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Allelopathic potential and ecotoxicity evaluation of gallic and nonanoic acids to prevent cyanobacterial growth in lentic systems: A preliminary mesocosm study

Didier Techer ^a, Pascal Fontaine ^a, Aline Personne ^a, Sandrine Viot ^a, Marielle Thomas ^a.

a : Université de Lorraine, UR AFPA, USC INRA 340, Campus Victor Grignard, Boulevard des Aiguillettes, BP 239, 54506 Vandœuvre-lès-Nancy Cedex, France

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Keywords

Allelopathy, cyanobacteria, gallic acid, nonanoic acid, biomarkers.

Abstract

The increase in anthropogenic nutrient loading affecting many freshwater ecosystems combined with global warming may lead to cyanobacterial blooms on an increasingly frequent basis. Among the various physicochemical and biological methods which have been proposed to rapidly control blue-green algae growth, the use of plant-derived substances such as allelochemicals has gained great interest as an environment-friendly approach. The primary aim of this work was to evaluate the efficiency of gallic and nonanoic acid application to preemptively inhibit cyanobacterial growth in lentic hydrosystems. In order to address the process feasibility under realistic exposure scenarios, thirteen outdoor freshwater mesocosms (unit volume: 3 m³) were designed, each containing phytoplankton (including local blue-green algae species) and various non-target organisms from higher trophic levels (Physa, Lymnaea, Gammarus, and Scardinius erythrophthalmus). After an 8-week mesocosm stabilization period, a full factorial design based on the presence/absence of gallic acid (GA) and nonanoic acid (NA) (including a control group) was implemented into the exposure tanks. Regular monitoring of major phytoplankton taxa was conducted during a 28-day experiment using an on-line fluorometer. The main results suggested that gallic acid was more efficient than nonanoic acid at limiting cyanobacterial growth at concentrations as low as 1 mg L⁻¹.

1. Introduction

The increase in anthropogenic nutrient loading observed in many inland aquatic ecosystems over the past decades has often led to cyanobacteria dominance in phytoplankton communities (Carpenter et al., 1998; Davis et al., 2009; Paerl and Huisman, 2008). In spite of integrated catchment management strategies to reduce external nutrient loading, substantial internal nutrient loading in the form of phosphorus and nitrogen release especially from shallow lake sediments may also occur which can promote growth of toxicogenic cyanobacteria (Orihel et al., 2013; Søndergaard et al., 2003). Several researchers have further agreed that global warming could act as a catalyst over the next few years for the occurrence of harmful algal blooms (Davis et al., 2009; Jöhnk et al., 2008; Paerl and Huisman, 2008). Recently, there has been renewed interest in using biologically derived substances (BDS) isolated either from aquatic or terrestrial microorganisms and plants to remediate cyanobacterial blooms in a more eco-friendly and less expensive way than

current physicochemical water treatment processes (Jančula and Mašálek, 2011; Shao et al., 2013). This kind of biological approach refers to “allelopathy” principles (Molisch, 1937). This term was coined by Molisch (1937) to describe naturally occurring inhibitory or stimulatory biochemical interactions among primary producers or between primary producers and microorganisms (Gross, 1999). Allelopathic interactions occur through the release of organic chemicals, i.e. allelochemicals (Whittaker and Feeny, 1971; Rice, 1984), in the environment. In recent decades, growing attention has been paid to allelopathy and its ecological significance both in terrestrial and aquatic ecosystems. Empirical evidence of allelopathic interactions has been reported in terrestrial plant community structures (and dynamics) as well as in aquatic autotroph communities of freshwater and marine habitats (Gross, 2003; Sotomayor and Lortie, 2012). Recent studies focusing on the role of allelopathy in cyanobacteria clearly suggested that cyanotoxins such as widespread and well-

studied cylindrospermopsin could have allelopathic action in sympatric phytoplankton (Rzyski and Poniedzialek, 2014). From an evolutionary viewpoint, such a biological role would be a beneficial trait allowing for the dominance and rapid expansion of several cylindrospermopsin-producing cyanobacterial species, particularly those belonging to the Nostocales such as *Cylindrospermopsis raciborskii* or *Aphanizomenon ovalisporum* (Sukenik et al., 2012).

Numerous studies in sustainable agriculture and forestry currently deal with allelopathic interactions or the use of pure allelochemicals as eco-friendly growth regulators or herbicides (Sotomayor and Lortie, 2012). In the aquatic environment, one of the major advantages of using allelochemicals against detrimental algae, and particularly cyanobacterial species, relies on the fact that these compounds are not usually persistent. Moreover, allelochemicals have lower ecotoxicity potential than former algicides derived from metal ions (e.g., aluminum, copper sulfate) or organochlorine herbicides (e.g., diuron) and they are also energy-independent treatment systems (Jančula and Maršálek, 2011; Shao et al., 2013). Gallic acid (CAS 149-91-7) and nonanoic acid (CAS 112-05-0) especially appear as promising BDS for allelopathic treatments against cyanobacterial proliferation. Both compounds, originally isolated from *Myriophyllum aquaticum* and *Myriophyllum spicatum*, have actually been shown to have a growth inhibitory effect at low concentrations towards *Microcystis aeruginosa*, one of the most common toxic bloom-forming cyanobacterium (Nakai et al., 2000, 2005; Saito et al., 1989). As a matter of fact, the effective concentration of gallic acid leading to 50% algae growth inhibition (EC₅₀) was reported to be of 1.0 mg L⁻¹ on average for *M. aeruginosa* NIES-87 strain (Nakai et al., 2000). For nonanoic acid, a slightly stronger growth inhibitory effect than that of gallic acid was reported by Nakai et al. (2005) towards *M. aeruginosa* (NIES-87) with an EC₅₀ of 0.5 ± 0.3 mg L⁻¹. Additionally, in the view of field application, nonanoic acid would be of particular interest since it is a “huge production volume” (HPV) chemical which is also available at highly competitive prices (TOXNET, 2008).

