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Evaluation of native acidophilic algae species as potential indicators of polycyclic aromatic hydrocarbon (PAH) soil contamination

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are pollutants that are potentially carcinogenic, are widely distributed in the environment, and accumulate in soils. The peroxydisulfate anion strategy for the remediation of PAH-contaminated soils has attracted widespread interest, despite its negative effects on soil microbial activity as a result of oxidative stress and a decrease in pH of the soil caused by the treatment. The acidification caused by the process can itself affect the growth of the normal flora, regardless of the presence of PAHs. For this reason, it is necessary to identify microorganisms that are capable of developing in acidic environments and are sensitive to the presence of PAHs. The objective of the present study was to identify native acidophilic/acid-tolerant algae isolated from the Agrio River-Lake Caviahue system, Argentina, that could possibly be used as bioindicators of soil PAH contamination. Two of the three acidophilic species assayed were identified as potential bioindicator species. *Cyanidium caldarium* and *Euglena mutabilis* were responsive to PAH contamination in the tested soils, while the response of *Keratococcus rhaphidioides* was dependent on the type of soil. The use of acidophilic and cosmopolitan species, such as *C. caldarium* and *E. mutabilis*, as bioindicators is a promising first step for assays of PAH contamination in soils.

Keywords PAH · *Cyanidium* · *Euglena* · *Keratococcus* · Phenanthrene · Bioindicator

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants that can enter the environment through the incomplete combustion of organic matter (such as wood) and fossil fuels (such as oil and coal). PAHs are hydrophobic compounds that are slightly soluble in water and have a high bioconcentration factor. PAHs have proven toxic, mutagenic, and carcinogenic properties (Moretto et al. 2005; Técher et al. 2012; González-Paredes et al. 2013). These features are related to their molecule structure, and the risk associated with compounds increases as the molecular weight increases (IARC 1998; Upham et al. 1998). An increase in molecule size is associated with an increase in hydrophobicity and electrochemical stability, two main factors that contribute to the persistence of PAHs in the environment (Cerniglia 1992; Kanaly and Harayama 2000).

It has been estimated that over 90 % of total PAHs released to the environment accumulate in soil (Wild and Jones 1995). Remediation strategies for contaminated sites have become a thoroughly investigated research field (Aprill and Sims 1990; Baud-Grasset et al. 1993; Bennett 1995; Gerhardt et al. 2009; Germida et al. 2002). In situ chemical oxidation processes consist of the injection of oxidants into both soils and superficial or groundwater of the contaminated area. Among in situ remediation techniques, the use of peroxydisulfate (PS) anion has attracted great interest, since this compound is stable enough not to react with the organic matter of the soil and is not significantly involved in sorption reactions; thus, it can persist for weeks in underground layers. It can be injected in high concentrations, transported through porous media, and is capable of being moved by diffusion or density difference toward low permeability materials (Huling and Pivetz 2006). PS treatment produces sulfate ions and H^+ as final products (Maurino et al. 1997). Sulfate is practically inert, and it is not considered a pollutant. The increase in H^+ concentration causes acidification of the environment (Huang et al. 2005; Liang et al. 2004), leading to a decrease in pH values as the concentration of persulfate increases (Tsitonaki et al. 2008).

Treatments with high PS concentrations have negative effects on the microbial activity of the system. The treatment itself exposes microorganisms to oxidative stress (Tsitonaki et al. 2008); however, the persistence of PS is limited (Johnson et al. 2008), and its concentration is depleted over time until no PS remains; thus, the effect is temporary. The treatment is also associated with a decrease in pH, which has a direct impact on microbial growth if the buffering capacity of the system is inadequate. This acidification persists even after the oxidative treatment is finished.

Given the toxicity of PAHs, microorganisms that exist naturally in the studied environment may be useful as biological indicators of degradation treatment efficiency. If the process is effective, it is expected that PAHs will be degraded to non-toxic levels, thereby enabling microorganisms to develop as they would in a non-contaminated site. Conversely, if the treatment is not effective, the presence of residual PAHs will result in the reduction of the natural microorganism population. With PS treatments, the acidification caused by the process can itself affect the growth of the normal flora, regardless of the presence of PAHs. For this reason, it is necessary to identify microorganisms that are capable of developing in acidic environments and that are also sensitive to PAH toxicity.

