Allelopathic effects among selected species of phytoplankton and macrophytes

Abstract

Aim: To understand the allelopathy among different taxonomic levels of freshwater photoautotrophs, the effect of aqueous extracts of *Phragmites australis* and *Schoenoplectus californicus* on monocultures of *Microcystis aeruginosa* and *Scenedesmus acutus* was investigated. In addition, the cell extract and filtered medium of each phytoplankton species were used and tested between them.

Methodology: In the macrophyte bioassays against alga, the allelopathic effect was evaluated separately using 10 and 20 mg of aqueous extract of leaf, stem and rhizome of *Phragmites*, as well as the stem and rhizome of *Schoenoplectus*. In the assays between the phytoplankton species, the cell extract (12.44 ± 0.16 µg l⁻¹ microcystin-LR equivalents) and the filtered culture medium (2.98 ± 0.03 µg l⁻¹ microcystin-LR equivalents) of *Microcystis* were used in 100 and 50% and were added to the *Scenedesmus* monocultures. In the same proportion, the cell extract and the filtered culture medium of *Scenedesmus* on monocultures of *Microcystis* were used.

Results: In *Phragmites*, leaf extract showed greater inhibition activity than the other plant organs on *Microcystis* cultures. The stem and rhizome showed a larger decrease for the growth of *Scenedesmus* cultures. For *Schoenoplectus*, the stem extract showed a greater inhibitory effect than the rhizome extract; this was observed on both alga and cyanobacteria. *Microcystis* when in contact with the cellular extract of *Scenedesmus*, it was observed that the bioactive containing substances in the extract had a greater inhibitory activity.

Interpretation: The leaf, stem and rhizome extracts of *Phragmites* and *Schoenoplectus* showed a different affinity of the allelopathic activity on *Microcystis* and *Scenedesmus*, suggesting a species-specific relationship.
Introduction

Allelopathy prevents competitors from making use of available resources, affecting growth and distribution of other species and therefore can control the environment in which they live (Thorpe et al., 2011). In aquatic systems, macrophytes released infochemicals through root, stem or leaf (Gross et al., 2007; Uddin et al., 2012). Species of phytoplankton release their bioactive compounds directly into water or are retained in the sediment (Bhadoria, 2011). The allelopathic effects of substances from macrophytes on the harmful algal blooms are gaining importance recently (Hong-Qiang et al., 2014). However, it is expected that active compounds released from macrophytes reach the recipient species and somehow affect their metabolism (Gao et al., 2011; Gross et al., 2012). From the macrophyte Phragmites, the most frequent allelochemicals are phenols, in particular gallic acid and to a lesser extent the organic acid ethyl 2-methylacetoacetate, which are obtained from exudates and roots. The leaf contains allelochemicals such as taraxerol and taraxerone. The allelopathic effect of these compounds has been evaluated on cultures of Chlorella pyrenoidosa and Microcystis aeruginosa (Li and Hu, 2005; Rudrappa et al., 2007). Phenolic compounds are also produced by Schoenoplectus, which have been isolated from the whole plant. The phenol (-) catechin showed a similar algaecide effect to that of CuSO₄ to Selenastrum capricornutum (D’Abrosca et al., 2006). However, its effect on cyanobacteria such as Microcystis aeruginosa has not been tested.

Cyanobacterial blooms generally produce secondary metabolites such as cyanotoxins, which can be released directly into the environment by senescence or cell lysis (Dittmann et al., 2013; Neilan et al., 2013). Cyanotoxins may cause damage directly (lethal) or indirectly (chronic) to different aquatic organisms due to accumulation effect. Their main function is the growth inhibition in other organisms, reducing the number of species that are potential competitors or grazers (Lin et al., 2014; Rao et al., 2015). In Microcystis aeruginosa the mechanism of action of its toxins affects several Chlorophytes such as Chlamydomonas neglecta, C. reinhardtii, Chlorella ellipsoidea, C. pyrenoidosa, C. vulgaris and Monoraphidium convolutum (Ishida and Murakami, 2000; Yang et al., 2014; Bittencourt-Oliveira et al., 2015).

