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Response of a salt marsh microbial community to metal contamination

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ABSTRACT

Salt marshes are important sinks for contaminants, namely metals that tend to accumulate around plant roots and could eventually be taken up in a process known as phytoremediation. On the other hand, microbial communities display important roles in the salt marsh ecosystems, such as recycling of nutrients and/or degradation of organic contaminants. Thus, plants can benefit from the microbial activity in the phytoremediation process. Nevertheless, above certain levels, metals are known to be toxic to microorganisms, fact that can eventually compromise their ecological functions. In this vein, the aim of present study was to investigate, in the laboratory, the effect of selected metals (Cd, Cu and Pb) on the microbial communities associated to the roots of two salt marsh plants. Sediments colonized by *Juncus maritimus* and *Phragmites australis* were collected in the River Lima estuary (NW Portugal), and spiked with each of the metals at three different Effects Range-Median (ERM) concentrations (1, 10×, 50×), being ERM the sediment quality guideline that indicates the concentration above which adverse biological effects may frequently occur. Spiked sediments were incubated with a nutritive saline solution, being left in the dark under constant agitation for 7 days. The results showed that, despite the initial sediments colonized by *J. maritimus* and *P. australis* displayed significant ($p < 0.05$) differences in terms of microbial community structure (evaluated by ARISA), they presented similar microbial abundances (estimated by DAPI). Also, in terms of microbial abundance, both sediments showed a similar response to metal addition, with a decrease in number of cells only observed for the higher addition of Cu. Nevertheless, both Cu and Pb, at intermediate metals levels promote a shift in the microbial community structure, with possibly effect on the ecological function of these microbial communities in salt marshes. These changes may affect plants phytoremediation potential and further work on this subject is in need.

1. Introduction

Salt marshes are among the most productive but also the most sensitive ecosystems on Earth (Costanza, 1997; Zhu, 2004). They serve as important sinks for contaminants, namely metals that tend to accumulate around plant roots and could eventually be taken up in a process known as phytoremediation. This process takes advantage of the ability of plants to uptake, adsorb and concentrate contaminants from the surrounding environment. This can be an important process for the recovery of those contaminated areas.

For the phytoremediation process to be efficient, plants rely on its interactions with microorganisms. Microbial populations that are associated to plant roots (rhizobacteria) can positively affect plants by improving growth and health, enhancing root development, or increasing plant tolerance to various environmental stresses (Glick, 2010). In fact, rhizobacteria have been reported to stimulate plants acquisition and recycling of nutrients and improve plant health and control plant pathogens (*e.g.* Rajkumar et al., 2010; Aafi et al., 2012). Nevertheless, above certain levels, metals are known to be toxic to microorganisms, and can eventually compromise their ecological functions. In fact, metal pollution can cause changes in the composition of the microbial community (Turpeinen et al., 2004), resulting in adverse effects on various parameters influencing plant growth and ecological remediation (Guo et al., 2009). Despite the fact that some works dealing with the impact of heavy metals on microbial communities from rhizosphere of plants associated to agricultural (Giller et al., 1998) or mine soils (Guo et al., 2009; Xu et al., 2012) are available, no studies could be found reporting metal effect on microbial communities associated to salt marsh plants.

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The aim of present study was to investigate, in the laboratory, the effect of selected metals (Cd, Cu and Pb) on the microbial communities associated to the roots of two salt marsh plants. Rhizosediments colonized by *Juncus maritimus* and *Phragmites australis* were collected in a temperate estuary (River Lima estuary, NW Portugal), and spiked with each of the metals at three different concentrations. Both plants are common examples of ubiquitous species in Portuguese estuaries (Costa et al., 2009): *J. maritimus* is a native plant with adventitious roots system and *P. australis* is an invasive with a fibrous root system. But these plants have different roles on metal distribution and retention in the salt marshes (Almeida et al., 2011).

2. Materials and methods

2.1. Study area and sampling

The salt marsh area of the Lima River estuary, in the northwestern coast of Portugal, was selected as case study. Sampling was performed in February 2011 at low tide. Rhizosediments (sediments around plant roots) colonized by *Juncus maritimus* and *Phragmites australis* were collected at the lower Lima estuary [41.418N; 08.518W (WGS84)]. Sediments were collected between 5 and 15 cm, the depth with the higher plant below-ground biomass, placed in individual sterile bags and immediately refrigerated in an ice chest for transportation. On arriving at the lab, a fraction of each sediment was frozen at −20 °C for total DNA extraction. Another fraction was dried until constant weight for sediment characterization. Remaining collected sediment was kept refrigerated, at 4 °C, until the beginning of the experiments (within 2 h).

2.2. Sediment characterization

Each type of sediment was analysed in triplicate for organic matter (OM) content, which was determined by loss on ignition (4 h at 500 °C), as well as for water (H_2O) content. Grain size analysis was performed by sieving (*Cisa*, stainless steel) in a mechanical shaker. The sediments were divided into five fractions, being each fraction weighted and expressed in percentage of the total dry weight: silt and clay (<0.063 mm), fine sand (0.063–0.25 mm), medium sand (0.25–1 mm), coarse sand (1–2 mm), and gravel (>2 mm). Initial metals concentrations in sediments were determined in dried samples and microbial abundance was estimated in wet samples as described below.