Phenanthrene has been used as a model compound for the study of the biodegradation of PAHs because (i) it is found in high concentrations in PAH-contaminated environmental samples; (ii) many PAHs containing a phenanthrene moiety are carcinogenic; and (iii) the regiospecificity and stereoselectivity of oxygenases can be determined in metabolic studies because phenanthrene is the smallest PAH to have both a "bay-region" and a "K-region" (Bezalel et al. 1996).

The Agrio River-Lake Caviahue system is a naturally acidified system located in Copahue-Caviahue Provincial Park (37° 53 S; 71° 02 W), in the Andean area of province of Neuquén, Argentina. The sources of the river are located near the crater of the active volcano Copahue; the acidic fluids of Copahue are responsible for the extreme acidity of the river (pH <2, Baffico et al. 2004) and of the lake that it is fed by the river (pH <3, Pedrozo et al. 2001). Acidophilic or acid-tolerant algae have been observed in the system (Baffico et al. 2004; Pedrozo et al. 2001), and they are potential candidates for bioindicators in acidic conditions. *Cyanidium caldarium* (Tilden) Geitler (Rhodophyta) and *Euglena mutabilis* Schmitz (Euglenophyta), both present in the Agrio River, have frequently been reported to inhabit extremely acidic and often metal-polluted sites (Nakatsu and Hutchinson 1988; Toplin et al. 2008). The effects of acidity and metal tolerance have been studied for both species (Nagasaka et al. 2004; Olaveson and Nalewajko 2000). *Keratococcus rhaphidiodes* (Hansgirg) Pascher (Chlorophyta) is the main species present in Lake Caviahue and has not been as widely studied as the above species (Beamud et al. 2007, 2010).

The objective of the present study was to search for native acidophilic/acid-tolerant algae in the Agrio River-Lake Caviahue system that are suitable for use as bioindicators of soil PAH contamination.

Materials and methods

Soil sampling and processing

Soil samples were taken from the littoral zone of Lake Caviahue at two sampling points (soil 1, 37° 51′48.1″ S, 71° 02′ 34.1″ W and soil 2, 37° 53′ 37.1″ S, 71° 01′ 28.6″ W) with a shovel and were bagged until further processing at the laboratory.

Soil humidity was determined as follows: 5 g of each soil sample was weighed and placed in a watch glass in the oven at 105 (±5)°C. Each sample was weighed until a constant weight was reached. Soil pH and electrical conductivity (EC) were measured according to EPA methods 9045D and 9050A, respectively: In a 50-mL glass container, 5 g of the soil sample was mixed with 25 mL of ultrapure water; the solution was then shaken for 5 min, and the soil pH and EC were measured.

Soil samples were dried in a furnace at 60 °C. A fraction of each sample was intended for texture analysis, which was performed using a set of sieves (Standard Series Sieves, USA), of three different mesh sizes: 2, 1, and 0.25 mm. The following fractions were determined according to the Wentworth scale, modified by Friedman et al. (1992): coarse/thick sand (<2 mm), medium sand (2–1 mm), fine sand (1–0.25 mm), and very fine sand (0.25–0.05 mm). The material obtained after sieving was used to determine the fractions of silt (0.05–0.002 mm) and clay (>0.002 mm) by the densimeter method (Forsythe 1985). On the remaining dried and sieved soil samples, total phosphorus (TP), total carbon (TC), and total nitrogen (TN) were measured. For the measurement of TP, a portion of sediment was digested with sulfuric acid and 30 % hydrogen peroxide (Carter 1993), and after digestion, the dissolved P content was determined according to Murphy and Riley (1962). TC and TN were determined using an automatic analyzer Thermo FlashEA 1112 (Thermo Fisher Scientific, USA).

Both soils, sieved through 2-mm mesh, were placed, separately, in a glass container. They were contaminated with 3,337±70 mg of phenanthrene kg^{-1} of dry soil. Phenanthrene was delivered in an acetone solution and mixed manually into the soil with a spatula, in accordance with the methods of Kulik et al. (2006). The organic solvent was removed by evaporation before inoculation.

