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Research Article

RESEARCH ARTICLE

Assessing environmental drivers of microbial communities in estuarine soils of the Aconcagua River in Central Chile

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One sentence summary: Microbial communities from Aconcagua River estuarine soils are mainly influenced by environmental factors such as pH, copper, arsenic and hydrocarbons, indicating inputs from mining and a crude oil refinery.
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ABSTRACT

Aconcagua River basin (Central Chile) harbors diverse economic activities such as agriculture, mining and a crude oil refinery. The aim of this study was to assess environmental drivers of microbial communities in Aconcagua River estuarine soils, which may be influenced by anthropogenic activities taking place upstream and by natural processes such as tides and flood runoffs. Physicochemical parameters were measured in floodplain soils along the estuary. *Bacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Pseudomonas*, *Bacillus* and *Fungi* were studied by DGGE fingerprinting of 16S rRNA gene and ribosomal ITS-1 amplified from community DNA. Correlations between environment and communities were assessed by distance-based redundancy analysis. Mainly hydrocarbons, pH and the composed variable copper/arsenic/calcium but in less extent nitrogen and organic matter/phosphorous/magnesium correlated with community structures at different taxonomic levels. Aromatic hydrocarbons degradation potential by bacterial community was studied. Polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenases genes were detected only at upstream sites. Naphthalene dioxygenase *ndo* genes were heterogeneously distributed along estuary, and related to *Pseudomonas, Delftia, Comamonas* and *Ralstonia*. IncP-1 plasmids were mainly present at downstream sites, whereas IncP-7 and IncP-9 plasmids showed a heterogeneous distribution. This study strongly suggests that pH, copper, arsenic and hydrocarbons are main drivers of microbial communities in Aconcagua River estuarine soils.

Keywords: microbial community; Aconcagua River; DGGE; 16S rRNA gene; PAH-RHD gene; IncP plasmid

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INTRODUCTION

Coastal estuaries are changing ecosystems influenced by several dynamic processes. Brackish water formed by the confluence of sea- and freshwater, and sediments carried by each fraction, provides a buffer zone with particular physicochemical and sedimentological properties. Organisms developing in this environment are usually euryhaline. Estuarine soils at river shore (also called floodplain soils) are submerged or not depending on the tide level and flooding regime. These soils are the result of interactions between the soil and its aquatic environment and are thus considered as semiterrestrial soils (Córdova-Kreylos *et al.* 2006; Du Laing *et al.* 2009). Additionally, most human activities develop close to rivers, and seaports are usually settled on estuaries. Therefore, these environments are of economic, social and ecological relevance. Estuaries can act as a repository of runoff pollutants, nutrients and pathogens (Córdova-Kreylos *et al.* 2006), and can thus be regarded as a summary of activities occurring upstream. Anthropogenic activities may adversely affect naturally occurring microbial communities. Microbial communities are largely affected by environmental disturbances, such as agriculture (Suzuki *et al.* 2012; Ding *et al.* 2013; Vasileiadis *et al.* 2013), pH changes (González-Toril *et al.* 2003; Lauber *et al.* 2009; Rousk, Brookes and Bååth 2009; Baffico 2010; Rousk *et al.* 2010), heavy metal pollution (Córdova-Kreylos *et al.* 2006; Altimira *et al.* 2012), pesticide application (Morgante *et al.* 2010; Hernández *et al.* 2011) and hydrocarbon pollution (Gomes *et al.* 2007; Ding *et al.* 2010; Yergeau *et al.* 2012).

Aconcagua River has a course of 140 km at the Valparaíso Region in Central Chile and has major economic and social importance for this area. Aconcagua River streamflow changes seasonally, with higher runoffs during spring when glacier melting at the Andes Cordillera takes place. In addition, year-to-year fluctuations occur, especially when precipitation increases by El Niño Southern Oscillation effect (Bown, Rivera and Acuña 2008). Anthropogenic activities along the Aconcagua river's basin and tributaries include agriculture, copper and molybdenum mining, a bronze factory, a slaughterhouse and a cement factory, among others (Dirección General de Aguas 2004). Effluents are treated before reaching the river's course under normal operating conditions. Treated domestic wastewater from several cities also flows into the Aconcagua River's course. Additionally, a petroleum refinery is located at the river's estuary, approximately 2 km from seashore. These anthropogenic activities may affect microbial diversity at the Aconcagua River estuary. In the Aconcagua River valley, the presence of agrochemicals and high copper levels in agricultural soils and their influence on microbial communities has been reported (Hernández *et al.* 2008a,b; Morgante *et al.* 2010; Altimira *et al.* 2012). In addition, high levels of PAH in the coastal reef close to the Aconcagua River mouth have been observed (Palma-Fleming *et al.* 2008). The presence of petroleum hydrocarbons could be associated with the crude oil refinery activity close to the Aconcagua River estuary. Noteworthy, two important spills have taken place during the last years. In 2002, 70 000 L of crude oil were accidentally released into Aconcagua River estuary. In late 2011, 5000 L of copper concentrate were discharged into the upper section of the Aconcagua River.