Few studies have assessed the effect of dissolved microcystins and cyanobacterial biomass containing toxins at concentrations similar those found in nature (1-10 μg l⁻¹ microcystin-LR) to phytoplankton species (Kearns and Hunter, 2001; Bittencourt-Oliveira et al., 2013). Many photoautotrophs are capable of producing bioactive substances (Erhard, 2006). For example, chemicals released by Scenedesmus are capable of inhibiting the growth of M. aeruginosa (Jia et al., 2008; Chen and Guo, 2014). Species interaction experiments usually are done to provoke the release of allelochemicals in the producer species (Bittencourt-Oliveira et al., 2015). However, bioactive substances are permanently present in algae and since they are fundamental part of the physiological processes, they are involved in the growth and development. These in turn may function as interacting substances with the environment in adaptation and defense, as is the case with cyanotoxins (Lefaive and Ten-Hage, 2007). Therefore, it is necessary to know the allelopathic effects of common photoautotrophic organisms in aquatic systems for understanding the role of allelopathic processes in nature.

The aim of the present work was to evaluate the allelopathic effect of aqueous extracts of P. australis and S. californicus to M. aeruginosa and S. acutus and to quantify the effect of conditioned medium from M. aeruginosa on S. acutus and vice versa.

Materials and Methods

Culture conditions: M. aeruginosa was isolated from Valle de Bravo reservoir, State of Mexico (Mexico) while S. acutus (Meyen) (strain no. 72) was obtained from the University of Texas, (Austin, Texas). Both the species were cultured on Z8 liquid medium (Staub, 1961; NIVA, 1972). The cultures were homogenized using an orbital shaker at 90 rpm (Labnet, Orbit 1900) at 28±2°C, pH 6.8 and a photosynthetic active radiation (PAR) of 90 μmol photons m⁻² s⁻¹, using cold white fluorescent light with a photoperiod of 14:10 h (light/dark). PAR was measured through a quantum sensor equipped with a semi-spherical sensor (Apogee Instruments, MQ-200). The emergent macrophytes, P. australis and S. californicus were collected from the La mintzita reservoir (State of Michoacán, Mexico). Whole plants were selected. Six plants of Phragmites and Schoenoplectus were taken with an average length of 1.61 ± 0.04 and 1.63 ± 0.09 m, respectively.

Aqueous extracts, cell extracts and filtered culture medium: The macrophytes were dried at 26±2°C for five days. Each of the three structures (leaf, stem and rhizome) plant were weighed (20 g), which were separately macerated with 50 ml water chromatographic grade (Sigma-Aldrich 34877) for 24 hr. Subsequently the first extracts were filtered by filter paper (Whatman qualitative, Grade 1) and lyophilized (Labcono, LYPH LOCK 4.5). The extract was filtered by Millipore system with 0.45 μm nitrocellulose membrane (Merck, MF-Millipore HAWP04700), that was used in the assays.

Algal aqueous extracts were separately obtained from the cultures in 350 ml of Z8 medium, whose cell density was 2.90 ± 0.46 x10⁶ cell ml⁻¹ for M. aeruginosa and 2.90 ± 0.46 x10⁶ cell ml⁻¹ for S. acutus. The cells were centrifuged at 10,000 rpm for 5 min (BOECO, SC-8). The sediment was lyophilized and 0.5 g dry weight of algal biomass was used and suspended in 150 ml of fresh Z8 medium. The extract was filtered with 0.45 μm membrane. The supernatant was filtered through the Millipore
fixing the samples in 20% Lugol. When cell counting was performed to determine the effect of the extracts and to compare it with the cell number of the control cultures, it was observed that the decrease of the organisms below the cell inoculum at day zero was due to the fragmentation and agglomeration of the cells, so that the cell portions were not considered in the cell count for the results.