2.3. Laboratory experiments

The controlled laboratory experiments were carried out for 7 days in 50 mL flasks under constant mechanical shaking in the dark. For that, 10 mL of sediment were mixed with 20 mL of one half-strength modified Hoagland nutrient solution (Hoagland and Arnon, 1950) supplemented with 80 mM glucose and salts, using the artificial seawater formula of Cavanaugh (1975). This medium was used in order to avoid limitation by nutrients or carbon, and to obtain the average salinity observed in the sampling site (12.5). To improve homogenization, the flasks were manually shaken once every day. Metals were added to the sediments in the form of chlorine salts ($CdCl_2$, $CuCl_2$ and $PbCl_2$, all pro analysis grade, Merck), previously dissolved in medium. Metal concentrations were added at three different concentrations (1 × ERM, 10 × ERM, 50 × ERM, Effects Range-Median (ERM) being 9.6, 270 and 218 $\mu g/g_{\text{dry sediment}}$ for Cd, Cu and Pb respectively, the sediment quality guideline that indicates the concentration above which adverse biological effects may frequently occur (Long et al., 1995)). Artificial contaminated sediments were used in the experiments to minimize unknown sources of variation that could interfere with the interpretation of the results. A control treatment, without metal addition to the medium, was also carried out. Three independent replicates were carried out for each treatment.

At the end of the experiments, supernatant sub-samples were collected for dissolved metal determinations. A portion of sediment was collected for microbial characterization and a portion was frozen at −20 °C for total DNA extraction.

To prevent contamination, all sampling and labware material was washed with Extran, rinsed with water, soaked in 10% (v/v) HCl solution for at least 24 h, rinsed several times with bi-deionized water (conductivity < 0.1 $\mu S\ cm^{-1}$) and dried in an oven.

2.4. Metal determinations

Metals concentrations were determined in all solutions at the end of the controlled laboratory experiments and in initial sediments. Dried sediment samples were digested with suitable amounts of suprapure concentrated HNO_3 (*Merck*) (5 mL) in closed poly(tetrafluoroethylene) (PTFE) vessels using a high-pressure microwave system (Advanced microwave digestion system, Ethos 1, *Milestone*). Atomic Absorption Spectrometry (AAS), either with flame atomization (PU 9200, Philips) or with electrothermal atomization (4100 ZL, Perkin–Elmer coupled to an AS-70 autosampler, provided with a Zeeman background correction), depending on the metal levels, was used for the analysis of Cd, Cu and Pb. More details can be found in Almeida et al. (2004).

2.5. Microbial abundance

To estimate microbial abundance in initial sediment and in sediments at the end of the experiments, Total Cell Counts (TCC) were obtained by the DAPI direct count method (Porter and Feig, 1980; Kepner and Pratt, 1994). For that, 0.1 g of homogenized samples were added to 2.5 mL of saline solution (0.2 μm-filtered, 9 $g\ l^{-1}$ NaCl), 200 μL of Tween 80 (0.2 μm-filtered, 12.5% (v/v)), and fixed with 1 mL of formaldehyde (0.2 μm-filtered, 4% (v/v)). Samples were stirred at 150 rpm for 15 min followed by sonication for 20–30 s at low intensity (0.5 cycle, 20% amplitude). Sub-samples of fixed solution or sediment samples were then stained with 4′,6′-diamidino-2-phenylindole (DAPI), and incubated in the dark for 12 min (Porter and Feig, 1980). Samples were filtered onto black Nucleopore polycarbonate filters (0.2 μm pore size, 25 mm diameter, Whatman, UK) under gentle vacuum and washed with autoclaved 0.2 μm-filtered distilled water. Membranes were set up in glass slides and cells counted at 1,875× on an epifluorescence microscope (Labphot, Nikon, Japan). Direct count of DAPI stained cells have been extensively used for estimation of microbial abundance in water and sediments (*e.g.* Radomski et al., 2011; Mucha et al., 2011).

2.6. Microbial community structure using Automated rRNA Intergenic Spacer Analysis (ARISA)

Total DNA was extracted from wet homogenized samples of initial sediment and samples from the end of the experiment, using a modification of the CTAB (bromide-polyvinylpyrrolidone-b mercaptoethanol) extraction protocol (Dempster et al., 1999) described by Barrett et al. (2006). Quality of extracted DNA was evaluated by visualization on 1.5% agarose gels and each DNA preparation was quantified with the Qubit fluorometer (Invitrogen). For ARISA, extracted DNA was amplified using ITSF (5′-GTCGTAACAAG GTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) primers set (Cardinale et al., 2004), which amplifies the ITS1 region in the rRNA operon plus ca. 282 bases of the 16S and 23S rRNA (Hewson and Fuhrman, 2004). ITSReub was labelled with the