In this study, microbial communities and environmental variables of estuarine soils from Aconcagua River were characterized in order to assess the correlation between environmental factors and the structure of microbial communities. Semiterrestrial estuarine soils along the last ∼1500 m of the river's mouth were studied. Denaturant gradient gel electrophoresis (DGGE) fingerprinting of 16S rRNA gene fragments amplified from total community DNA was used to analyze the bacterial and fungal structure at five soils, from ∼10 m from seashore until ∼1500 m upstream. To analyze not only dominant bacteria, DGGE was performed at different taxonomic levels, *Bacteria*, *Actinobacteria*, *Alpha-* and *Betaproteobacteria*. In order to get deeper insights into bacterial communities, DGGE fingerprinting was also conducted for specific taxonomic genera (*Pseudomonas* and *Bacillus*) commonly associated with anthropogenic environmental disturbances. Environmental parameters including variables related to human activities taking place along the river's basin were measured and used as the constraining matrix for distance-based redundancy analysis (db-RDA). This constrained multivariate analysis using microbial communities and environmental metadata was performed in order to find the best descriptors (i.e. the drivers) of microbial community structure at these estuarine soils. In order to obtain information about the catabolic potential for aromatic compounds of the microbial community, polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase (PAH-RHD) genes, naphthalene dioxygenase (*ndo*) genes and catabolic IncP plasmids were also studied as indicators of anthropogenic impact. This study yielded insights into the environmental factors either natural or anthropogenic that significantly correlated with the microbial community structure of estuarine soils located along the last ∼1500 m from the Aconcagua River in Central Chile.

MATERIALS AND METHODS

Site description and sampling

Five riverside sites (∼350 m distance between neighbor sites) were sampled along the Aconcagua River estuary located in Central Chile (Valparaiso region, 32°55′S, 71°29′W) in November 2011 (Fig. 1). Site I is located ∼10 m from seashore, whereas site V was sampled ∼1500 m upstream. Five composite samples, comprised by randomly collected subsamples with 1–2 m distance from each other, were taken from each site. Subsamples from the surface stratum (0–20 cm depth) were taken 1–3 m from the river shoreline. After removing stones and vegetable fragments, samples were homogenized and sieved through a 10-mm mesh prior to further analyses.

Physicochemical characterization of soils

Soil analyses were performed by the Soil Laboratory at the Agronomy Faculty, Pontificia Universidad Católica de Valparaíso (Quillota, Chile), following protocols established by the Chilean National Institute of Agriculture and Livestock Research (INIA). Organic matter (OM) was determined by weight loss at 360°C (Sadzawka 1990). Nitrogen was determined by digestion with sulfuric acid, salicylic acid, potassium sulfate, copper sulfate and titanium dioxide, and titration (Sadzawka 1990). Potassium and sodium were determined by extraction with ammonium acetate and atomic emission spectroscopy (Sadzawka 1990). Zinc, manganese, iron and copper were quantified by diethylenetriaminepentaacetic acid extraction and atomic absorption spectroscopy (AAS) (Sadzawka 1990). Arsenic was determined by acid extraction and AAS (Sadzawka 1990). Soil pH and electrical conductivity were determined in aqueous soil suspension with a pH meter and a conductivity meter, respectively (Sadzawka *et al.* 2006). Calcium and magnesium were determined by extraction with ammonium acetate and AAS (Sadzawka *et al.* 2006). Boron was extracted with calcium chloride and determined by the azomethine H colorimetric method (Sadzawka *et al.* 2006).

