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Influence of the interaction between phosphate and arsenate on periphyton's growth and its nutrient uptake capacity

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HIGHLIGHTS

- As affected structural and functional parameters of periphyton starved of P.
- Effects of As were detected in *noP* communities, but not when P was available.
- Intracellular As contents were higher in communities starved of P.
- As tolerance was induced by the combination of As and P but not by As or P alone.
- Chronic exposure to realistic As levels can lead to changes in stream ecosystems.

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ABSTRACT

Periphyton communities grown in microcosms were studied under the exposure to different arsenate (As) and phosphate (P) regimes with the aim of revealing the effect of chronic exposure to As on periphyton physiological and structural characteristics. Also, we aimed to study periphyton changes on sensitivity to As, exposed to different P and As regimes. As affected structural and functional parameters of periphyton communities starved of P, inhibiting algal growth, photosynthetic capacity, changing community composition and reducing the ability of the community to retain P. The effects of As on these parameters were only detected in P starved communities, showing that chronic exposure to As led to changes in the photosynthetic apparatus under the conditions of P-limitation, but not when P-availability was higher. This fact reveals a lower toxicity and/or a higher adaptation of the P-amended community. Intracellular As contents were higher in communities starved of P. However, As tolerance was only induced by the combination of As and P but not by As or P alone indicating that tolerance induction may be an ATP-dependent mechanism. This study reveals that chronic exposure of natural communities to environmentally realistic As concentrations will damage periphyton communities affecting key ecosystem processes, as P uptake, leading to changes in stream ecosystems, as these organisms play a key role in nutrient cycling through nutrient uptake and transfer to higher trophic levels.

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1. Introduction

Arsenic is a metalloid naturally occurring in aquatic ecosystems (Smedley and Kinniburgh, 2002) and it is toxic to human health. Its presence has been studied in freshwaters and groundwaters from all over the world, including Latin America (Bundschuh et al., 2004; Rosso et al., 2011; López et al., 2012), Asia (Hossain, 2006; Li et al., 2012) and Europe (Garcia-Sanchez and Alvarez-Ayuso, 2003; Aloupi et al., 2009).

Primary producers and microbial communities play a key role on the biogeochemistry of arsenic (Sanders and Windom, 1980; Hasegawa et al., 2009). These organisms take up arsenate (As(V)) and incorporate it into the algal cell. Most of As(V) is reduced, methylated and then released to the surrounding media. The processes involved have been well studied in marine systems but poorly addressed in freshwater systems (Rahman et al., 2012), especially in running waters. This fact brings concern in terms of ecologic integrity and human health since both arsenic detoxification and arsenate reduction have been attributed to microbial activity in freshwater systems (Hasegawa et al., 2010; Guo et al., 2011; Rahman et al., 2012). Also, microbial communities are the main primary producers in most fluvial systems and the first to interact with dissolved substances such as toxicants. These facts make them suitable as early warning systems for detection of aquatic

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contamination (Sabater et al., 2007). Exposure to toxicants may alter its normal physiology or structure, leading to changes in trophic relations and ecosystem functioning (Barranguet et al., 2003).

Many biotic and abiotic factors influence the sensitivity of algae to arsenic. While arsenite (As (III)) is considered to be more toxic than arsenate (As (V)) in marine phytoplankton, their toxicity is reversed in freshwater algae (Knauer et al., 1999; Karadjova et al., 2008). In addition to speciation, arsenic toxicity varies depending on chemical conditions such as pH, oxygen levels or phosphate concentration and biotic factors such as the species type and the duration and intensity of prior pre-exposure leading to different detoxification mechanisms (Levy et al., 2005) and hence to a large range of sensitivities (Jurewicz and Buikema, 1980; Vocke, 1980; Fargasová, 1994; Goessler et al., 1997; Wang et al., 2013). However, the underlying mechanisms of arsenate tolerance remain unclear. Theoretically, algae may respond in several ways to palliate arsenate toxicity: via regulation of uptake (reducing the affinity or number of arsenate transporters on the surface), intracellular reduction to arsenite and excretion, scavenged by sulfur-containing compounds, or methylated to less toxic organic species (Karadjova et al., 2008; Zhao et al., 2009; Gupta et al., 2011; Wang et al., 2013).

Investigations performed with freshwater microalgae suggest that bioaccumulation and subsequent methylation, a detoxification mechanism commonly reported in marine phytoplankton, is not the primary mode of detoxification. Levy et al. (2005) proposed that arsenic is taken up by cells using a phosphate transport system, reduced to As III in the cell and then excreted into the growth medium, probably by an active transport system.

Since natural arsenic pollution in streams is frequently linked to high phosphate concentration, a strong interaction between both factors is anticipated (Guo et al., 2011). The structure of a molecule of As (V) is chemically similar to that of the nutrient phosphate (PO_4^{3-}). The inability of the PO_4^{3-} receptors to discriminate against As (V) (Button et al., 1973) is a major reason for As (V) toxicity. Also, As (V) has been shown to competitively inhibit PO_4^{3-} transport system in *Euglena gracilis* (Blum, 1966). Levy et al. (2005) showed that phosphate enrichment reduced arsenate toxicity to algal cultures in the laboratory suggesting that phosphate reduced arsenic uptake due to extracellular competition. Further, Wang et al. (2013) support that arsenic toxicity is phosphate condition-dependent. In their investigations both arsenate bioaccumulation and intracellular phosphorus, more specifically the As/P ratio, determined arsenate toxicity. Toxicity differed among algal species depending on their ability to regulate arsenate accumulation. Therefore, it is important to explore the effect of phosphate concentrations on the uptake of arsenate as well as the effect of arsenate concentrations on growth of freshwater periphyton under different phosphate conditions.

Environmental effects of arsenic cannot be derived from knowledge of existing toxicity data based on algal monocultures, which have a five orders of magnitude range of variability (Knauer et al., 1999; Kramárová et al., 2012; Silva et al., 2013; Wang et al., 2013). Instead, the use of periphyton, complex microbial communities composed of algae, bacteria and fungi attached to substrata, has higher ecological realism because it integrates the diversity of physiological responses of the species that comprise them (Sabater et al., 2007). Microcosm settings allow exposure of these communities under controlled conditions including P and As exposure to experimentally address the interaction between factors, allowing isolation of the factors that want to be tested. The aim of this study was to reveal the effect of arsenate on periphyton physiological and structural characteristics and community tolerance under different phosphate conditions. Also, we aimed to study the role of periphyton on phosphate retention under exposure to different phosphate and arsenate regimes. The main hypotheses of our investigation are that arsenate impairs the normal functioning and alters the structure of periphyton communities starved of phosphate and that chronic exposure to arsenate and phosphate would increase tolerance to arsenate.