This study sought to address the specific issue of gallic and nonanoic acid use in order to inhibit growth of cyanobacteria

at their early stages of proliferation. Indeed, for pond and freshwater quality management purposes, several authors agree that one of the most promising approaches to circumvent blue-green algae blooms would rely on BDS application before massive cell growth (Shao et al., 2013). Besides, allelochemical activity may largely vary depending on laboratory vs environmental conditions (Laue et al., 2014; Shao et al., 2013). Thus, the objectives were twofold: (i) to screen broadly potential dose-dependent (or synergistic) allelopathic effects, and (ii) to test the overall efficiency of such a substance application procedure that could be routinely implemented in situ to “timely” control cyanobacterial proliferation in small freshwater systems. Moreover, in order to address the process feasibility under more realistic exposure scenarios as well as the aspects of ecotoxicological risk assessment, outdoor freshwater len-tic mesocosms were designed in which phytoplankton species (encompassing local cyanobacteria species) and several other non-target model organisms representing different trophic groups were introduced. These latter included rudds (*Scardinius erythrophthalmus*) – a cyprinid fish species widely found in European freshwaters (Georgieva et al., 2014) –, Gammarus (*Gammarus roeseli*) – crustacean amphipods often used as sentinel species for water quality monitoring in Western Europe (Gerhardt et al., 2011) –, and two other common invertebrate species belonging to the gastropod class (Caquet, 1990), i.e. Physa (*Physa marmorata*) and Lymnaea (*Lymnaea stagnalis*). Since (i) experiments were designed to be conducted just at the beginning of the cyanobacterial proliferation period, and (ii) all reported cyanobacteria concentration levels in the mesocosms remained below what is expected to cause adverse cyanotoxin-related health and environmental effects, i.e. b 10 µg chlorophyll a/L (WHO, 2015), enzymatic biomarkers were measured in non-target species on the assumption that it would mainly reflect the allelopathic substance ecotoxicity potential. Thus, following a 28-day allelochemical applications into mesocosms, a set of exposure biomarkers consisting of lactate dehydrogenase (LDH), glutathione-S-transferase (GST), and total glutathione peroxidase (GPx) activities was assessed at the sub-cellular level in the non-target species (Coelho et al., 2011; Diamantino et al., 2001; Georgieva et al., 2014; Hermes-Lima, 2004; van der Oost et al., 2003).

2. Material and Methods

2.1. Ethical statement

All animal treatment and procedures used in this study complied with the general guidelines of the European Union Directive 2010/63/ EU and the French Animal Care Guidelines. In addition, three of the co-authors were authorized by the competent French authorities to perform animal experiments and particular attention was paid to fish welfare. In situ investigations were permitted by the animal welfare authority of the Regional council (agreement number C54-547-18).

2.2. Test chemicals

Gallic acid (98% purity, powder form) was purchased from Acros Organics (No. 410860010). Gallic acid quantities

required for each series of allelopathic treatment were weighed to the nearest milligram and spread onto the surface of each mesocosm concerned by this allelochemical treatment. With a water solubility of 11.9 g L⁻¹, gallic acid powder was practically instantaneously dissolved into the 2.6 m³ of water volume per mesocosm. Nonanoic acid (97% purity, liquid form) was obtained from Alfa Aesar (Lot. 10,146,705) and directly added into mesocosms to achieve proper dilutions. Each compound was added in sufficient quantities into mesocosms to achieve final water concentrations of 1, 2 or 4 mg L⁻¹ respectively for A1, A2 and A3 allelochemical application series (cf. Section 2.4). All the other chemicals required for biomarker assessment in non-target organisms were provided by Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.3. Mesocosm design

