificial river water; 5 adults of *D. magna* were used in 9 different experiments for all the experimental condition, and we used 100 specimens in the total study of motility. We exposed in acute manner, at meditated manner 30 min of incubation or a chronic manner after 72 hrs of incubation, to the different condition. We measured the absorbance at 328 nm on a Peak C-7100 Series instrument spectrophotometer. The absorbance peaks were continuously recorded for two minutes, as an indication of the passage of the *D. magna* versus the light emission. The number of peaks per unit of time was counted, and the frequency of events was calculated as an indication of the motility event. Under these conditions, five adults were exposed to increasing concentrations of V/V of river water contaminated with *D. geminata*, and we used a river water without *D.geminata* has control. Increasing concentrations of polyphenols extracted from *D. geminata* between 5 and 500 ppm were used, and gallic acid (1 to 1000 ppm) was added to an artificial river water solution, has positive control.

Data analysis

Unless otherwise indicated, the results, including image analysis, are presented as the means ± SEM. Statistical comparisons were performed using Student's t-test or ANOVA with the software graphpad prism 4. A probability level (p) of less than 0.05 was considered statistically significant.

Results

D. geminata- **contaminated water effects on** D. magna **viability**

The newborn and adult forms of *D. magna* were exposed to dilution by water contaminated with *D. geminata* and polyphenol extract from *D. geminata* and gallic acid. Figure 1A shows a curve of the concentration dilution V/V of the river water (Negative Control) or contaminated with *D. geminata*. The figure shows a reduction of over 50% in the percentage of life when the concentration of the river water contaminated with *D. geminata* is increased, with an IC_{50} of 0.023 V/V. We explored the effect of polyphenols obtained from *D. geminata* and observed a decrease in viability (Fig. 1B) when the polyphenols were increased, with an IC_{50} of 52 ppm. We compared the effect with a polyphenol standard, i.e., gallic acid (Positive control), and Fig. 1B shows the inhibition of viability when the concentration is increased, with an IC_{50} of 4.5 ppm. Finally, we exposed *D. magna* to the IC_{50} values in a chronic manner from the previous experiment, 72 hrs of incubation (Fig. 1C), and we observed a reduction in viability over 50 % when *D. magna* was exposed to river water with *D. geminata*, a polyphenol from *D. geminata* or gallic acid, this date help to us suggested the polyphenols present in the samples can altered the viability of the *D.magna*.

Effect of D. geminata**-contaminated water on** D. magna **motility**

We explored the impact of the polyphenol obtained from *D. geminata* on the motility of *D. magna*. In Fig. 2A, we show example traces of the motility observation of *D. magna* in the spectrophotometer; the absorbances are expressed in Y and time in X, the number of events is between 10 to 15 in the control condition are reduce two events in the experimental condition. The quantification of the motility is shown in Fig. 2B when *D. magna* are exposed to increased concentrations of the *D. geminata* polyphenol extract; reduction form 0.1 Hrs to 0.05 Hz. The figure shows a reduction in the frequency of the peak when the concentration of polyphenol increases above 50 ppm, with an IC_{50} of 52 ppm. We used this concentration to explore the time effect. We exposed the samples to 52 ppm of polyphenol extract from *D. geminata* at different times. In Fig. 2C, we show example traces of the absorbance peak, relation between absorbance and time. Figure 2D shows the quantification of the motility at different times. We observed that 52 ppm did not induce a change in the frequency of events when used acutely; however, when *D. magna* were incubated longer than 5 minutes, the frequency of activity was reduced. The data in the figure proposed a model of action of the *D. geminata*-contaminated water over the *D. magna*, explored in the next result

Effect of D. geminata-**contaminated water on** D. magna **motility after acute incubation**

We used the previous IC_{50} value from water contaminated with *D. geminata* (0.023 V/V) polyphenol extract (52 ppm) and gallic acid (4.5 ppm) to observe the change in the frequency of *D. magna* after incubation for 30 minutes. Figure 3A shows examples of trace absorption changes in the relation between absorbance y time. Figure 3B shows the quantification of the motility, and we observed a reduction of over 40% in the frequency when the *D. magna* samples were exposed to the different

compounds. River water contaminated with *D. geminata* reduced the frequency by over 40%, and the effects of the polyphenol extract from *D. geminata* and gallic acid were similar. All the data suggested the *D. geminata*-contaminated water contained polyphenosl and this altered the motility of the *D. Magna* and finally induce the death of the sample