2. Materials and methods

2.1. Experimental design

The experiment was performed in microcosms placed inside growth chambers following Bonnineau et al. (2010). Briefly, microcosms consisted of 2 L rounded crystallizing dishes of 25 cm diameter. Water pumps (Hydor, Pico 300, 230 V 50 Hz, 4.5 W) were placed inside the dishes to recirculate water, mimicking lotic ecosystems, stimulating periphyton attachment, growth and to avoid temperature or nutrient gradients. Periphyton inoculum was collected from the Llémena River, a small calcareous tributary of the Ter River (North-East Spain). This river is known to be unpolluted by arsenic (Baig et al., 2010; ACA). Inoculum was spiked into each crystallizing dish and allowed to colonize forty 1.4 cm^2 and four 17 cm^2 etched glass substrata placed at the bottom of the crystallizing dishes and covering the whole bottom. Small glasses were used for PAM measurements during colonization and during dose–response experiments. Large glasses were scrapped for accumulation measurements at the end of the experiments. Six treatments were applied from the beginning of periphyton colonization with three replicates per treatment. Treatments consisted of the exposure of periphyton communities to different levels of PO_4^{3-} and As (V) and the combination of the two. Final concentrations of PO_4^{3-} and As (V) in each treatment were selected according to environmentally relevant concentrations found in the literature (Feijoó and Lombardo, 2007; Rosso et al., 2011). Three treatments were spiked with 10 μg/L PO_4^{3-} as KH_2PO_4 (Merck, Darmstadt, Germany) (a limitant P concentration, referred to as *NoP*) and three with 100 μg/L PO_4^{3-} (a non-limiting P concentration, referred to as *P*). 130 μg/L of As (V) as $NaH_2AsO_4.7H_2O$ (Merck, Darmstadt, Germany) were added to one of the *P enriched* treatments and to one of the *P limited* treatments (High As concentration, referred to as *AsH*). 15 μg/L of As (V) were added to one of the three *P enriched* and to one of the *P limited* treatments (*low As*, referred to as *AsL*). This concentration is close to the guidelines for human consumption (World Health Organization et al., 1984). The six treatments are referred to as *noP noAs*, *noP AsL*, *noP AsH*, *P noAs*, *P AsL*, *P AsH*. Solutions were made with dechlorinated tap water filtered with an active carbon (AC) filter in order to minimize possible solutes present in it. A 12:12 dark/light period and a temperature of 21 °C were obtained by placing the crystallizing dishes in the growth chambers (Radiber AGP-570).

The experiment lasted for 4 weeks: metalloid exposure started since the inoculum was placed in the crystallizing dishes for the first time. At the end of the colonization, a P uptake rate experiment and a pollution induced community tolerance (PICT) experiment were performed (Blanck et al., 1988).

2.2. Water sampling and analysis

Medium was renewed 3 times a week to minimize water chemistry changes during the whole experiment. In order to monitor water physical parameters, water temperature, pH, dissolved oxygen and conductivity were measured with appropriate multi-parameter sensor probes (HACH LANGE GMBH, Germany). These measurements were performed three times per week in three different crystallizing dishes each time, before and after every water renewal. While the water was renewed, and in the same crystallizing dishes where physical parameters were monitored, water samples were collected to monitor chemical parameters. To analyze P levels, 10 ml of water was filtered with GF/F glass fiber filters (Whatman). To analyze As levels, 5 ml of water was filtered using 0.2 μM nylon membrane filters (Whatman), and acidified with 1% HNO_3 (65% HNO_3, Suprapur, Merck, Germany). Samples were stored at 4 °C. On the last week of colonization 10 ml of water was taken from each crystallizing dish after water renewal and 3 days later, to analyze As speciation. Samples were filtered using 0.2 μM nylon membrane filters (Whatman), and stored at −20 °C (APHA, 2004).

The phosphate concentration was analyzed using the ascorbic acid method modified to avoid arsenate interference (Carvalho et al., 1998). Total dissolved As concentration in water was measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, 7500c Agilent Technologies, Inc. Wilmington, DE). Arsenic species (particularly As (III) and As (V)), were determined. The separation of As (V) and As (III) was performed by pasing the solutions through disposable cartridges packed with a selective aluminosilicate sorbent (MetalSoft Center, Highland Park, USA). The sorbent selectively adsorbs As (V) while arsenite remains in the percolated samples. As (V) concentration was obtained from the difference between total As and As (III). For As determination in all the solutions, ICP-MS equipped with collision cell was used following the operational parameters described in Colon et al. (2011). The detection limit for As was 0.08 μg l^{-1}. Rh was used as internal standard. The accuracy of the analytical method was checked periodically using certified water reference (SPS-SW2 Batch 113, Oslo, Norway).

2.3. Periphyton sampling and analysis

Samples of the periphyton community were taken during the experiment to follow temporal variations on structural and functional parameters of the community. The amount of the samples and frequency of collection are detailed below.

2.3.1. PAM sampling and analysis

In order to monitor changes in algal biomass during the colonization period, colonized substrata were sampled 16 times (4 times a week for 4 weeks). Each sampling time, three colonized glasses were randomly removed from each crystallizing dish. Glass removal was performed gently with fine-tipped forceps, so as not to disturb other substrates. The glasses were used to measure the minimum fluorescence yield (Fo) using a Pulse of Amplitude Modulated Fluorometer (PHYTO-PAM Heinz Walz GmbH, Effeltrich, Germany), as Corcoll et al. (2011) procedures. According to Rysgaard et al. (2001), Fo of dark-adapted cells at 665 nm, is proportional to the chlorophyll-a (chl-a) concentration. Fo can be used as a structural parameter of the community, as it relates to algal biomass. In order to calibrate Fo with algal biomass, chl-a was extracted from the periphyton samples with 90% acetone, and measured spectrophotometrically (Jeffrey and Humphrey, 1975). The linear relationship between the two parameters was confirmed (Schmitt-Jansen and Altenburger, 2008).

The following fluorescence parameters were also measured. Maximum quantum yield ($_{M}$) and effective quantum yield ($_{M'}$) measurements were performed twice a week. The $_{M}$ parameter represents the maximum photosynthetic potential and the $_{M'}$ represents the photosynthetic efficiency at a steady state electron transport (Genty et al., 1989). The Fo of the different algal classes composing the periphyton communities were recorded three times during the last week of colonization. The Fo of the different algal classes was used to evaluate the relative contribution of each one to the whole communities in relation to the effect of the toxicant.

As PAM fluorometry is a fast, non-destructive technique, colonized glasses were returned into the crystallizing dishes after measurements.

2.3.2. Arsenic accumulation

In order to measure the total amount of As accumulated in the periphyton, samples from each crystallizing dish were taken at the end of the experiment. The four large substrata (a total area of 68 cm^2) of each crystallizing dish were scratched and suspended in 100 ml of filtered water obtained from the corresponding crystallizing dish. The amount of biomass collected from the four substrata was always above 10 mg dry weight, which was enough for digestion and analysis of As accumulation. The suspension was divided into two fractions. One fraction was treated with EDTA to remove arsenic adsorbed to the cell wall and most of the inorganic complexes embedded in the biofilm. This process allowed for the measurement of the intracellular arsenic content in periphyton. The other fraction was used to measure the total amount of arsenic accumulated in periphyton. The difference between total and intracellular arsenic content is considered to be adsorbed arsenic on periphyton. Three aliquots of each fraction were filtered with sterile and pre weighed filters (cellulose nitrate 0.45 μm, Whatman) to obtain the dry weight (DW) of each sample after arriving to a constant weight of the sample. Then the filters were digested with 4 ml of concentrated HNO_3 (65% HNO_3, Suprapur, Merck, Germany) and 1 ml of H_2O_2 (31% H_2O_2, Suprapur, Merck, Germany). A 75-time dilution with Milli-Q water and acidification (1%) of the samples was performed. For a more detailed procedure of this protocol, see Meylan et al. (2003). Digested samples were analyzed following the procedure described for total arsenic in water. Arsenic bioaccumulation was expressed as dissolved arsenic per dry weight (μg As·g DW^{-1})

2.3.3. Diatom community analysis

To analyze diatom community, biomass from 1 colonized glass from each treatment was scraped and resuspended in Milli-Q water at the end of the colonization period. Organic matter was removed by 10 ml of hydrogen peroxide (40%) and heat to obtain clean frustules. Frustules were then washed with distilled water, dehydrated on cover glasses, and mounted in Naphrax (Refractive index of 1.74). Up to 600 valves were counted and identified in each sample with a light microscope (Nikon Eclipse 600W, Tokyo, Japan) using Nomarski differential interference contrast optics at a magnification of 1000×. In order to describe the diatom community, diatoms were primarily identified following Krammer and Lange-Bertalot (1986, 1988, 1991a, 1991b), as well as from more current literature (Lange-Bertalot, 1993, Krammer, 2000, Lange-Bertalot, 2001, Krammer, 2002).