The mesocosms consisted of thirteen polyester tanks (2.14 m long × 2.14 m wide × 0.65 m deep) for a total volume of 3 m³ each, filled with 2.6 m³ of continuously aerated tap water. Continuous water mixing and aeration was ensured through air bubble introduction at the bottom of the tanks. They were all located at the outdoor facility of INRA – La Bouzule farm (Laneuvelotte, Meurthe-et-Moselle, France) – and exposed to naturally varying climate conditions (from May to July). Half of the surface of each mesocosm was covered with a PVC shade cloth and the other half with a net to prevent bird predation on fish. A 25 L volume of surface sediments collected from a reference site in a non-polluted area (“wild” Moselle River, Tonnoy, 54210, France) was packed into rectangular PVC containers and laid at the bottom of the tanks in order to simulate more natural environmental conditions especially in ponds. Several plankton enrichments (sampled from a pond located near the experimental platform) were conducted weekly during the mesocosm stabilization period. For this purpose, a 20-μm mesh size plankton sampling net was regularly deployed aboard a motor boat in the nearby pond. Then, the net content was split into equal volumes of one liter and introduced into each mesocosm. Such an operation aimed at favoring the biodiversity and rapid development of plankton communities (Caquet et al., 2005). Previous microscopic examination of water samples had revealed the presence of *Merismopedia elegans* as the major cyanobacterial species in the sampled pond (personal observation). After a two-week stabilization period, rudds (*S. erythrophthalmus*) in the juvenile stage as mainly planktivorous filter-feeding and herbivorous fish, Physa (*P. marmorata*) and Lymnaea (*L. stagnalis*) as biofilm, algae and plant feeders, and Gammarus (*G. roeseli*) as shredder representative were successively introduced into the artificial ponds. Rudds (20 individuals per mesocosm; 6.7 ± 0.2 cm) were obtained from a fish farming pond (Lorraine Region, France). Adult Lymnaea (30 specimens per mesocosm) were sampled from the reference site (“wild” Moselle River, Tonnoy, 54210, France) whereas *P. marmorata* (100 specimens per mesocosm) were obtained from a local fish farmer (Lorraine Region, France). In order to avoid Gammarus predation by rudds and for easier later sampling, the amphi-pods were encaged. For this purpose, a transparent polyethylene water bottle was cut in the mid-part and filled with a 2 cm layer of natural sediments; a willow branch was also added so as to serve as a feeding-source. 20 specimens of *G. roeseli* collected from the reference site were introduced into each bottle which was enclosed with a plastic mesh before total immersion into the artificial ponds. The total duration of mesocosm layout (including pond stabilization and organism acclimation) was eight weeks after which subsequent treatments with allelopathic substances started.

2.4. Allelopathic treatments

A full factorial design based on the presence/absence of gallic and nonanoic acids was established, leading to four treatment combinations: control mesocosms (without any allelopathic substances; n = 4 tanks), [+ gallic acid] treated mesocosms (n = 3), [+ nonanoic acid] treated mesocosms (n = 3), and [+gallic acid + nonanoic acid] treated mesocosms (n = 3). Sequential allelochemical treatments were conducted in the exposure tanks according to a geometric concentration

series (on the assumption that most allelochemical activity of the selected substances could be neglected after a five-day exposure; Laue et al., 2014; Shao et al., 2009). Our experimental strategy aimed at working in a proactive way, as soon as the first signs of cyanobacterial proliferation would occur (i.e. when the relative percentage of cyanobacterial biomass compared to the total phytoplankton community biomass tended to increase overall among the mesocosms of a given treatment, cf. supplementary material), by applying gallic acid and/or nonanoic acid directly onto water. Regular monitoring of major phytoplankton taxa biomass development was conducted afterwards in all the experimental units using a chlorophyll fluorometer (as described in the next section). Depending on the growth of phytoplankton biomass in the mesocosms, different doses (1 mg L⁻¹ [on the basis of literature data, Nakai et al., 2000], 2 mg L⁻¹, or 4 mg L⁻¹) were applied at different times (respectively identified as A1 for the first BDS application, A2 at T+13 days, and A3 at T+19 days). At T+28 days of the experiments, the global health status of the non-target species (snails, gammarids, and fish) was finally assessed through measurement of a set of exposure biomarkers.

2.5. Phytoplankton analysis

Water samples were regularly collected from each mesocosm using a PVC tube equipped with a bottom-closing rubber cap. Series of 25 mL subsamples were used for direct chlorophyll fluorescence measurement and algae class differentiation using an AlgaeLabAnalyser instrument (ALA bbe Moldaenke, Kiel, Germany) (Beutler et al., 2002). The ALA is an on-line laboratory cuvette spectrofluorometer which allows separate determination of chlorophyll a contents (expressed in μg chlorophyll a/L) from blue-green algae (Cyanophyceae), green algae (Chlorophyceae), brown algae (Bacillariophyceae, Dinophyceae), and Cryptophyceae (Beutler et al., 2002).

2.6. Physicochemical parameters

Water temperature was continuously recorded in all the exposure tanks using a HOBO data logger. Conductivity, pH, and dissolved oxygen were monitored twice a week using an appropriate digital probe (PONSEL ODEON® Digital Handheld Multiparameter). Ammonia, nitrite, nitrate, and orthophosphate ion concentrations were determined colorimetrically using a portable spectrophotometer and specific proprietary reagents (HANNA Instruments, HI 3873, HI 93700-01). Use of a digital probe and portable spectrophotometer was particularly appropriate for direct, rapid, and reliable in situ monitoring of water physicochemical parameters in the outdoor mesocosms (Caquet et al., 1996; Orellana et al., 2011).

2.7. Biomarker determination in non-target organisms

Lactate dehydrogenase (LDH), glutathione-S-transferase (GST), and total glutathione peroxidase (GPx) activities were determined to investigate the potential adverse effects of allelochemicals on the higher aquatic organisms. All of these enzymes are expressed in tissues of most organisms, being involved in energy production, xenobiotic phase II biotransformation, and detoxification, respectively, as well as

in cellular protection against oxidative damage (Coelho et al., 2011; Diamantino et al., 2001; Hermes-Lima, 2004; van der Oost et al., 2003). LDH, total GPx, and GST activities were assessed as standard exposure biomarkers in total per-mesocosm homogenates of *Lymnaea* (n = 30), *Physa* (n = 20), and *Gammarus* (n = 20), as well as in fish livers (n = 6) and gills (n = 6). Following anesthesia in an ice bath, rudds were killed by a blow to the head and tissue (gills and liver) were collected, whereas invertebrates were immediately snap-frozen into liquid nitrogen and stored at -80°C until further laboratory processing. Homogenate preparation was conducted according to the procedure described in (Techer et al., 2015a). Protein content, LDH activity (EC 1.1.1.27), total GPx activity (EC 1.11.1.9), and GST activity (EC 2.5.1.18) were respectively determined on appropriate S9 fraction dilutions according to the methods of (Bradford, 1976; Drotar et al., 1985; Habig and Jakoby, 1981; Jesus et

al., 2013) adapted to microtiter plates (as reported in Techer et al., 2015a).