Effect of D. geminata**-contaminated water on** D. magna **motility after chronic incubation**

We experimented again using the previous IC_{50} value from water contaminated with *D. geminata* (0.023 V/V) polyphenol extract (52 ppm) and gallic acid (4.5 ppm) to observe the changes in the frequency of *D. magna*; however, we increased the incubation time to 72 hours to observe the chronic effects. The values were similar to those in the previous figure. Figure 4A shows examples of traces of the absorption change. And the relation between absorbance and time, show an event over 10 when the control condition is observed and reduction to 5 events when used polyphenols. Figure 4B shows the quantification of motility, and we can observe over 50% of reduction in the frequency when *D. magna* samples were exposed to the different compounds. River water contaminated with *D. geminata* decreased almost 50% of the frequency, and the effects of the polyphenol extract from *D. geminata* and gallic acid were similar. Confirm in this result the idea of the reduction on the motility of the *D. magna*, this effect induces the death of the samples

Discussion

Our result present in this work are continued of result in previous work about the *D. geminata* toxicity, and suggested the polyphenols present in the river water contaminated whit the microalgae induce cells alteration, cellular death and now reduction in the viability of the *D. magna*. Since the introduction of *D. geminata* in local freshwater ecosystems, it has been a cause of concern to potential consequences on stream communities (Ladrera et al. 2018). In attempts to understand the *D. geminata* toxicity in a small organism, we study it effect on a standardised model. In a previous work for our group (Olivares et al. 2015), we proposed that the toxic effects are secondary to the presence of polyphenols in river water contaminated with *D. geminata*. Our results suggest that water contaminated with *D. geminata* can be mediated reduce effects on *D. magna* viability and motility by the presence of polyphenols and in recently work we suggested a toxic effect a cellular level, are secondary to the present of polyphenols present in the river contaminated with D. geminate (Olivares-Ferretti et al. 2019). The experiment design in the present work, utilised the *D. magna* standardised model to evaluate the effects of artificial river water contaminated with *D. geminata*, which previously reported (Parodi et al. 2015). The water samples included the presence of a biological compound that has been observed in *D. geminata* cells, where the pigment was measured at 440 nm identified by HPLC(Olivares-Ferretti et al. 2019), it is present in our samples. Previous studies have reported the *D. geminata* effects on aquatic organisms and spermatozoa (Brand and Grech 2020; Larned and Kilroy 2014; Olivares et al. 2015). Viability observations in river water with increasing V/V concentrations resulted in typical *D. magna* values; however, the level of *D. magna* mortality increased when the river water was contaminated with *D. geminata* (Fig. 1A). We observed the lethal doses of river water contaminated with *D. geminata* and polyphenols from *D. geminata* and used a

standard, gallic acid (Fig. 1B). It has been reported, the presence of epiphytic cyanobacteria in blooms, which are microcystins promoter (Whitton et al. 2009), higher microcystin dosage on *D. magna* caused chronic toxicity (Chen et al. 2005). Our results suggest that river contamination with *D. geminata* may have a chronic effect on aquatic organisms (Fig. 1C).

However, we followed the idea of subtoxic effects, and in particular, we explored a mechanism to explain the deadly impact. We evaluated *D. magna* motility using it is endogenous fluorescence particularity by a UV light protocol, recorder the absorbance of the solution (Teplova et al. 2010). The traces obtained from the spectrophotometer (Figs. 2A and 2C) shows every change registered, considered the peaks as a movement of *D. magna*. We observed a reduced *D. magna* motility when the concentration of the polyphenol extracts from *D. geminata* was increased in an acute manner (Fig. 2B). It has been reported an increasing basic metabolism in *D. magna* to counteract a prolonged exposure of cytotoxins in freshwater ecosystems (Grzesiuk et al. 2018). Our results showed a reduced motility when *D. magna* was incubated with polyphenol DL_{50} for a longer period of time (Fig. 2D). We suggest that *D. geminata* and the polyphenol produced by *D. geminata* alter the motility in *D. magna*, and this lead a reduction in viability in longer exposition.