Organisms and culture

Algae species were collected from the Agrio River and Lake Caviahue and were isolated and cultivated in the laboratory. *C. caldarium* and *E. mutabilis* were grown in a Satake medium (Satake and Saijo 1974), while *Keratococcus rhaphidioides* was grown in an "A" medium (modified from Hill 1980). Monospecific cultures were maintained in Erlenmeyer flasks at a pH of 3 and a constant temperature of 20 °C under continuous light with cool-white fluorescent illumination of 150 μmol photons m^2 s^{-1}.

Experiment

For three algae species and two soil samples, control and treatment triplicates were incubated for 20 days under the lab conditions indicated above in 50-mL angled-neck flasks (Nunclon, Denmark). Control flasks contained 0.5 g of soil, 20 mL of filtered Lake Caviahue water (0.22-μm pore size, cellulose acetate membrane filters, Schleider and Schuell, Germany), and 5 mL of algal inoculum. Treatment flasks contained 0.5 g of phenanthrene- contaminated soil, 20 mL of filtered lake water, and 5 mL of algal inoculum. At the end of the incubation, each treatment was filtered by glass microfiber filters (MG-F, Munktell, USA), and algal biomass was estimated by the concentration of chlorophyll *a*, determined by 90 % acetone extraction, and a spectrophotometer after correction for phaeophytin (APHA 1985).

Statistics

Data presented are the mean of three independent experiments, each consisting of two soils run in parallel for each species of alga. Treatments were compared by two-way ANOVA with soil (soil 1 and soil 2) and treatment (control and PAH contamination) as factors. The confidence level was set at 5 %.

Results

The characteristics of the soil samples are shown in Table 1. Soil 1 had higher conductivity and higher carbon content than soil 2, while pH values were similar between the two samples. Both soils are classified as sandy-loam. In both soils, the clay and silt fractions represented less than 0.4 % of the total sample, and sand was the most abundant fraction. Despite these similarities, the soils were not identical. Thick sand made up an important fraction of soil 1 (28.03 %), in contrast with soil 2 (7.52 %). In addition, the content of very fine sand was five times higher in soil 2 (8.46 %) than in soil 1 (1.67 %).

After 20 days of incubation, the three assayed species showed substantial growth under the experimental conditions, and the average biomass (estimated as the concentration of chlorophyll *a*) was different for each species (Fig. 1). *E. mutabilis* growing in uncontaminated soils (control) reached the highest biomass in both soils, followed by *K. rhaphidioides* (only soil 1) and *C. caldarium*. In soils contaminated with PAH, the final biomass was lower than the respective control for all three species; *K. rhaphidioides* had the lowest biomass followed by *C. caldarium* and *E. mutabilis*. ANOVA analysis of the biomass of *C. caldarium* and *E. mutabilis* showed no interaction between factors and no significant difference between soil types; however, there was a significant difference between the treatments (Table 2). In the *K. rhaphidioides* experiment, there was a significant interaction between soil and treatment, suggesting that the response of the species is dependent on the soil type (Table 2).

Table 1 Characteristics of the soil samples used in the study

	Soil 1	Soil 2
pH	4.4	4.6
EC (μS cm^{-1})	109.0	30.1
Humidity (%)	9	6.6
P (μg L^{-1})	345.4	444.5
N	n.d.	n.d.
C (%)	0.154	0.113
Texture		
Thick sand (%)	28.03	7.52
Medium sand (%)	23.70	23.83
Fine sand (%)	46.60	59.82
Very fine sand (%)	1.67	8.46
Silt (%)	0.00	0.01
Clay (%)	0.00	0.36

n.d. not detectable

Discussion

The toxic potential of soils before, during, and after remediation processes is determined primarily by chemical techniques such as HPLC and GC/MS (N'Guessan et al. 2004; Flotron et al. 2005). The size of a particle is related to some soil properties; therefore, it is important to determine and compare soil properties, such as the capacity of the soil to absorb pollutants, which increases as particle size decreases (Temporetti et al. 2013). However, several factors are not assessed by these chemical procedures: the mobility of pollutants in the soil matrix, metabolizing reactions within the soils, and the bioavailability of the compounds (both the initial contaminant and the intermediate products). Because organisms are affected by all these factors and are capable of reacting to them, a bioindicator may give more comprehensive information about the fate of pollutants, their by-products, and