2.8. Statistical analysis

All data were expressed as mean \pm SE. Normality and homogeneity of variance were tested with the Kolmogorov–Smirnov test and Levene's test, respectively. The significance of the differences observed between treatments (at a level of $p < 0.05$) was determined by ANOVA for parametric distributions or by Kruskal–Wallis for nonparametric distributions followed by Holm–Sidak multiple pairwise comparison method (with an overall significance level of 0.05). For unequal treatment group sizes the Dunn's test was used (also with an overall significance level of 0.05). All statistical analyses were carried out using Statistica v.10.

3. Results

3.1. Water quality

Overall, no significant differences were reported in any of the water parameters measured between mesocosms just before experimentation (T0) (Table 1). During the experimental period (Exp), substantial increases in ammonia and orthophosphate concentrations were observed in all the mesocosms compared to T0 (Table 1). Moreover, a slight but significant decrease in pH was reported in [+gallic acid] and [+gallic acid + nonanoic acid] treated mesocosms compared to the control group (Table 1). Finally, water temperature in all the mesocosms was found to show similar daily variations according to the time of day, with the highest temperatures recorded around noon and the lowest temperatures around midnight (Fig. 1A).

3.2. Blue-green, green, and brown algae, and cryptophyceae growth

At T0 (before A1), no significant differences were noted in phyto-plankton community biomass regardless of the taxa and allelopathic treatment considered (Fig. 1B, C, D, E). By contrast, the day following A1 (Day 1), a slight but significantly lower concentration of cyanobacteria was measured in [+gallic acid] treated mesocosms compared to control (ANOVA $p = 0.028$ followed by Holm–Sidak method at $p < 0.05$; Fig. 1B). Overall, this trend was maintained until A2 despite the lack of statistical significance between the two categories of mesocosms (i.e. control vs [+gallic acid] treated mesocosms) (Fig. 1B). Similarly, a trend towards a lower cyanobacteria concentration was observed in [+gallic acid + nonanoic acid] tanks during the first seven days of the experiments compared to control and also to [+nonanoic acid] mesocosms (Fig. 1B). From Day 11 to Day 13, an overall decrease in cyanobacteria concentration was observed for all treatment modalities (Fig. 1B), and a change in temperature was recorded during the same time interval (on average from 23°C on Day 11 to 15.5°C on Day 13, Fig. 1A). Between A2 and A3, in [+ gallic acid] treated mesocosms, cyanobacteria biomass dropped to trace level with no more than 0.5 Eig chlorophyll a/L (Fig. 1B). A similar trend was reported in [+gallic acid + nonanoic acid] mesocosms (Fig. 1B). On Day 20, just after A3, cyanobacteria concentrations were significantly lower in [+gallic acid] mesocosms compared to the control ones

(ANOVA $p = 0.034$ followed by Holm–Sidak method at $p < 0.05$), being below the limit for spectrofluorometric detection (Fig. 1B). Overall, relative cyanobacteria concentrations in [+gallic acid] and [+gallic acid + nonanoic acid] treated mesocosms remained well below those observed in control (and in [+nonanoic acid]) mesocosms until the end of the experiments on Day 28 (Fig. 1B). On the contrary, for control and to a lesser extent for [+nonanoic acid] exposure tanks, overall cyanobacteria concentrations in water kept increasing (Fig. 1B) and temperatures were found to be higher during this time period (Fig. 1A). The highest cyanobacteria concentration was recorded in the control group with up to 8 Eig/L of chlorophyll a on average on Day 28, which was significantly higher (Kruskal–Wallis $p = 0.018$ followed by Dunn's method at $p < 0.05$) than that reported in [+gallic acid] treated mesocosms (0.4 ± 0.2 Eig chlorophyll a/L) (Fig. 1B).

Regarding green algae growth (Fig. 1C), changes in biomass were quite similar in all treatment types including the control group, mainly reflecting temperature variations (Fig. 1A). Major differences between mesocosms were reported at the end of the experiments with higher green algae concentrations for the two experimental modalities using gallic acid (i.e. [+gallic acid] and [+gallic acid + nonanoic acid] mesocosms). The relative amounts (14 Eig chlorophyll a/L) were thus significantly greater in [+gallic acid + nonanoic acid] mesocosms compared to control (4.6 Eig chlorophyll a/L) and [+ nonanoic acid] ones (2.9 Eig chlorophyll a/L) (Fig. 1C). It is worth noting that the latter findings were the opposite of what was previously mentioned for cyanobacteria biomass (Fig. 1B).