The toxicity biotests are carried out by checking organisms parameters (Walker et al. 2012). We recorded whether this effect occurred when river water contaminated with *D. geminata*, a standard sample of polyphenols and acid gallic, was used (Maheshwari et al. 2017), acutely and chronically. Figure 3 shows a reduction on the *D. magna* motility in all the conditions; likewise, the acid gallic had a stronger effect on the motility, supporting the idea that the polyphenols had an effect on this parameter. It has been described a hypo-locomotion response to water-born toxicants in *D. magna* (Huang et al. 2017), our data suggest an acute impact over the motility mediated by the polyphenol present in the river water contaminated with *D. geminata*.

The anthropogenic influence generates biodiversity loss by increase toxicants on natural freshwater (Tickner et al. 2020), allows us to consider in possible of toxic components from the *D. geminata* invasion. Our data show a reduction in the *D. magna* motility without a more substantial impact, exhibiting that the chronic effects at sublethal doses were the same as those observed in the acute experiment suggesting that the effects on viability are more complex (Fig. 4). It has been reported that *D. geminata* changes in macroinvertebrate composition blooms show a decrease density in determinate trophic groups (Larned and Kilroy 2014; Whitton et al. 2009). However, the *D. magna* enzymatic metabolism increase to chronic toxicity adaptation mechanism has been described (Chen et al. 2005).

Conclusion

Our study aimed to assess the harmful effects of *D. geminata* at the macroinvertebrate level, as well as the other effects of this diatom as observed in a previous report from our group. These effects are of great interest when considering whether the same effects are noted in the cell lines of native freshwater species, other species or biological models when representing a bioindicator for water quality (Venugopal

2002) and when evaluating whether *D. geminata* contamination can induce changes in the biota of rivers (Cifuentes et al. 2012; Montoya et al. 2012). Furthermore, there are few records of the biota or species being affected by microalgae in Chilean rivers or other places (Ladrera et al. 2018), representing a lack of research on the effects of *D. geminata* in cell models or using native river species. For example, a recent study indicated a change in the microalgae composition when *D. geminata* was present in a river (Zamorano et al. 2019). Our data e propose that at sublethal concentrations, the presence of *D. geminata* polyphenols affect the viability of *D. magna*, because induced a reduction of the motility clouding when long-term exposure occurs and we suggest a mechanism for explain toxicity over macroinvertebrates present on rivers contaminated with *D. geminata*. Our results suggest toxic and complicated implications of *D. geminata* contamination; specifically, the effect of polyphenols in *D. geminata* had a direct impact on macroinvertebrates in rivers and finally affected all the biota of the river.

Declarations

Acknowledgements

We thank SERNAPESCA (Araucanía Region) and EcoHyd for support in obtaining and maintaining the *D. geminata* samples. We also thank IO 101340 and 101322 from Universidad Mayor for research support. P.O.F. acknowledges the CONICYT-PCHA/2017/21170159 support.

Compliance with Ethical Standards:

Funding: This study was not privately funded.

Conflict of Interest: The authors declare that they have no conflicts of interest.

Ethical approval: No animals or humans were used in the present work.