Diatom density in relation to the colonized surface area was quantified by means of subsequent dilutions of the digested fraction. For each sample, drops of suspended diatom material were prepared, and up to 50 fields in each drop were counted. These numbers were later converted into diatom cell per cm^2 and used to estimate the diatom community density (Veraart et al., 2008).

Diatom biovolume was also determined. A set of geometric shapes was selected for determining biovolume of diatoms as proposed by Hillebrand et al. (1999). A total of 25 randomly selected cells for each species were measured but this cannot be applied for rare species like *Aulacoseira ambigua* (Grunow) *Simonsen* or *Caloneis alpestris* (Grunow) Cleve so they were measured as they occurred. The linear dimensions were measured manually using a micrometer during the identification of the diatom cells. The cell volume of each species was computed by applying average dimensions for each species from each sample to the geometrical shape that most closely resembled the species. An elliptic prism shape was chosen for some pennate diatoms as those belonging to the genus *Achnanthidium* and a cylinder shape was chosen for some central diatoms as those belonging to the genus *Cyclotella*.

2.4. Phosphate uptake rate

P uptake rates (U) were calculated by measuring the decay of P throughout time after spiking. After the colonization period, a P spike was performed to each crystallizing dish in order to measure the uptake rate after each treatment. P concentration in each crystallizing dish was previously analyzed. Subsequently, KH_2PO_4 was spiked to each crystallizing dish in order to increase by two times the background P concentration. Samples for P concentration (10 ml) were then taken at 1, 5, 10, 15, 30, 45, 60, 90 and 120 min after spiking, and filtered through 0.2 μm Nylon Membrane filters (Whatman). After 120 min of exposure, the medium of each treatment was reestablished to its basal levels of P and As. The following experiments were performed after acclimatation of the periphyton to basal conditions (Rhee, 1972; Jansson, 1993; Hwang et al., 1998; Scinto and Reddy, 2003).

2.5. PICT

In order to assess periphyton's changes in sensitivity to As PICT, short-term toxicity tests were performed to the communities exposed to the different treatments after the colonization period. Photosynthetic efficiency was used as endpoint in these toxicity tests. Stock solution containing 1.10^{-1} M As ($NaH_2AsO_4.7H_2O$, Merck, Darmstadt, Germany) was prepared in Milli-Q water and logarithmic dilutions of the concentrations tested were prepared dissolving the stock solution in filtered dechlorinated tap water (3 blanks and 3 replicates for each of the 6 increasing concentrations). Final test concentrations ranged from 0 to 10^{-2} M. Periphyton from each crystallizing dish (1 cm^2 substrata) were exposed to 10 ml of increasing concentrations of As during 24 h under the same conditions of light and temperature as in their colonization period with no phosphate addition. Response measurements were performed with PhytoPAM fluorometer, using $_{M'}$ as endpoint. Effective concentrations inhibiting the photosynthetic efficiency were obtained from dose–response curves.

2.6. Statistical analysis

Changes in algal biomass (Fo) from the whole colonization period were fitted to a sigmoid curve (*1*) to characterize and compare growth among treatments. Data from each crystallizing dish corresponding to three repetitions were fitted together using SigmaPlot 11.0 and curve parameters were obtained from each treatment by triplicate.

$$y = \frac{a}{1 + e^{-\left[\frac{x - x_0}{b}\right]}} \tag{1}$$

The x_o parameter represents the time when maximal community growth is achieved. The *a* parameter represents the carrying capacity and the 1/*b* parameter represents the growth rate (1). Differences among the parameters for each treatment were tested with two-way ANOVA in order to test if changes in the growth of the communities were statistically significant. The factors were As, with three levels (*noAs*, *AsL* and *AsH*) and P, with two levels (*noP* and *P*). Also, post-hoc multiple comparisons were performed (Holm–Sidak method).

Two-way ANOVAs were applied to examine differences on each fluorescence parameter (Fo, $_M$, $_{M'}$,%F of each algal class) among treatments on weeks 3 and 4. Two-way ANOVAs were also applied to analyze the arsenic accumulation differences and the physical and chemical conditions among treatments.

Diatom samples were classified into groups using a hierarchical cluster analysis, group average method and Bray–Curtis similarity distance. Version 6 of the PRIMER-E statistical package was used (Clarke and Gorley, 2005). Differences in diatom species contribution to total biomass (in terms of biovolume) among treatments were tested by means of two-way ANOVAs.

P uptake rate (U, %P cm^{-2} h^{-1}) was calculated as the percentage of P removed from water column per area per time. Decay of P concentration over time in each treatment was fitted to an exponential decay curve using Sigmaplot 11.0. P uptake rate coefficient (B, min^{-1}) was the coefficient represented by the decay of P concentration in time. Abiotic controls were performed and showed no decay of P during the same experimental time and conditions. Data were transformed to ensure that distribution of the parameters was normal. Differences among treatments for U and B parameters were tested by two-way ANOVAs and post-hoc multiple comparisons.

Half maximal effective concentrations (EC50) were calculated for each treatment exposed to the gradient of concentrations in the dose–response test by triplicate. This concentration was calculated by fitting each replicate to a four parameter logistic curve using the SigmaPlot 11.0 software, obtaining the EC50 parameter in triplicate for each treatment. EC50 differences among treatments were tested by two-way ANOVA and post-hoc multiple comparisons.

3. Results

3.1. Physical and chemical characteristics

The physical and chemical characteristics of water of each treatment remained stable during the whole period of colonization and were similar among treatments (Table 1). Dissolved oxygen, temperature, pH and conductivity showed no significant differences among treatments in the two-way ANOVA. There was a statistical difference among As levels ($P < 0.001$). In *AsL* treatments, As concentration remained around 15 μgAs L^{-1} and in *AsH*, it remained approximately 136 μgAs L^{-1}. In *P* treatments, there was a statistical difference among treatments ($P < 0.001$). The PO_3^{-4} concentration remained around 100 μg L^{-1} and in *noP* treatments it remained below 30 μg L^{-1}.

At the last week of the colonization, As (V) was the only species of As found in all treatments except for *noPAsL*, that had a 21% of As(III).

3.2. Biomass accrual and growth curves

Two-way ANOVA of Fo made day by day show a clear effect of P on biomass accrual from week 2 onwards: $p < 0.001$, $F = 32.686$ for week 2; $p < 0.01$, $F = 10.964$ for week 3 and $p < 0.005$, $F = 8.416$ for week 4. The highest biomass was obtained by *P noAs* on day 21 (64% more growth than the control) and the lowest was obtained by *noP AsH* (68% lower than the control).