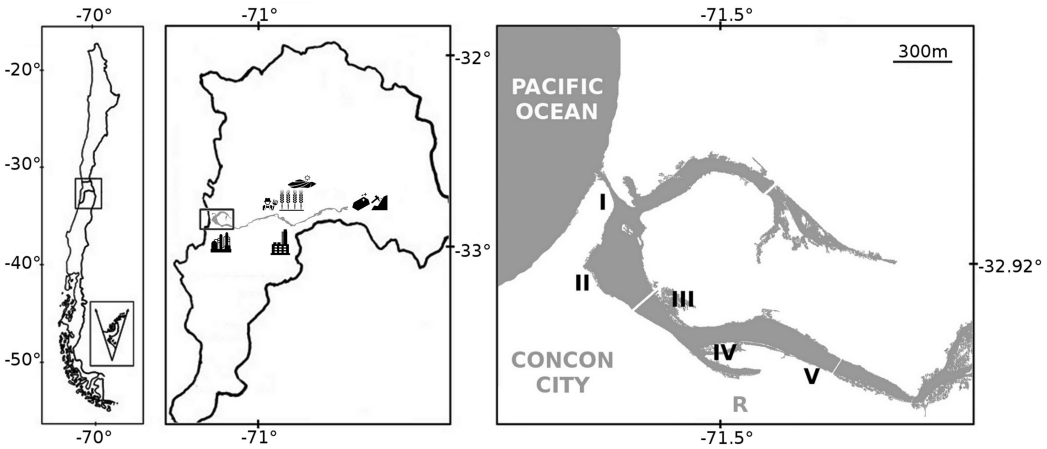

Figure 1. Aconcagua River estuary at Central Chile. Anthropogenic activities such as mining (copper and molybdenum), agriculture, industrial activities (e.g. bronze factory, cement factory and slaughterhouse) and a petroleum refinery probably influencing estuarine soils microbial communities are depicted in the central panel. Sites are enumerated from I to V starting from the seashore. Five replicates were taken from each site. R: crude oil refinery.

Phosphorus was extracted with calcium bicarbonate and determined by the molybdenum blue method (Sadzawka *et al.* 2006). Total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAH) were quantified by a gas chromatograph coupled to mass spectrometer (GC-MS) (Bravo-Linares *et al.* 2011). Briefly, samples were extracted with hexane:dichloromethane (1:1) in an ultrasonic bath and quantified using as internal standard 1-chlorooctadecane.

Total community DNA extraction from soils

Total community (TC) DNA was extracted from 0.5 g of dry soil with the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA) with mechanical disruption by the FastPrep-24 beadbeater instrument (MP Biomedicals, Santa Ana, California, USA), and subsequent purification with the GeneClean II Spin Kit (MP Biomedicals, Solon, Ohio, USA), following the manufacturer's instructions.

PCR-DGGE of bacterial 16S rRNA gene and fungal internal transcribed spacer (ITS)

For bacterial community analyses, the universal primer pair (GC)-F984/R1378 was used to amplify the V6–V8 segment from 16S rRNA gene (Heuer *et al.* 1997). Nested and semi-nested PCR approaches were performed for amplification of 16S rRNA gene segments from *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Pseudomonas* and *Bacillus*. The product obtained in a specific amplification of each taxonomic subgroup was used as template for the amplification with the universal primer pair described above (Table S1, Supporting Information). This strategy allowed the use of the same amplicon (V6–V8 segment) and DGGE conditions for all groups as described previously (Babin *et al.* 2013). PCR products were submitted to DGGE using a PhorU2 apparatus (Ingeny, Goes, Netherlands) and silver stained according to Weinert *et al.* (2009). Briefly, a double gradient gel composed of 46.5–65% denaturant (urea and formamide) and 6.2–9% (w/v) acrylamide:bisacrylamide (37.5:1) was run at 140 V for 17 h at 58°C in Tris-acetate-EDTA buffer. For *Fungi*, the ribosomal ITS was amplified by a nested PCR with the universal primer pairs ITS1F/ITS4 and (GC)-ITS1F/ITS2 (Weinert *et al.* 2009). An 8% (w/v) acrylamide:bisacrylamide (37.5:1) gel with a gradient consisting of 23–58% denaturant was run at 100 V for 18 h at 60°C in Tris-acetate-EDTA buffer. Digitalized gels were analyzed with GelCompar II 6.5 (Applied Maths) to get a square diagonal matrix of pairwise Pearson correlations (*r*) between each pair of DGGE profiles.