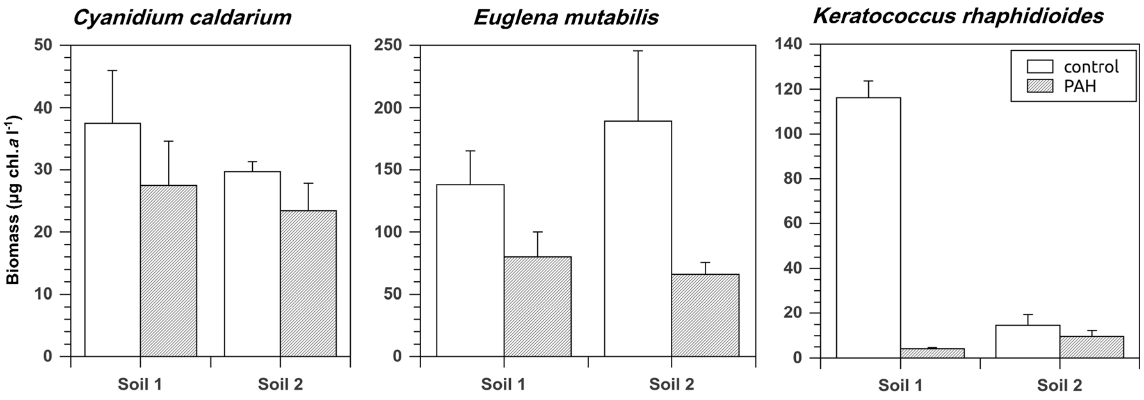

Fig. 1 Average biomass (indicated by the concentration of chlorophyll *a*) of the three acidophilic algae growing in soils contaminated with PAH. *Error bars* represent standard deviation (n=3)

the effectiveness of decontamination methods than does a chemical analysis. Employing several commonly used aquatic test organisms (*Scenedesmus subspicatus*, *Daphnia magna*, *Vibrio fischeri*, and *Pseudomonas putida*), Maxam et al. (2000) checked their applicability as indicators of soil samples contaminated with different organic and inorganic substances. The authors concluded that not all organisms are equally useful as bioindicators because of the differential level of response to contaminants. Nevertheless, organisms were useful as indicators of toxicity in the extracted soil water.

In the particular case of PS treatment, the pronounced decrease in pH negatively impacts the growth of native organisms or organisms usually assessed in tests (Baker et al. 1982); thus, it is necessary to have an acidophilic organism that is sensitive to the tested pollutant. Two of the three acidophilic species assayed in the present study were suitable as potential indicators of PAH contamination in soils. *C. caldarium* and *E. mutabilis* were responsive to the treatment in the tested soils, while the response of *K. rhaphidioides* was dependent on the type of soil. *C. caldarium* and *E. mutabilis* are very well-known cosmopolitan species, found in highly acidic environments (Whitton and Diaz 1981; Johnson 1998), with a high tolerance to metal toxicity (Nakatsu and Hutchinson 1988; Nagasaka et al. 2004). For these reasons, they are capable of growing in contaminated soils despite their reduced growth in treatment soils, as compared with the control soils. *K. rhaphidioides* showed a growth response dependent on the type of soil; for this reason, it would not be a good bioindicator of PAH contamination in soil. This acidophilic species is not common in other acidic environments, and the information about its physiological requirements is poorly known. In addition to this, *K. rhaphidioides* belongs to the planktonic community of Lake Caviahue (Pedrozo et al. 2001), and the interaction of this species with the soil particles may negatively affect its growth, resulting in a non-responsive species.

Table 2 Results of multifactor ANOVA for biomass of the three acidophilic species

	C. caldarium p	*E. mutabilis* p	*K. rhaphidioides* p
soil	0.124	0.526	**1.00 E-07**
treatment	**0.046**	**0.003**	**2.11 E-08**
soil × treatment	0.594	0.122	**4.24 E-08**

Probabilities (p) and numbers in bold type denote a significant effect of the corresponding factor (95 % of confidence)

In summary, the use of acidophilic and cosmopolitan species, such as *C. caldarium* and *E. mutabilis*, as bioindicators is a promising first step for assays of PAH contamination in soils.

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