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

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Figures

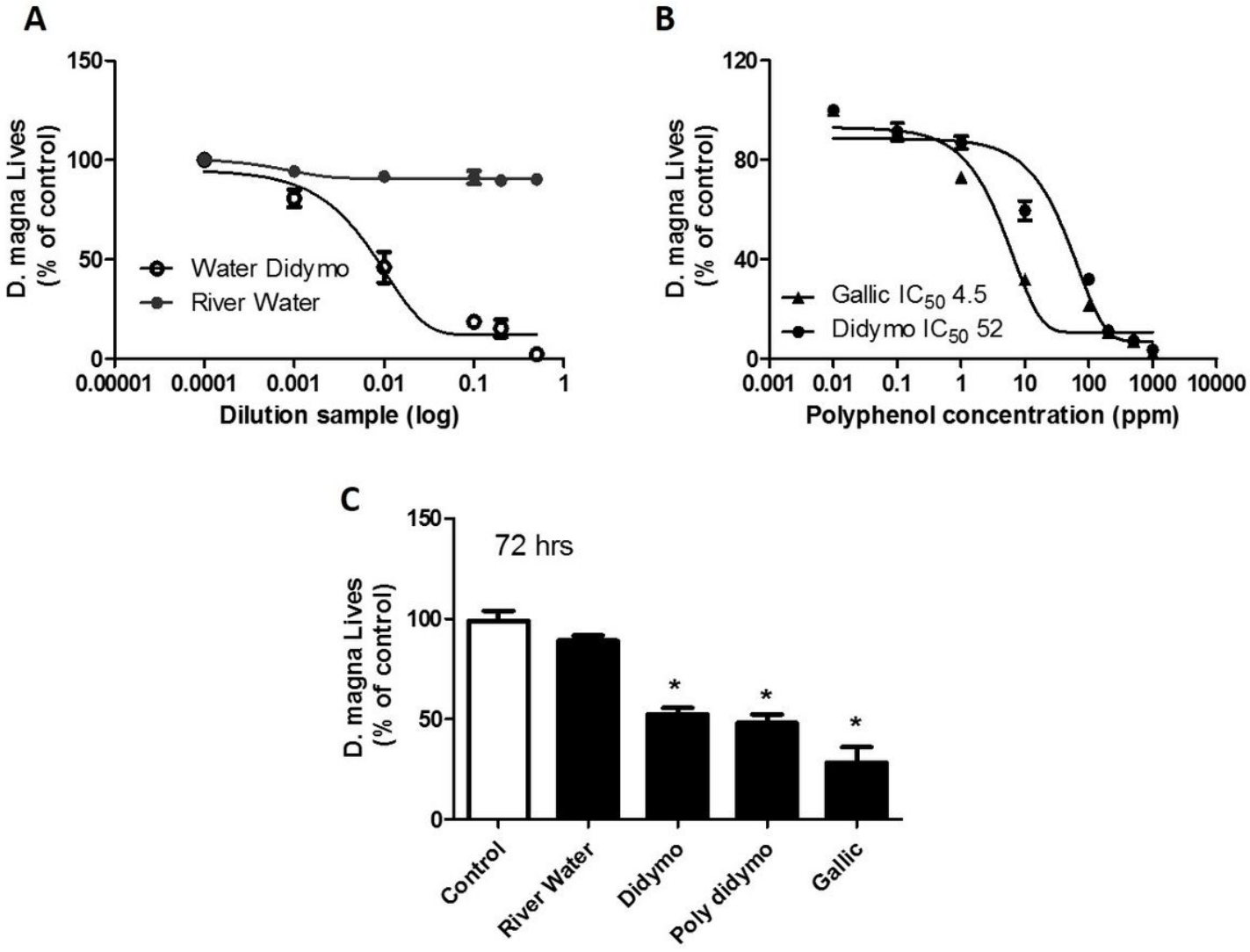

Figure 1

Effect of polyphenols on D. magna viability. A show the effect on the number of D. magna lives after 24 hours of exposure to river water contaminated with D. or artificial river water. B shows the effect on the number of D. magna alive after 24 hours of exposure to polyphenol extract (poly didymo) or gallic acid (Galic). C shows the effect on the number of D. magna lives after 72 hours of exposure under different conditions, control, Negative control (River water) water contaminated (Didymo) to polyphenol extract

(poly didymo) or gallic acid (Galic) at the IC50. Each bar or point represents the measurements from at least 5 independent cultures and experiments (mean ± SEM). The asterisk indicates $p < 0.05$ (ANOVA).