Growth parameters were obtained by fitting Fo temporal series to a sigmoidal model. In addition to P effects observed by the analysis of Fo changes reported above, growth parameters were also affected by As (Fig. 1, Table 2). Chronic exposure to As inhibited growth, and the effect was attenuated by the presence of phosphate. According to the two-way ANOVA, *P* treatments had higher Xo values, reaching half of its maximum growth up to 5 days before than the *noP* treatments (Table). *AsH* separates *P* from *noP* treatments, being *P AsH* the first reaching its maximum growth. Growth rate, represented by the reciprocal of *b*, was similar among treatments showing significant differences for *As* treatments (Table 2). Growth rate was only affected by As. The

Table 1
Mean and standard error (SE) of dissolved oxygen (Oxy.), water temperature (Temp.), pH, conductivity (Cond.), phosphate (PO_4^{3-}) and total dissolved arsenic (As_T) concentrations during the colonization for each treatment. P values were obtained by two Way ANOVA (NS = not significant). Upper case letters indicate difference between treatments. Treatments are different combinations of phosphate (PO_4^{3-}) and arsenic (As (V)). Concentrations used are 10 μg/L PO_4^{3-}, referred to as *NoP*; 100 μg/L PO_4^{3-}, referred to as *P*; 130 μg/L of As (V), referred to as *AsH* and 15 μg/L of As (V), referred to as *AsL*. Some treatments do not have As and are referred to as *NoAs*. The six treatments are referred to as *noPnoAs*, *noPAsL*, *noPAsH*, *PnoAs*, *PAsL*, *PAsH*.

	noP noAs			noP AsL			noP AsH			P noAs			P AsL			P AsH			
	MEAN	SE	N	MEAN	SE	n	MEAN	SE	n	MEAN	SE	N	MEAN	SE	n	MEAN	SE	N	2 W ANOVA
Oxy. (mg L^{-1})	8.24	0.07	20	8.25	0.07	20	8.21	0.06	20	8.50	0.14	20	8.51	0.14	20	8.05	0.13	20	NS
Temp. (ºC)	22.02	0.23	19	22.17	0.16	19	22.21	0.16	19	21.97	0.17	19	22.49	0.20	19	21.88	0.25	20	NS
pH	8.55	0.06	16	8.56	0.06	16	8.58	0.06	16	8.77	0.11	15	8.61	0.14	15	8.62	0.09	17	NS
Cond. (μS cm^{-1})	410.10	7.13	20	408.45	5.55	20	424.20	3.58	20	393.55	12.81	20	392.95	12.89	20	402.55	11.68	20	NS
PO_3^{-4} (μg L^{-1})	31.6^a	15.9	20	11.3^a	6.9	20	5.0^a	2.2	20	80.9^b	16.2	20	110.3^b	16.2	20	109.7^b	16.9	20	$P < 0.001$ (*P factor*)
As_T (μg L^{-1})	1.96^a	0.03	4	14.78^b	0.59	16	136.76^c	3.20	22	1.91^a	0.02	4	16.02^b	0.39	22	136.01^c	3.24	22	$P < 0.001$ (*As factor*)

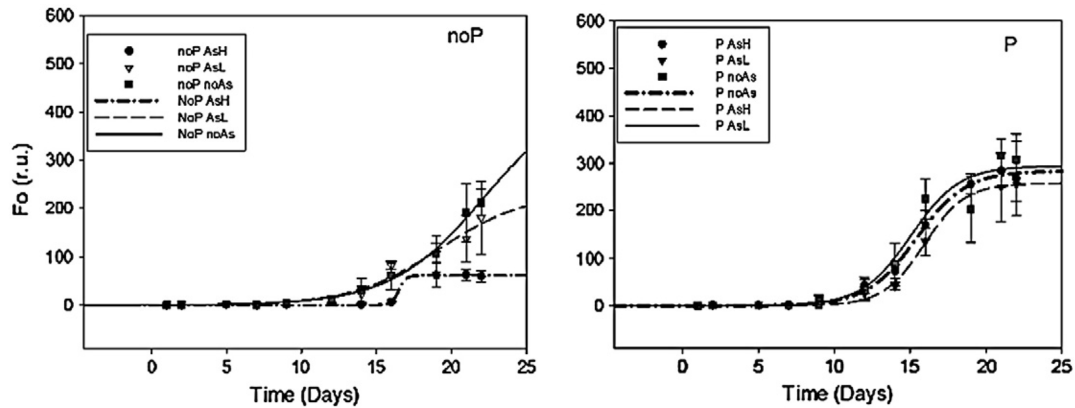

Fig. 1. Mean growth curves for each treatment with standard error (n = 3). The different lines show fitting of the treatments to the sigmoidal model. Treatments are different combinations of phosphate (PO_4^{3-}) and arsenic (As (V)). Concentrations used are 10 μg/L PO_4^{3-}, referred to as *NoP*; 100 μg/L PO_4^{3-}, referred to as *P*; 130 μg/L of As (V), referred to as *AsH* and 15 μg/L of As (V), referred to as *AsL*. Some treatments do not have As and are referred to as *NoAs*. The six treatments are referred to as *noPnoAs*, *noPAsL*, *noPAsH*, *PnoAs*, *PAsL*, *PAsH*. Units are expressed as random units of minimal fluorescence and time in days. Left graph shows *noP* treatments. Right graph shows *P* treatments.

maximum growth rate was reached by *P AsH*, being 42% higher than the control treatment, *noP noAs*.

The carrying capacity (*a*) was enhanced by P-addition. *P noAs* had the highest *a*, being 34% higher than the control treatment (Table 2), and *noP AsH* the lowest, 61% below the control treatment. Arsenic effects were also significant on this parameter and more marked in *noP* treatments, where *noP AsH* was separated from the other *noP* treatments (Fig. 2).

3.3. Photosynthesis parameters

Maximum quantum yield ($_{M}$) and effective quantum yield ($_{M'}$) were also affected by treatments. $_{M'}$ in P treatments was 50% higher than $_{M'}$ in *noP* treatments at weeks 3 and 4 (P < 0.001 in both cases). The parameter $_{M}$ at week 3 increased in P enriched treatments with no dependencies toward As (P = 0.013). On the other hand, $_{M}$ was also reduced by arsenic on week 4, mainly in the *noP* treatments with a significant interaction of the factors (p = 0.016 for As, P = 0.013 for the interaction, Table 2).

The percentage of fluorescence of cyanobacteria, diatoms and green algae groups, measured at week 4 of the experiment, was influenced by both treatments. Two-way ANOVA showed a significant interaction between P and As for diatoms' fluorescence, with a 17% decrease in fluorescence in two treatments with low phosphate: *AsH* and *noAs* (Table 2). This decrease was compensated by an increase in the percentage of green algae. The fluorescence of cyanobacterium did not change on week 4.

3.4. Total and intracellular arsenic concentrations

Total and intracellular As concentrations do not show significant differences in all samples. Intracellular As was higher in all *As* treatments compared to *noAs* treatments which had background As levels (Two-way ANOVA, P < 0.001 for As treatment, Fig. 3). Post-hoc comparison

Table 2
Mean and standard error of the growth curve parameters (Xo, a, 1/b), the fluorescence parameters (Φ_M is the maximum quantum yield, $\Phi_{M'}$ is the effective quantum yield), the percentage of algal fluorescence (%Bl is % of blue algae, %Gr is percentage of green algae, %Br is percentage of brown algae) and EC50 (effect concentration 50) expressed in mol L^{-1} for each treatment. Treatments are different combinations of phosphate (PO_4^{3-}) and arsenic (As (V)). Concentrations used are 10 μg/L PO_4^{3-}, referred to as *NoP*; 100 μg/L PO_4^{3-}, referred to as *P*; 130 μg/L of As (V), referred to as *AsH* and 15 μg/L of As (V), referred to as *AsL*. Some treatments do not have As and are referred to as *NoAs*. The six treatments are referred to as *noPnoAs*, *noPAsL*, *noPAsH*, *PnoAs*, *PAsL*, *PAsH*. P and F values for each treatment were obtained by two-way ANOVA. Upper case letters indicate difference among treatments. ° Indicates that the *AsH* factor separates groups with *P* from *noP*.