Two-way repeated measures ANOVA was applied with a post hoc means analysis using a Bonferroni’s multiple comparisons test with an alpha of 0.05 level of significance to compare growth differences among treatments.

**Results and Discussion**

The 10 mg aqueous extract of *Phragmites* on *Microcystis* cultures showed that the leaf had an inhibiting effect. This decrease was maintained during the 4 days of the trial. A significant difference ($p<0.01$) was observed in the cell density in cultures with the extract vs *Microcystis* controls. The stem and rhizome extracts showed no significant effects ($p>0.05$).

*Scenedesmus* was sensitive to the extracts from *Phragmites*. The 12.44±0.16 µg l$^{-1}$ microcystin-LR equivalents of *Microcystis* (as 100 and 50%) were added to the *Scenedesmus* cultures. In the same proportion, the cell extract and the filtered medium of *Scenedesmus* were added to the cultures of *Microcystis*. Both the phytoplankton species were cultured in triplicate in 25 ml volume of Z8 medium. The cell densities of *Scenedesmus* and *Microcystis* were separately estimated using a hemocytometer at initial, day 2 and day 4 after fixing the samples in 20% Lugol. When cell counting was performed to determine the effect of the extracts and to compare it with the cell number of the control cultures, it was observed that the decrease of the organisms below the cell inoculum at day zero was due to the fragmentation and agglomeration of the cells, so that the cell portions were not considered in the cell count for the results.

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Fig. 1: The effect of 10 mg concentration in dry weight of the aqueous extracts of each macrophytes structural parts *P. australis* and *S. californicus* to *M. aeruginosa* and *S. acutus* are shown. Error bars are based in three replicates.
significant ($p<0.01$) inhibitory effects were observed during the four days of the experiment. The stem and rhizome extracts of Schoenoplectus decreased the growth of Microcystis. A significant difference ($p<0.01$) was observed only on day 2 as compared to control. At day 4, both extracts lost their inhibition effect, so that Microcystis was able to reach densities similar to control. In Scenedesmus it was observed that the low growth was maintained to a greater degree by the stem extract during the experiment. The negative effect of both extracts were significantly different ($p<0.01$) from controls and by day 4 the effect of the extracts was lower (Fig. 1).

At 20 mg concentration, the leaf extract showed a greater negative effect on the growth of Microcystis, followed by stem and rhizome, the lower effects being on day 2. The three extracts had a negative effect on Microcystis and there were significances among them ($p<0.01$) to the cell growth in the control cultures. The growth of Scenedesmus differed significantly ($p<0.01$) from controls with the three extracts of 20 mg of Phragmites. The extracts of Schoenoplectus also showed a tendency of inhibition on both Microcystis and Scenedesmus. However, it was observed that Microcystis is able to increase its cell number despite exposure to extracts unlike Scenedesmus where the inhibition effect was maintained during the assay (Fig. 2).

The recurrent tendency of decreasing effects of the extracts from both macrophytes is due to bioactive substances, mainly phenols. These compounds are prone to oxidation, so their persistence once extracted from the plant tissues is about 4 days and hence experiments longer than this duration do not reflect the bioactivity of the compound (Sampietro et al., 2009; Li et al., 2012). Our results are consistent with the inhibitory effect of Phragmites and Schoenoplectus on different species reported in literature (D’Abrosca et al., 2006; Addisie and Medellin, 2012; Chang et al., 2012). Phragmites leaf extract showed greater inhibition activity than the other plant organs on Microcystis cultures. The stem and rhizome showed a stronger decrease on Scenedesmus. For Schoenoplectus, the stem extract showed a greater inhibitory effect than the rhizome for both phytoplankton species tested.

The differences in allelopathic effects produced by aqueous extracts are partly due to the site where the bioactive substances are produced in the macrophytes. Phragmites has well developed leaf blades, where the synthesis of most of the
secondary metabolites occurs. *Schoenoplectus*, on the other hand, lacks leaf blades, so the site of greatest production of secondary metabolites is the stem (Weston et al., 2012).