Finally, brown algae (Fig. 1D) and to a lesser extent cryptophyceae (Fig. 1E) growth profiles were also similar to those of green algae (Fig. 1C) in terms of biomass variation following temperature changes during the same time period. Besides, at the end of the experiments higher concentrations of brown algae and cryptophyceae were also measured in [+gallic acid] and [+gallic acid + nonanoic acid] treated mesocosms when compared to control and [+nonanoic acid] exposure tanks (Fig. 1D, E).

3.3. Biomarker evaluation of non-target higher organism species

3.3.1. Physa

As reported in Fig. 2A and C, no significant variations were found either in LDH or in GST activities in *Physa* collected from [+ gallic], [+ nonanoic acid], or [+ gallic acid + nonanoic acid] treated mesocosms. By contrast, a significant decrease in GPx activity was noted in specimens from the mesocosms previously subjected to [+ gallic acid + nonanoic acid] allelopathic treatment compared to those from the control group, in the range of 6.0 ± 0.6 vs 9.3 ± 0.8 U/mg proteins, respectively (Fig. 2B).

3.3.2. Lymnaea

Only a significant decrease in GST activity in organism homogenates obtained from [+gallic acid + nonanoic acid] mesocosms could be reported when compared to those collected from control and [+nonanoic acid] exposure tanks (Fig. 2F).

3.3.3. Gammarus

Measurements of LDH, GPx, and GST activities in total *Gammarus* homogenates did not allow any significant enzymatic activity variations to be sorted out according to the exposure conditions (Fig. 2G, H, I).

3.3.4. Fish

In fish livers, no significant differences in enzyme activities were noted between organisms from control and allelochemically-treated mesocosms (Fig. 3A, B, C). Biomarker investigation in gills (Fig. 3D, E, F) revealed a lower GPx activity in samples from [+ nonanoic acid] treated mesocosms compared to those from the control group, i.e. 13.2 ± 2.5 vs 21.1 ± 2.0 U/mg proteins, respectively (Fig. 3E).

4. Discussion

The present study is the first to address the issue of the allelopathic potential of gallic and nonanoic acids at the early stage of cyanobacterial proliferation, when water temperature has not yet reached its seasonal maximum (20 °C–25 °C for the present study site, located in northeastern France), and importantly in an ecologically relevant context, i.e. through an outdoor multi-species mesocosm approach. Due to their allelopathic potential (Nakai et al., 2000, 2005), one may hypothesize that applying gallic or nonanoic acids during a harmful algal bloom could lead to “massive” cyanotoxin release. Thus, acting preemptively by limiting cell growth at their early stages of proliferation could be a promising approach for allelochemical in situ application. Traditionally, most experiments focusing on allelopathy against cyanobacterial growth have been done under laboratory conditions due to complex chemical synthesis or prohibitive pricing to obtain sufficient substance amounts for field application (Jančula and Maršálek, 2011; Shao et al., 2013). By contrast, accounting for their allelopathic activity (Nakai et al., 2000, 2005) and because of their use in a wide variety of industrial or agrochemical areas with concomitant commercial availability (Coleman and Penner, 2008; Dena et al., 2012; Galati and O'Brien, 2004; Niho et al., 2001; Ow and Stupans, 2003; Techer et al., 2015a; TOXNET, 2008), gallic and nonanoic acids were therefore considered as “good candidates” for an early in situ cyanobacterial growth control approach. For instance, for a pond of 2 m depth on average and depending on nonanoic or gallic acid application (e.g. 1 mg L⁻¹), one could hypothesize minimum prices varying from 20 to 200 US \$ dollar per hectare (based on personal estimations only).

Similar environmental conditions in all the exposure tanks at T0 and throughout the experiments were required in order to rule out any physicochemical parameters interference which could indirectly or unexpectedly affect cyanobacterial growth in the designed mesocosms.

Thus, it has to be pointed out that most of mesocosms tended to have low initial PO₄ content, despite disparities in all tested groups and regardless the assigned treatment

(Table 1). As reported by Caquet et al. (1996, 2001), phosphorus- and nitrogen-containing nutrient concentrations often display high inter-system variability, depending closely on primary producer biological activity which is rarely synchronous between such replicated outdoor tanks. Water quality analyses confirmed the homogeneity of the measured parameters (absence of statistical significance in nutrient contents) in all the tanks prior to the experiments and also its compatibility with phytoplankton growth (including cyanobacteria) during the experimental phase (Table 1).

Regarding the allelopathic activity of gallic and nonanoic acids, overall the latter did not seem to induce any significant cyanobacterial growth inhibition, and no synergistic effect could be observed when the two allelochemicals were added together to mesocosms (Fig. 1B). Although *M. elegans* (previously identified in the water samples used for initial mesocosm enrichments) are unicellular blue-green algae belonging to the Chroococcales order (like *M. aeruginosa*), results suggested that nonanoic acid would be an inappropriate substance to inhibit their growth in situ. Rather, the experimental observations would indicate that only gallic acid had the potential to slow down initial cyanobacterial proliferation (Fig. 1B). According to the literature, the allelopathic activity of polyphenols like gallic acid may be due to alkaline phosphatase inhibition or photosynthetic process disruption, along with reactive oxygen species formation and cellular oxidative damage in cyanobacterial cells (Dziga et al., 2007; Gross et al., 1996; Hoppe, 2003; Leu et al., 2002; Nakai et al., 2000). However, these results had to be mitigated. Indeed, when considering the magnitude of the changes observed following gallic or nonanoic acid application after A2 (i.e. the “slopes” of corresponding cyanobacterial biomass, Fig. 1B), the possibility of a nonanoic acid allelochemical effect cannot be ruled out. Furthermore, using fluoroprobe was a well-suited method for a rapid monitoring of phytoplankton communities during in situ experiments (as previously reported by Beutler et al., 2002). Nevertheless, decreases in total cyanobacterial biomass as observed in the case of gallic acid application did not necessarily reflect the depression of species which could pose potential environmental hazards and health threats,