		Treatments						Two-way ANOVA		
Day		noP noAs	noP AsL	noP AsH	P noAs	P AsL	P AsH	P	As	P * As
Growth curves' parameters	Xo	*18.8 ^{a}* *±1.98*	*19.73 ^{a}* *±3.15*	*19.68 ^{a}* *±2.60*	*18.46 ^{a,b}* *±4.02*	*17.14 ^{a,b}* *±0.55*	*14.42 ^{b}* *±1.26*	*p = 0.042°* *F = 5.19*	*n.s.*	*n.s.*
	A	*229.8 ^{a}* *±54.11*	*272.74 ^{a}* *±122.97*	*88.68 ^{b}* *±32.50*	*307.97 ^{a}* *±34.30*	*265.69 ^{a}* *±67.60*	*242.43 ^{a}* *±34.78*	*p = 0.033°* *F = 5.82*	*p = 0.027* *F = 4.932*	*n.s.*
	1/b	*0.55* *±0.08*	*0.32* *±0.05*	*0.42* *±0.01*	*0.51* *±0.13*	*0.38* *±0.03*	*0.92* *±0.24*	*n.s.*	*p = 0.046* *F = 4.115*	*n.s.*
$_{M}$	*Week 3*	*0.64 ^{a,b}* ±0.01	*0.62 ^{a,b}* ±0.02	*0.61 ^{b}* ±0.02	*0.66 ^{a}* ±0.03	*0.66 ^{a}* ±0.01	*0.65 ^{a}* ±0.04	*p = 0.013°* F = 8.476	*n.s.*	*n.s.*
	Week 4	*0.66 ^{a}* ±0.01	*0.62 ^{b,c}* ±0.01	*0.61 ^{b}* ±0.01	*0.64 ^{a}* ±0.01	*0.63 ^{a,c}* ±0.01	*0.65 ^{a}* ±0.03	*n.s.*	p = 0.016 F = 5.89	p = 0.013 F = 6.35
$_{M'}$	*Week 3*	*0.38 ^{a}* *±0.01*	*0.32 ^{a}* *±0.01*	*0.24 ^{a}* *±0.03*	*0.47 ^{b}* *±0.01*	*0.47 ^{b}* *±0.01*	*0.462 ^{b}* *±0.01*	*P < 0.001* *F = 95.42*	*n.s.*	*n.s.*
	Week 4	*0.31 ^{a}* *±0.01*	*0.27 ^{a}* *±0.01*	*0.28 ^{a}* *±0.01*	*0.40 ^{b}* *±0.01*	*0.38 ^{b}* *±0.02*	*0.39 ^{b}* *±0.01*	*P < 0.001* *F = 102.67*	*n.s.*	*n.s.*
Algal fluorescence	*%blue*	39.25 ±2.29	*36.47* ±3.16	*36.93* ±0.83	*41.53* ±2.01	*37.42* ±1.51	*39.16* ±1.55	*n.s*	*n.s*	*n.s.*
	%green	37.02 ^{a} ±8.40	32.18 ^{a} ±5.15	44.20 ^{a} ±8.95	18.61 ^{b} ±3.79	36.30 ^{a,b} ±5.43	24.88 ^{b} ±4.86	*P = 0.016* *F = 6.28*	*n.s.*	*n.s.*
	%brown	23.73 ^{a} ±6.22	31.35 ^{a} ±3.75	18.86 ^{a} ±8.12	40.38 ^{b} ±2.22	26.28 ^{a,b} ±4.15	35.96 ^{b} ±6.18	*P = 0.017* *F = 6.15*	*n.s.*	*P = 0.035* *F = 3.60*
EC50 (M)	[As(V)] (mol L^{-1})	*8.11*10^{-6} ^{a}* *±1.8*10^{-6}*	*9.84*10^{-6} ^{a}* *±5.5*10^{-6}*	*20.9*10^{-6} ^{a}* *±6.3*10^{-6}*	*11.1*10^{-6} ^{a}* *±1.4*10^{-6}*	*398*10^{-6} ^{b}* *±200*10^{-6}*	*933*10^{-6} ^{b}* *±84*10^{-6}*	*p < 0.001* *F = 92.304*	*p < 0.001* *F = 34.70*	*p < 0.001* *F = 17.92*

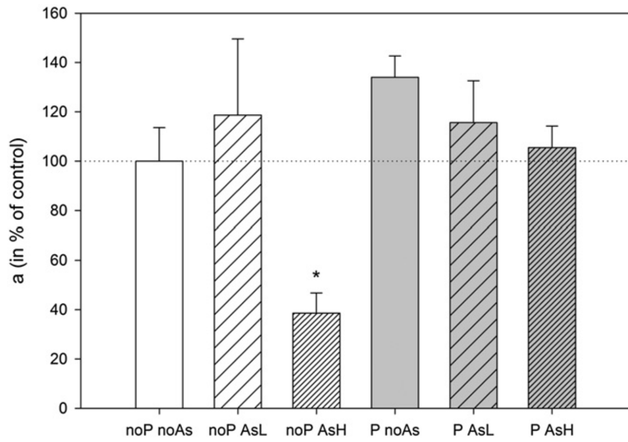

Fig. 2. Relative carrying capacity (a) of each treatment obtained from the fitting to a sigmoidal model. Treatments are different combinations of phosphate (PO_4^{3-}) and arsenic (As (V)). Concentrations used are 10 μg/L PO_4^{3-}, referred to as *NoP*; 100 μg/L PO_4^{3-}, referred to as *P*; 130 μg/L of As (V), referred to as *AsH* and 15 μg/L of As (V), referred to as *AsL*. Some treatments do not have As and are referred to as *NoAs*. The six treatments are referred to as *noPnoAs*, *noPAsL*, *noPAsH*, *PnoAs*, *PAsL*, *PAsH*. Bars indicate standard error. * shows significant differences from the control treatment (two-way ANOVA).

showed that accumulated As followed the expected pattern, increasing in *AsL* treatments and reaching the highest values in *AsH* treatments (Holm–Sidak method, $P < 0.001$ for noAs vs. AsL, $P < 0.001$ for AsL vs. AsH, $P < 0.001$ for AsH vs. noAs, Fig. 3). There was also a significant difference in As intracellular concentration between *P* and *noP* treatments (two-way ANOVA, $P = 0.001$). P enriched treatments showed lower intracellular accumulation. *P AsH* communities had 53% less intracellular As than *noP AsH*.

3.5. Diatom community

A total of 85 diatom species were found in samples from all treatments (Table 3). Diatom community in P treatments was dominated by *Mayamaea permitis* (Hustedt) Lange-Bertalot (3–50%), while in *noP* treatments it was dominated by *Achnanthidium minutissimum* Kützing (20–70%).

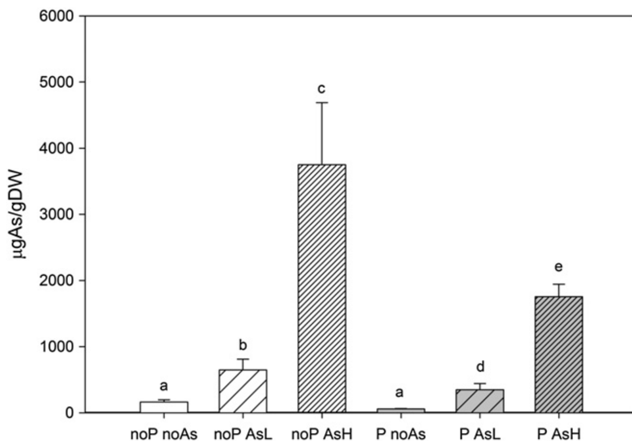

Fig. 3. Mean and standard error of intracellular contents of arsenic in periphyton biomass (g of dry weight) for the different treatments (two-way ANOVA $p = 0.001$ for P, $p < 0.001$ for As.). Higher case letters show differences among treatments in the two-way ANOVA. Treatments are different combinations of phosphate (PO_4^{3-}) and arsenic (As (V)). Concentrations used are 10 μg/L PO_4^{3-}, referred to as *NoP*; 100 μg/L PO_4^{3-}, referred to as *P*; 130 μg/L of As (V), referred to as *AsH* and 15 μg/L of As (V), referred to as *AsL*. Some treatments do not have As and are referred to as *NoAs*. The six treatments are referred to as *noPnoAs*, *noPAsL*, *noPAsH*, *PnoAs*, *PAsL*, *PAsH*.