In this work we observed that the leaf, stem and rhizome extracts of *Phragmites* and *Schoenoplectus* showed different affinities for allelopathic activity on *Microcystis* and *Scenedesmus*, suggesting a species-specific relationship. Variability in sensitivity to bioactive substances has been reported even within species of the same genus. For example, *Chlorella pyrenoidosa* is susceptible to *Phragmites* compounds but *Chlorella vulgaris* is not (Li and Hu, 2005). The degree of interaction that a bioactive substance has is related to the particular characteristics of recipient species (Nakai et al., 2008; Gao et al., 2011; Sheng-hua et al., 2015). In addition, some recipient species may also develop resistance, while others do not. For example, only some recipient species in a given ecosystem exhibit a weak or no sensitivity to allelochemicals (Švanys et al., 2014).

Allelopathic activity in this work is also based on macrophyte exudates which are the allelochemicals released directly into the medium. Under conditions of water depletion the macrophytes activate various signaling mechanisms for the synthesis of new bioactive substances or retain the compounds already produced in a particular tissue (Choi et al. 2009). This condition may occur in this work and therefore the same kind of allelochemicals were consistently present in our test jars. Further we recorded that the inhibition activity of extracts from *Phragmites* and *Schoenoplectus* was most noticeable at four days of bioassay. This can be explained by exposure of the bioactive substances to other chemical forms dissolved in water, which hinder the inhibiting effect on sensitive recipient species. The dilution affects the intensity of allelopathic effects and the allelochemicals should have hydrophilic properties to reach the receptor cells (Macías et al., 2008; Gross et al., 2012). Therefore, the concentration at 20 mg in the extracts of both macrophytes was able to cause negative effects on the growth of *Microcystis* as compared to 10 mg concentration.

In the bioassays where the allelopathic effect of dissolved microcystin (2.98±0.03 μg l⁻¹) was evaluated on *Scenedesmus* cultures, no significant differences (p>0.05) were observed when...
50% of the filtered medium was used, with respect to controls. Only on the fourth day the density of Scenedesmus decreased. The allelopathic effect was constant during the experiment period when 100% of the filtered medium was used, resulting significantly different (p <0.01) from control (Fig. 3). The filtered medium was still effective in inhibiting Scenedesmus despite the low levels of microcystin. These results contrast with other studies where microcystin at 1-10 μg l⁻¹ did not cause a negative effect on S. quadricauda and S. obliquus: the allelopathic effect was observed at microcystin concentration of 50 to 25000 μg l⁻¹ (Babica et al., 2007; El Sheekh et al., 2010).

The allelopathic effect in our study could was compounded by the presence of other substances in the filtered medium of Microcystis (Bittencourt-Oliveira et al., 2013). The cell extract of 100% Microcystis (12.44 ± 0.16 μg l⁻¹) had a stronger inhibitory effect than the proportion of 50% on the growth of Scenedesmus at day 2 of the assay. However, for day 4, the 100% extract reduced its effect until reaching the proportion of 50% (Fig. 3), suggesting that the decrease on Scenedesmus was due to a chronic effect by the accumulation of microcystins. Bittencourt-Oliveira et al. (2015) mentioned that S. acuminatus cultures did not suffer from allelopathic effects when exposed to crude extracts of M. aeruginosa at 5-10 μg l⁻¹ of microcystin-LR. The allelochemicals released by Scenedesmus negatively affect cyanobacteria (Jia et al., 2008; Chen and Guo, 2014), as also observed here. The inhibitory effect was more pronounced when using the cell extract. This implies that the susceptibility of cyanobacteria to the bioactive substances of Scenedesmus is high, since the low proportion was sufficient enough to cause an adverse effect similar to the 100% of the extract. These results indicate the interactions between algae and Microcystis in nature are probably more diverse than those derived from laboratory tests.

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References


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