such as for instance well-known *M. aeruginosa* species (Straub et al., 2011). Besides, *M. elegans* which has been identified in mesocosm enrichment samples can be involved into bloom formation (Qu et al., 2015), but to our knowledge it has not yet been recognized as a harmful cyanobacterial species (Morski Instytut Rybacki - Państwowy Instytut Badawczy, Poland, 2011). Because a fish pond was located near the outdoor facility where the mesocosm experiments took place, from an ecological viewpoint (and due to legislative limitations) these latter could not involve the use of potentially toxic cyanobacterial species like *M. aeruginosa*, which has not been previously identified in that nearby pond. The primary objective of the present study was to investigate the technical feasibility of using gallic or nonanoic acid directly applied in the pure form especially before bloom occurs at a field scale, taking account their allelochemical activity towards *M. aeruginosa* (previously tested under laboratory conditions) and their commercial availability (Nakai et al., 2000, 2005). With this in mind, our results showed a potential of gallic acid ingrowth control of an ubiquitous cyanobacterial species, i.e. *M. elegans* (even if not belonging to toxic species), in a more realistic scenario than controlled laboratory assays. However, there is no doubt that further studies involving harmful bloom-forming algal species along with additional microscopic morphological examination of cyanobacterial diversity would help to clarify to which extent the tested allelochemicals in their pure form (particularly gallic acid) can prevent from massive blue-green algae growth. Moreover, in the view of a global pond management strategy, it has to be pointed out that only an allelopathic approach would certainly not be sufficient for an effective long-term water resource management. Rather it should be considered as a complementary tool for improving the success of an integrated landscape management strategy involving notably anthropogenic nutrient loading reduction.

Following this idea, still in the view of global implementation of freshwater pond management strategies, it is worth noting that green algae and to a lesser extent brown algae and cryptophyceae, while showing overall temperature-driven biomass growth (Fig. 1C, D, E), also tended to compete with cyanobacteria (Fig. 1B). On the one hand successive gallic acid treatments (applied at the very beginning of summer) did

not allow the complete elimination of cyanobacteria from the mesocosms (Fig. 1B), but on the other hand they did not prevent the growth of other competitive photosynthetic microorganisms (Fig. 1C, D, E). The relative specificity of the allelochemical towards cyanobacteria did not alter the primary productivity in the treated ecosystems which is of importance for ecosystem functioning.

Last, regarding the ecotoxicity potential of gallic and nonanoic acids, previous studies dealing with their acute toxicity in model invertebrate (*Daphnia magna*) and fish species (*Danio rerio*) reported LC50 values respectively of 28.1 mg L⁻¹ and 100 mg L⁻¹ for gallic acid on the one hand (Kamaya et al., 2005; Techer et al., 2015a), and of 96 mg L⁻¹ and 81.2 mg L⁻¹ for nonanoic acid on the other hand (Techer et al., 2015a; US EPA, 1992). These “threshold values” well above the EC50 reported for gallic and nonanoic acid allelopathic activity towards cyanobacteria (1 and 0.5 mg L⁻¹, respectively, Nakai et al., 2000, 2005) suggested that biological effects concomitant to the allelochemical exposure in a concentration range compatible with their algicidal properties would not be harmful for higher organisms introduced into the mesocosms. Such assumptions were in accordance with our findings, leading to the assessment of standard subcellular biomarkers. Slight but significant decreases in total GPx activities were reported in *Physa* and fish gills respectively exposed to [+gallic acid + nonanoic acid] (Fig. 2B) and [+nonanoic acid] (Fig. 3E). Both allelochemicals have been reported to induce oxidative stress (Techer et al., 2015b). Moreover, due to its medium chain length, nonanoic acid is especially efficient to intercalate into plasma cell membrane, leading ultimately to reactive oxygen species production when used as a herbicide in higher terrestrial plants (Lederer et al., 2004). The fact that nonanoic acid especially may be involved into antioxidant enzyme activity disruption on the longterm cannot be completely ruled out. Despite this assumption and as illustrated by the absence of any other biomarker induction signal in snails, gammarids, and fish tissues (Figs. 2, 3), the toxicological evaluation overall did not indicate any harmful effects of both compounds (either alone or combined) in these non-target species.

5. Conclusion

According to our mesocosm exposure conditions and contrasting with laboratory data, gallic acid appears as a more potent and promising allelochemical than nonanoic acid to control cyanobacterial growth at their early stage of proliferation. Moreover, in our experimental conditions, no ecotoxicity was reported in non-target test species, thereby making it even more relevant to test the application of this

polyphenolic compound in naturally occurring freshwater systems, possibly paving the way for further allelopathy-based aquatic ecotechnology development.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2015.12.164>.

Table 1.