Phosphate enrichment caused a clear separation among diatom communities (Fig. 4). Cluster analysis shows that communities can be separated by P regime, but no similarity was observed within As treatments.

Two-way ANOVA showed 11 species affected by the treatments: 5 species with higher contribution to total biomass (in terms of biovolume) in *P* treatments, 5 with higher biovolume in *noP* treatments and 2 with lower biovolume in the *AsH* treatments (Table 4).

3.6. Phosphate uptake

As and P influenced P uptake. Uptake rate (U, %P cm^{-2} h^{-1}) was 2 times higher in *noP* treatments than in P treatments (two-way ANOVA, $P < 0.001$) and lower with As than without (two-way ANOVA, $P = 0.032$. Table 1). Moreover, As effects have a tendency to be more evident without P. When U is calculated in relation to biomass instead of the area (%P $mgDW^{-1}$ h^{-1}), differences among treatments were attributed to P. Uptake rate coefficient (B) was affected by both treatments. It was one order of magnitude higher in no P treatments than

Table 3
List of the 85 diatom taxa found in all samples after colonization including rare species.

Achnanthes curvirostrum Brun
Achnanthes imperfecta Schimanski
Achnanthes lemmermannii Hustedt
Achnanthidium minutissimum (Kützing) Czarnecki
Achnanthidium pyrenaicum (Hustedt) H. Kobayasi
Adlafia minuscula var. *muralis* (Grunow) Lange-Bertalot
Amphora inariensis Krammer
Amphora libyca Ehrenberg
Amphora ovalis (Kützing) Kützing
Amphora pediculus (Kützing) Grunow ex A. Schmidt
Aulacoseira ambigua (Grunow) Simonsen
Caloneis alpestris (Grunow) Cleve
Caloneis bacillum (Grunow) Cleve
Cocconeis pediculus Ehrenberg
Cocconeis placentula Ehrenberg
Cocconeis placentula var. *lineata* (Ehrenberg) Van Heurck
Cyclotella atomus Hustedt
Cyclotella meneghiniana Kützing
Cymbella affinis Kützing
Cymbella cymbiformis C. Agardh
Cymbella lanceolata (C. Agardh) C. Agardh
Denticula tenuis Kützing
Diploneis modica Hustedt
Diploneis oblongella (Nägeli ex Kützing) Cleve-Euler
Diploneis ovalis (Hilse) Cleve
Encyonema minutum (Hilse) D.G. Mann
Encyonema prostratum (Berkeley) Kützing
Encyonema silesiacum (Bleisch) D.G. Mann
Encyonopsis microcephala (Grunow) Krammer
Epithemia adnata (Kützing) Brebisson
Fallacia lenzii (Hustedt) Lange-Bertalot
Fallacia pygmaea (Kützing) A.J. Stickle & D.G. Mann
Fistulifera saprophila (Lange-Bertalot & Bonik) Lange-Bertalot
Fragilaria capucina Desmazières
Fragilaria capucina Desmazières var.*gracilis* (Oestrup) Hustedt
Fragilaria capucina var. *vaucheriae* (Kützing) Lange-Bertalot
Geissleria decussis (Østrup) Lange-Bertalot & Metzeltin
Gomphonema angustatum (Kützing) Rabenhorst
Gomphonema angustum C. Agardh
Gomphonema lateripunctatum Reichardt & Lange-Bertalot
Gomphonema minutum (C. Agardh) C. Agardh
Gomphonema parvulum (Kützing) Kützing
Gomphonema rhombicum Fricke
Grunowia tabellaria (Grunow) Rabenhorst
Gyrosigma acuminatum (Kützing) Rabenhorst
Gyrosigma nodiferum (Grunow) Reimer
Halamphora coffeaeformis (C. Agardh) Levkov
Halamphora montana (Krasske) Levkov
Halamphora veneta (Kützing) Levkov
Kolbesia ploenensis (Hustedt) J.C. Kingston

(continued on next page)

Table 3 (*continued*)

Lemnicola hungarica (Grunow) F.E. Round & P.W. Basson
Mayamaea permitis (Hustedt) K. Bruder & L.K. Medlin
Melosira varians C. Agardh
Navicula antonii Lange-Bertalot
Navicula capitatoradiata Germain
Navicula cari Ehrenberg
Navicula cryptocephala var. *veneta* (Kützing) Rabenhorst
Navicula cryptotenella Lange-Bertalot
Navicula gregaria Donkin
Navicula meniscus Schumann
Navicula reichardtiana Lange-Bertalot
Navicula schroeteri Meister
Navicula subminuscula Manguin
Navicula tripunctata (O. F. Müller) Bory de Saint-Vincent
Navicula viridula var. *rostellata* (Kützing) Cleve
Nitzschia alpina Hustedt
Nitzschia amphibia Grunow
Nitzschia dissipata (Kützing) Grunow
Nitzschia fonticola Grunow
Nitzschia frustulum (Kützing) Grunow
Nitzschia gessneri Hustedt
Nitzschia heufleriana Grunow
Nitzschia inconspicua Grunow
Nitzschia linearis W.M. Smith
Nitzschia microcephala Grunow
Nitzschia palea (Kützing) W.M. Smith
Nitzschia palea var. *major* Rabenhorst
Planothidium delicatulum (Kützing) Round & Bukhtiyarova
Planothidium frequentissimum (Lange-Bertalot) Round & L. Bukhtiyarova
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot
Rhoicosphenia abbreviata (C. Agardh) Lange-Bertalot
Sellaphora seminulum (Grunow) D.G. Mann
Staurosirella pinnata (Ehrenberg) D.M. Williams & Round
Surirella brebissonii var. *kuetzingii* Krammer & Lange-Bertalot
Ulnaria ulna (Nitzsch) P. Compère

in *P* treatments and lower with As, showing a higher affinity for P in no P treatments and a lower affinity for P in As treatments (Table 5).

3.7. PICT

$_{M'}$ showed a good response as endpoint in the dose–response tests. As and P conditions during growth influenced community tolerance to As, and the effect of different levels of As depended on what level of P was present (Table 2, Fig. 5). Communities from P *AsH* and P *AsL* treatments had higher EC50s in acute expositions than the control (*noP noAs*). The most tolerant community was *P AsH*, with a EC50 of 9.33 10^{-4} M, two orders of magnitude higher than the control, which had the lowest EC50. Communities enriched with P but not exposed to As during growth showed similar EC50 values than the controls.

4. Discussion

4.1. Arsenate generates toxicity to the periphyton community

Arsenate affected structural and functional parameters of periphyton communities starved of phosphate, as our hypothesis stated. Chronic exposure to 130 μgAs/L, a concentration commonly found in fluvial systems naturally enriched with arsenic (Rosso et al., 2011), inhibited algal growth up to 61%, compared to non-exposed communities and changed diatom sizes. It also caused changes in the community composition with a reduction in the relative abundance of diatoms and some diatom species (*Amphora pediculus*) and community adaptation. The decrease in diatom biomass concurs with observations from Wängberg et al. (1991) in marine phytoplankton and with Tlili et al. (2011) in freshwater periphyton.

Chronic exposure to 15 μg/L As (*AsL* treatment), concentration close to drinking guidelines, had a subtle effect on periphyton, which was not reflected in the structural or physiological variables measured. This differs from studies regarding marine periphyton (Blanck and Wängberg, 1991; Eisler, 1994), where a clear sensitivity to low concentrations of As was observed, indicating that sensitivity toward arsenate is different in these environments. However, a clear tolerance induction was observed in this treatment in the PICT experiment with results similar to AsH suggesting that this low dose may trigger community adaptation with no detectable changes in most of the structural or physiological variables measured. It is in agreement with Tlili et al. (2011) observations showing community adaptation after exposure to 16 μg L^{-1}.