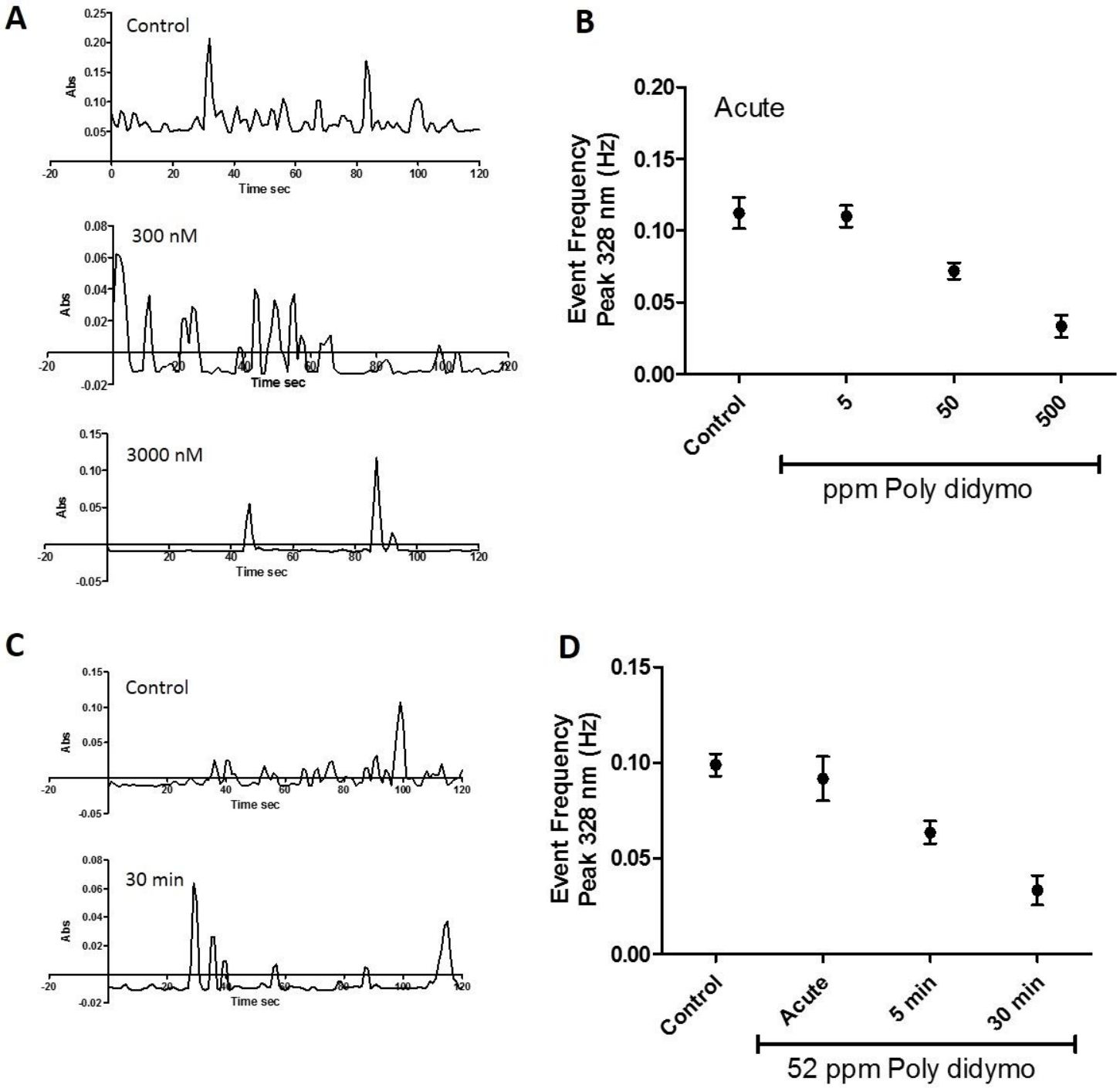

Figure 2

Effect of polyphenols on D. magna motility. A shows the effect on the motility record of D. magna after acute exposure to different concentrations of polyphenols (poly didymo) from D. geminata. B shows the dot graph of the motility record of D. magna after acute exposure to different concentrations of polyphenols (poly didymo) from D. geminata. C shows the effect on the motility record of D. magna after different times of exposure to polyphenols from D. geminata. D shows the dot graph of the motility record

of D. magna after different times of exposure to polyphenols (poly didymo) from D. geminata. Each point represents the measurements from at least 5 independent cultures and experiments (mean ± SEM). The asterisk indicates p < 0.05 (ANOVA).