Measurement of water physicochemical parameters in mesocosms before experiments (T₀), and during allelopathic treatments (Exp.);

		Control	+Gallic acid	+ Nonanoic acid	+Gallic acid + nonanoic acid
O ₂ (mol L ⁻¹)	T ₀	9.9±0.1	93±0.2	9.8±0.3	93±0.1
	Exp.	9.6±0.1 [^]	8.9±0.1 ^α	9.1 ±0.1 ^a	8.8±0.1 ^a
pH	T ₀	8.5±0.0	8.4±0.0	8.6±0.0	8.5±0.0
	Exp.	8.5±0.1 [^]	8.3±0.0 ^a	8.3 ± 0.0	8.2 ± 0.0 ^a
Conductivity (μS cm ⁻¹)	T ₀	1112±4	1100±9	1096±13	1099±12
	Exp.	1064±7	1061±7	1060±7	1061±7
NH ₄ (mg L ⁻¹)	T ₀	0.18 ± 0.04	0.18 ± 0.03	0.11 ± 0.01	0.16 ± 0.07
	Exp.	0.27 ± 0.02	0.29 ± 0.04	0.22 ± 0.02	0.22 ± 0.03
NO ₃ (mg l ⁻¹)	T ₀	0.0 ± 0.0	0.2±0.2	0.0 ± 0.0	0.17 ± 0.17
	Exp.	0.02 ± 0.02	0.07 ± 0.04	0.11 ± 0.05	0.09 ± 0.06
PO ₄ ⁻ (mgL ⁻¹)	T ₀	0.48±0.28	0.03 ± 0.03	0.1 ± 0.1	0.2 ± 0.2
	Exp.	0.77 ± 0.13	0.52 ± 0.1	0.93 ± 0.18	0.54 ± 0.1

Control: non-allelochemically treated mesocosms; +Gallic acid: mesocosms treated with gallic acid solely; +Nonanoic acid: mesocosms treated with nonanoic acid solely; +Gallic acid + nonanoic acid: mesocosms treated with the combination of gallic and nonanoic acids. Data are expressed as mean ± SE (average for all parameters at all time points). Significant differences between groups are indicated by capital letters against lower case ones (p < 0.05).

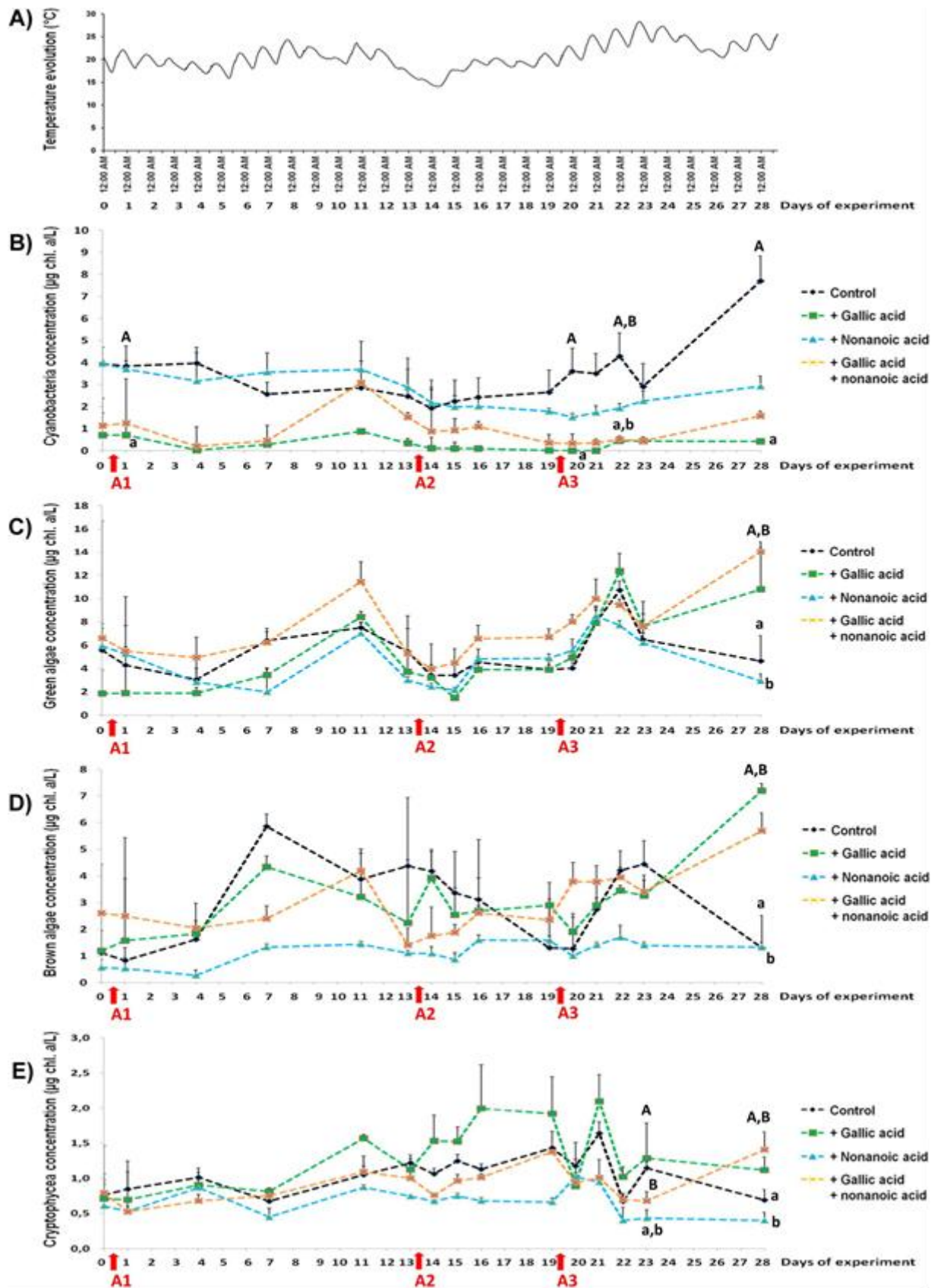


Figure 1.