The observed growth inhibition concurred with a marked reduction on the ability of the community to retain P as observed in the uptake rate parameter U, but also on the affinity of the community toward P (B parameter). Uptake rate is usually negatively related to the size of the internal P pool (Harrison et al., 1989) and surface adsorption (Wilhelmy et al., 2004; Yao et al., 2011). Even though the fraction of As that enters the cell is supposed to be low compared to the internal P pool (according to the affinity of both molecules to the transporters), the presence of As may affect this relation. Increases in arsenic have been shown to decrease phosphate uptake in five freshwater algae (Planas and Healey, 1978), but it may not occur in other species having a specific phosphate transport systems (*Synechoccocus*, Budd and Craig, 1981). The decrease of phosphate uptake by the periphyton due to chronic As pollution may lead to changes in stream ecosystems, as these organisms play a key role in nutrient cycling through nutrient uptake (Von Schiller et al., 2007).

Chronic exposure to 130 μgAs/L leads to As bioaccumulation in the periphyton up to 3750 μgAs/gDW. Arsenic accumulation is maximum in lower trophic levels (Maeda et al., 1992a; Kuroiwa et al., 1994). Compared to water concentrations, arsenic concentrations one order of magnitude higher are found in shrimps and fishes (Maeda et al., 1992b; Shah et al., 2009) and three orders of magnitude higher (Concentration Factors of 10^3) are commonly reported in sediments (e.g. Aloupi et al., 2009). In freshwater algae and macrophytes CFs range between 10^2 and 10^4 (Chen et al., 2000; Kuwabara et al., 2003; Robinson and Ayotte, 2006; Schaeffer et al., 2006; Favas et al., 2012). In our study, periphyton samples showed CFs ranging between 13,000 and 65,000.

4.2. Phosphate influences toxicity and modulates the response of the community

Our experimental settings allowed simulating the conditions of P limitation and no P limitation. As expected, P-limitation effects were very clear and of higher magnitude than the effects caused by As. Periphyton communities growing under both treatments differed in their P-uptake rate, being 5 times more efficient in the no P treatment, thus demonstrating that P was limiting (Riegman and Mur, 1984; Yao

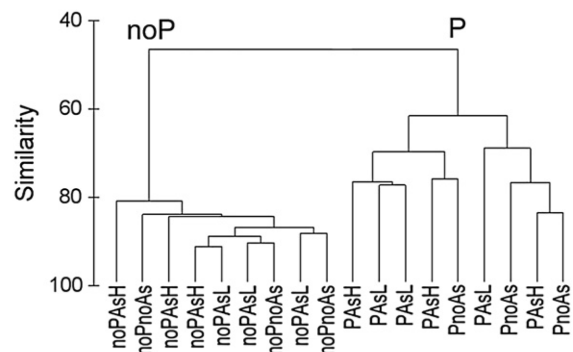

Fig. 4. Cluster representation of replicates of the different treatments with similarity (%) analyses among groups of diatoms. Treatments are different combinations of phosphate (PO_4^{3-}) and arsenic (As (V)). Concentrations used are 10 μg/L PO_4^{3-}, referred to as *NoP*; 100 μg/L PO_4^{3-}, referred to as *P*; 130 μg/L of As (V), referred to as *AsH* and 15 μg/L of As (V), referred to as *AsL*. Some treatments do not have As and are referred to as *NoAs*. The six treatments are referred to as *noPnoAs*, *noPAsL*, *noPAsH*, *PnoAs*, *PAsL*, *PAsH*.

Table 4
Mean and standard error (n = 3) of biovolumes (μm^3/cm^2) of diatom species showing significant responses to P or As treatments. P and F values for each treatment were obtained by two-way ANOVAs. Treatments are different combinations of phosphate (PO_4^{3-}) and arsenic (As (V)). Concentrations used are 10 μg/L PO_4^{3-}, referred to as *NoP*; 100 μg/L PO_4^{3-}, referred to as *P*; 130 μg/L of As (V), referred to as *AsH* and 15 μg/L of As (V), referred to as *AsL*. Some treatments do not have As and are referred to as *NoAs*. The six treatments are referred to as *noPnoAs*, *noPAsL*, *noPAsH*, *PnoAs*, *PAsL*, *PAsH*.

Diatom taxa	Treatment						Two-way ANOVA		
	noPnoAs	noP AsL	noP AsH	P noAs	P AsL	P AsH	P	As	P * As
Achnanthidium minutissimum (Kützing)	17607	18047	20645	6132	6313	7433	P < 0.001	n.s.	n.s.
	±684	±277	±1561	±479	±2437	±2376	F = 89,6		
Encyonopsis microcephala (Grunow) Krammer	857	1045	777	0.00	304	27	P = 0.018	n.s.	n.s.
	±687	±395	±444	±0,00	±152	±27	F = 7,52		
Diploneis modica (Hustedt)	2022	755	434	0.00	0.00	54.5	P < 0.001	P = 0.023	P = 0.017
	±0.00	±435	±434	±0.00	±0.00	±54.5	F = 26.1	F = 5.28	F = 5.83
Fragilaria capucina Desmazières	1160	243	835	0.00	143	326	P = 0.041	n.s.	n.s.
	±614	±121	±276	±0.00	±143	±326	F = 5.25		
Gomphonema lateripunctatum Reichardt & Lange-Bertalot	7257	6047	0.00	0.00	0.00	0.00	P = 0.002	n.s.	n.s.
	±2094	±4361	±0.00	±0.00	±0.00	±0.00	F = 7.56		
Mayamaea permitis (Hustedt) Lange-Bertalot	111	78	269	1467	758	1473	P = 0.001	n.s.	n.s.
	±46	±43	±144	±297	±96	±673	F = 18.2		
Planothidium frequentissimum (Lange-Bertalot) Round & Bukhtiyarova	96	0.00	396	724	500	1267	P = 0.006	n.s.	n.s.
	±96	±0.00	±219	±209	±400	±318	F = 11.0		
Nitzschia palea (Kützing) W.M. Smith	986	708	1169	5994	6293	4146	P = 0.007	n.s.	n.s.
	±190	±563	±870	±2062	±2901	±1874	F = 10.6		
Nitzschia amphibia Grunow	734	904	1157	3471	911	1036	P = 0.024	n.s.	n.s.
	±183	±407	±579	±1204	±260	±308	F = 6.67		
Halamphora veneta (Kützing) Levkov	0.00	0.00	0.00	206	299	662	P = 0.012	n.s.	n.s.
	±0.00	±0.00	±0.00	±266	±299	±191	F = 8.09		
Amphora pediculus (Kützing) Grunow	1728	2145	1217	1946	2149	994	n.s.	P < 0.031	n.s.
	±451	±107	±245	±498	±451	±88	F = 4.68		

et al., 2011). In addition, P limited communities had lower photosynthetic efficiency ($_{M'}$) than those non-limited leading to lower growth and the development of a diverse but different periphyton community dominated by *A. minutissimum* in the *noP* treatment, and by *M. permitis* and *Nitzschia palea* in the *P* treatment. *A. minutissimum* has been classified as sensitive to organic load and nutrient concentration in the specific pollution index (SPI, CEMAGREF, 1982). *N. palea* and *M. permitis* have been linked to waters with high nutrient loads (Van Dam et al., 1994) and are considered a pollution tolerant species (SPI, CEMAGREF, 1982).