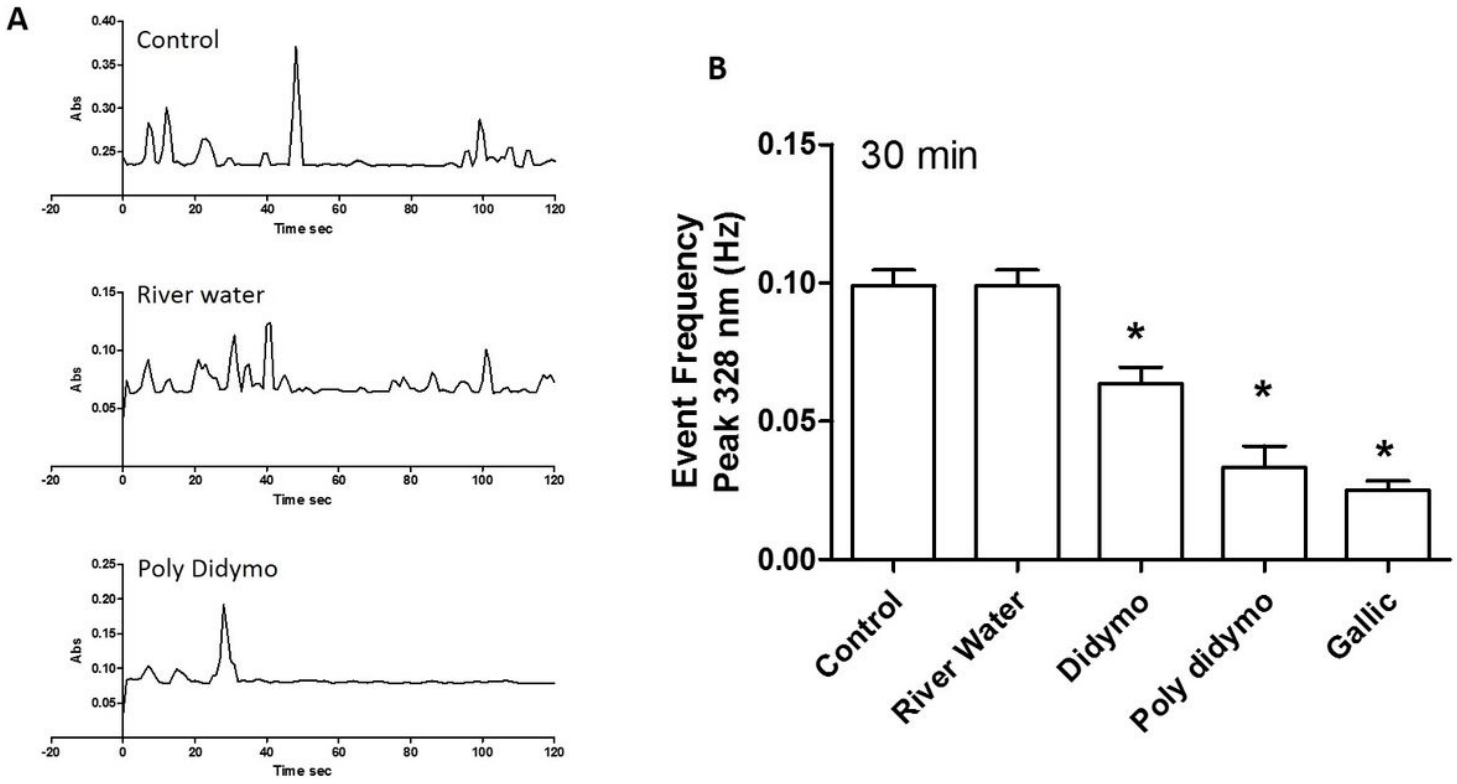

Figure 3

Effect of different polyphenols on D. magna motility. A shows the effect on the motilityrecord of D. magna after 30 minutes of exposure under different experimental conditions. B shows the bar graph of the motility record of D. magna after 30 minutes of exposure in different experimental conditions, control, Negative control (River water) water contaminated (Didymo) to polyphenol extract (poly didymo) or gallic acid (Galic). Each bar represents measurements from at least 5 independent experiments (mean ± SEM). The asterisk indicates p < 0.05 (ANOVA).