Variations of (A) temperature and (B) cyanobacteria, (C) green algae, (D) brown algae, (E) cryptophyceae biomass concentration (expressed in µg chlorophyll a L⁻¹) in control and allelochemically treated mesocosms. Allelopathic treatments (A1, A2, A3) are indicated by red arrows. Data are expressed as mean ± SE. Groups marked by capital letters are significantly different from those marked by lower case ones (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

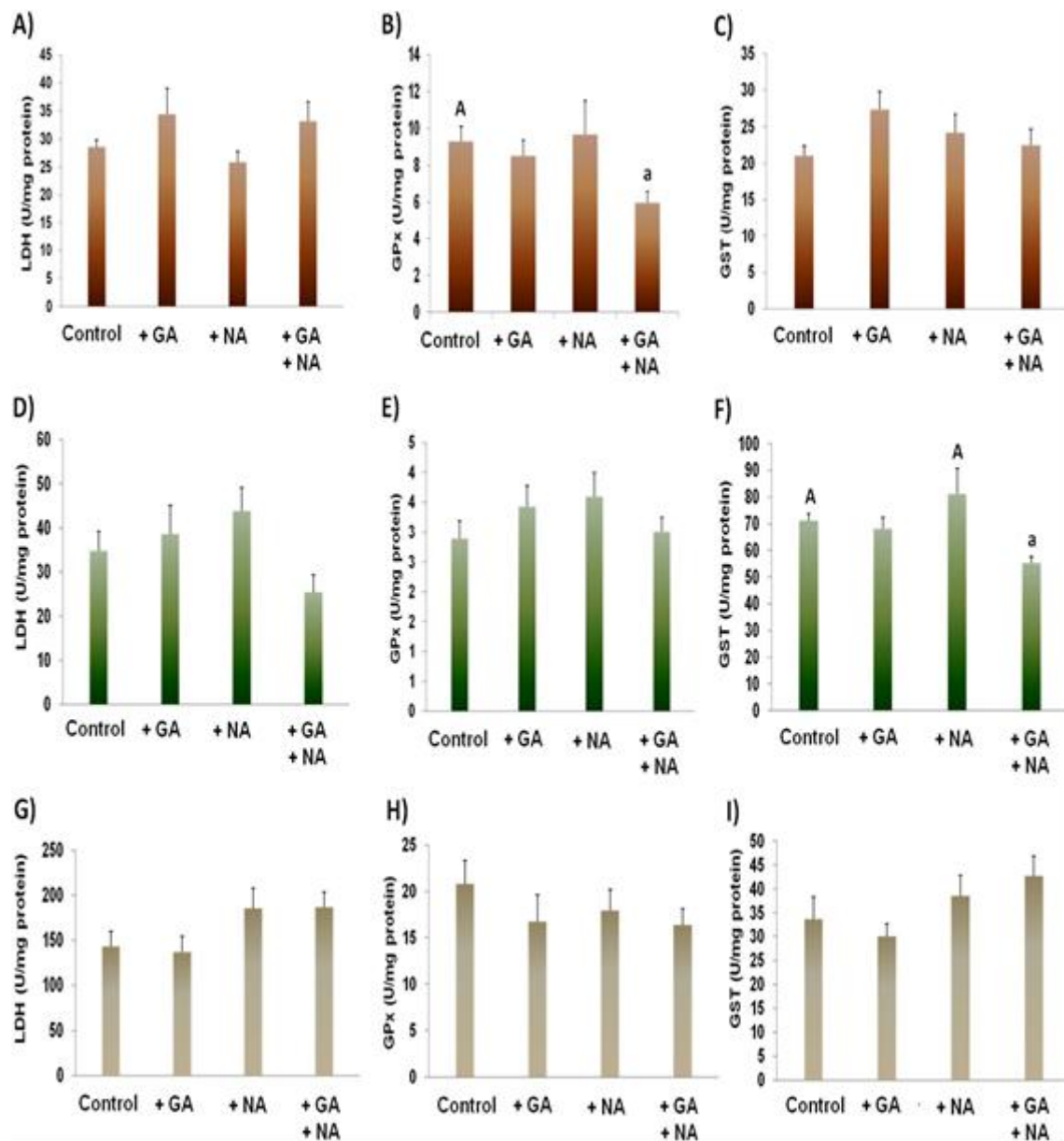


Figure 2.

LDH, GPx, and GST activities measurement in whole invertebrate homogenates at the end of allelopathic treatments (t+ 28 days): A, B, C) *Physa* homogenates; D, E, F) *Lymnaea* homogenates; G, H, I) *Gammarus* homogenates; +GA: mesocosms treated with gallic acid solely; +NA: mesocosms treated with nonanoic acid solely; +GA + NA: mesocosms treated with the combination of gallic and nonanoic acids. Data are expressed as mean \pm SE. Groups marked by capital letters are significantly different from those marked by lower case ones (p < 0.05).

6. References

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