While arsenic toxicity was observed in most endpoints measured, the effects differed between *P* treatments leading to a significant interaction among treatments in the two-way ANOVA. Effects of arsenic on the photosynthetic capacity ($_{M}$) were only detected in the *noP* treatment, showing that chronic exposure to arsenate led to changes in the photosynthetic apparatus under conditions of P-limitation, but not when P-availability was higher. This fact reveals a lower toxicity and/or a higher adaptation of the P-amended community. Changes in community composition linked with a recovery of $_{M}$ after chronic exposure are commonly observed when communities adapt to toxic exposure (Corcoll et al., 2012). This observation is in agreement with the acute toxicity results showing a clear increase in community tolerance (from 11 up to 933 μM) in the *P* treatments.

In spite of the fact that arsenic concentration was similar among treatments, intracellular arsenic contents were higher in the *noP* treatments. This difference, observed after chronic exposure, could be explained by lower uptake, higher excretion or both. Wang et al. (2013) found that, under low phosphorus conditions, arsenate toxicity was aggravated because there was little phosphate to compete with arsenate extracellularly for the transport systems and, at the same time, more transporters were synthesized in order to alleviate P-limitation, leading to higher bioaccumulation.

On the other hand, the high P-uptake rates measured in *noP* communities indicate that their cells may have increased the number of transporters. The lower concentration of arsenic in *P* treatments could also be reached by active excretion, a detoxification mechanism acquired after chronic exposure, thus the lower toxicity being related with the acquisition of community tolerance. This mechanism was suggested by Levy (2005), described previously in the models of Cullen et al. (1994) and Rosen (1999) where arsenic is taken by algal cells using a

Table 5
Mean, standard error and number of samples of the different P uptake parameters. Uptake rate (U) is expressed in percentage of uptake per hour per surface (cm^2) and by dry weight (mgDW). Uptake rate coefficient (B) is in min^{-1}. Higher case letters show differences among treatments in the two-way ANOVA. *n.s.* means not significant (p > 0.05).

	U (%P cm^{-2} h^{-1})			U (%P mgDW^{-1} h^{-1})			B (min^{-1})		
	MEAN	SE	*n*	MEAN	SE	*n*	MEAN	SE	*n*
noPnoAs	0.553^a	0.047	3	2.036	0.468	3	0.014^a	0.001	3
noPAsL	0.440^b	0.029	3	1.778	0.503	3	0.013^b	0.002	3
noPAsH	0.428^b	0.020	3	2.160	0.230	3	0.014^{a,b}	0.003	3
PnoAs	0.275^c	0.026	3	0.623	0.140	3	0.005^c	0.000	3
PAsL	0.256^c	0.020	3	0.630	0.079	3	0.004^c	0.001	3
PAsH	0.217^c	0.018	3	0.552	0.110	3	0.004^c	0.000	3

Two-way ANOVA	P	F	P	F	P	F
P	<0.001	78.1	<0.001	22.78	<0.001	88.707
As	0.032	4.63	n.s.		0.019	5.5900
*P*As*	n.s.		n.s.		n.s.	

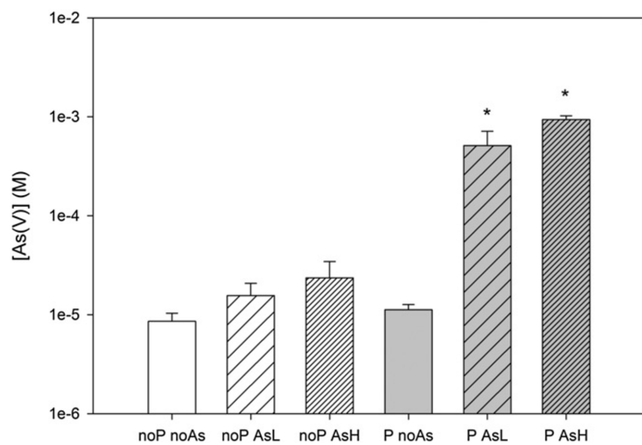

Fig. 5. Mean and standard errors of EC50 values for each treatment exposed to a gradient of As(V) concentrations in a dose–response test. This concentration was calculated by fitting each replicate to a four parameter logistic curve. * shows significant differences toward the control treatment (*noP noAs*) ($p < 0.05$).

phosphate transport system, reduced to As III in the cell and then excreted by and active transport system, using energy from an existing ion gradient or via an ATP-coupled pump.

It is also important to point out that arsenic tolerance was only induced by the combination of arsenate and phosphate but not by phosphate or arsenate alone. Community tolerance was low and similar in P treatments with no arsenic, indicating that phosphorus pre-exposure was not enough to cause an increase in the tolerance of the community, thus there was no co-tolerance between phosphate and arsenate. An increase in community tolerance due to competition between phosphate and arsenate for uptake was not expected since short-term tests were performed in a media with no phosphate, and the duration of the test was long enough for cells coming from P-media to reduce their intracellular P-levels. However a lower uptake-rate of arsenic and hence a lower toxicity was expected in the high-P community. In spite of a lower uptake rate, intracellular arsenic levels and, also, the As/P ratio reached similar levels thus causing similar effects.

While community tolerance was expected after chronic exposure in both P treatments, it is also true that the cost and mechanisms of adaptation are very complex depending, among other factors, on the community and mode of action of the toxic compound investigated (Amiard-Triquet et al., 2011). Our results suggest that the pressure exerted by chronic arsenic exposure selected species with a better capacity to regulate intracellular arsenic concentration by means of energy-dependent active transport systems and that this selection did not occur under P-limiting conditions.

Arsenic exposure reduced the P-uptake capacity in the P-limited community but not in the high-P one. This can be observed in the uptake rate coefficient B and in the uptake rate U, expressed per surface (%P cm^{-2} h^{-1}). It could be considered a protection mechanism since lower P-uptake will lead to lower arsenic uptake, thus allowing the community to cope with exposure by reducing growth. It is also possible that these communities responded to arsenic exposure by adjusting their P-uptake and hence reducing their growth rate but were not able to adapt by means of energy-dependent active transport systems due to P-depletion. Phosphate uptake was much lower in the high-P treatments and was not affected by arsenic supporting the point that community adaptation was following the mechanisms described above.

On the other hand, a clear effect of P-availability on arsenic toxicity was also demonstrated. Periphyton communities were less affected by arsenic under high P-conditions and this difference is attributed to lower arsenic uptake but also to the acquisition of a P-dependent detoxification mechanism and the health status of the community, which is reflected in the increase in biomass and the changes in diatom sizes in *P* communities.

Concerning speciation, arsenic concentration was mainly arsenate. It is in agreement with the results of Wang et al. (2013) who found that arsenite was the minor species and that arsenate played the dominant role, causing toxicity by its disturbance on phosphate metabolism thus being exacerbated under low-P conditions. The fact that *noP AsL* treatment has more arsenite than other treatments could be due to a detoxification mechanism. This detoxification strategy can be inhibited in metabolically damaged communities exposed to high As levels, preventing reduction–excretion pathway.

5. Conclusions

Overall, our results demonstrate that chronic exposure of natural communities to environmentally realistic arsenic concentrations will damage periphyton communities affecting a key ecosystem process, nutrient uptake. The fact that P-enrichment reduces P-uptake capacity is generally accepted, losing the depuration ability of the periphyton, a very important ecosystem function (Proia and Osorio, 2013; Guasch et al. subm.). Two of the parameters relative to P uptake present in our study indicate that chronic arsenic pollution will also contribute to lose it, an observation not reported before for these molecules. In addition, we have also shown that arsenic retention is reduced under high-P conditions. We can anticipate that periphyton communities in streams with high phosphate and arsenic concentration will not be very retentive, thus exporting both solutes downstream. Also, we can expect changes in diatom sizes in these communities. This study brings new arguments for the use of real measurements. However, since our hypotheses are based on microcosm experiments, field investigations will be required to support our experimental observations (Guasch et al., 2012).

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