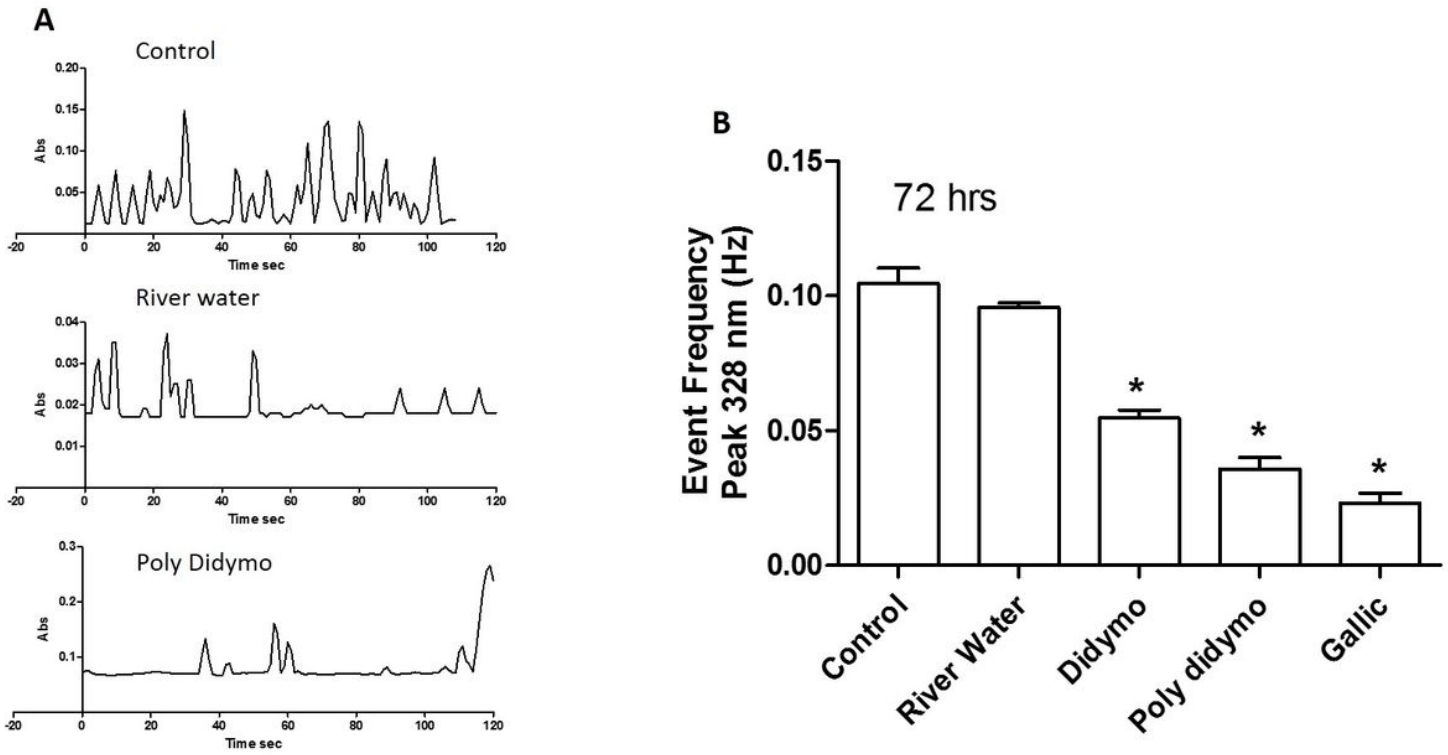

Figure 4

The chronic effect of different polyphenols on D. magna motility. A shows the effect on the motilityrecord of D. magna after chronic exposure under different experimental conditions. B shows the bar graph of the motilityrecord of D. magna after chronic exposure under different experimental conditions, control, Negative control (River water) water contaminated (Didymo) to polyphenol extract (poly didymo) or gallic acid (Galic). Each bar represents measurements from at least 5 independent experiments (mean ± SEM). The asterisk indicates $p < 0.05$ (ANOVA).