Statistical analyses of community and environmental data

Correlation matrices of DGGE fingerprints were used for UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering and dendrogram construction. Dissimilarity and significance of the differences among DGGE fingerprints were computed on these matrices using the permutation test described by Kropf *et al.* (2004). Dissimilarity values were calculated by *d*-value = $r_{within} - r_{between}$, where r_{within} is the average of pairwise Pearson correlation (*r*) values calculated for the five replicate samples within the same site, and $r_{between}$ is the average of pairwise Pearson correlation (*r*) values calculated for the 10 samples from the two sites to be compared. Pairwise distances (*D*), obtained by $D = 1 - r$, were used as community data matrix for db-RDA (Legendre and Anderson 1999). Environmental data was used as the explanatory matrix in db-RDA. Calcium, copper and arsenic concentrations were highly correlated ($R^2 > 0.98$), as well as organic matter, phosphorus and magnesium ($R^2 > 0.98$). They were grouped into the composed variables Ca.Cu.As and OM.P.Mg, respectively. Selection of explanatory variables was accomplished by automatic forward selection with vegan's *ordiR2step* function, which minimizes the inclusion of false positives into db-RDA model (Blanchet, Legendre and Borcard 2008; Szöcs, Kefford and Schäfer 2012). Each model proposed by *ordiR2step* was further tested for significance (vegan's anova function) and only constraints with $P < 0.01$ were selected for the final model. Finally, db-RDA was performed on principal coordinates of *D* matrices using the significant environmental variables obtained by *ordiR2step* as constraints. All steps were performed with R-software's Vegan package v.2.0–8 (Oksanen *et al.* 2013).

Analysis of genes coding for PAH-RHD and naphthalene dioxygenase (NDO)

RHD gene fragments were amplified using the degenerated primers 396F/696R described by Ding *et al.* (2010) and detected by Southern blot hybridization. PCR products (320 bp) were transferred to a nylon membrane, hybridized with a

Table 1. Physicochemical properties of soils from the five sites along the Aconcagua River estuary. Average and standard deviation values are shown.

	Site				
Soil parameter	I	II	III	IV	V
Texture	Sand	Sand	Loam	Loam	Loam
pH	8.65 ± 0	8.7 ± 0	8.03 ± 0.02	7.92 ± 0.01	7.84 ± 0.01
Conductivity (dS m^{-1})	1.83 ± 0.01	1.12 ± 0.01	1.35 ± 0.03	0.89 ± 0	1.98 ± 0.01
Nitrogen (mg kg^{-1})	12.70 ± 0.03	12.10 ± 0.03	16.20 ± 0.02	13.60 ± 0.02	14.00 ± 0.02
Potassium (mg kg^{-1})	130 ± 1	129 ± 1	167 ± 1	152 ± 0	285 ± 3
TPH (mg kg^{-1})	0.5	0.57	1.99	0.58	0.78
PAH (μg kg^{-1})	0	17.3	24.34	24.18	17.79
Organic Matter (%)	0.05 ± 0.01	0.01 ± 0	1.00 ± 0.01	2.00 ± 0.02	3.43 ± 0.11
Phosphorous (mg kg^{-1})	5.58 ± 0	4.9 ± 0.4	11.8 ± 0.5	16.4 ± 0.2	29.7 ± 0.6
Magnesium (mg kg^{-1})	224 ± 4	214 ± 5	332 ± 3	520 ± 2	874 ± 2
Calcium (mg kg^{-1})	712 ± 24	715 ± 20	1614 ± 30	3908 ± 28	5010 ± 28
Copper (mg kg^{-1})	3.34 ± 0.01	2.56 ± 0.04	10.9 ± 0.2	108 ± 2	147 ± 1
Arsenic (mg kg^{-1})	6.09 ± 0.07	7.41 ± 0.25	9.9 ± 0.7	26.25 ± 1.35	34.25 ± 0.75
Sodium (mg kg^{-1})	740 ± 15	418 ± 8	455 ± 16	275 ± 8	1044 ± 16
Zinc (mg kg^{-1})	0.53 ± 0.05	0.44 ± 0.02	3.74 ± 0.03	4.45 ± 0.1	6.54 ± 0.1
Manganese (mg kg^{-1})	6.11 ± 0.03	6.4 ± 0.01	26.1 ± 0.3	26.8 ± 0.6	32.6 ± 0.1
Iron (mg kg^{-1})	6.94 ± 0.06	9.93 ± 0.07	77.6 ± 0.2	93.1 ± 1.3	127 ± 1
Boron (mg kg^{-1})	0.62 ± 0.02	0.46 ± 0.01	1.43 ± 0.02	1.18 ± 0	2.23 ± 0.01

digoxigenin-labeled amplicon of RHD from pNF142 plasmid and the *nahAc* and *phnAc* genes (Table S2, Supporting Information), and revealed with the DIG DNA Labeling Kit (Roche) (Babin *et al.* 2013). PCR-DGGE and cloning of naphthalene dioxygenase genes (*ndo*) were performed according to Gomes *et al.* (2007). Briefly, a nested PCR with the NAPH-1F/NAPH-1R and NAPH-2F/NAPH-2R primer pairs was performed. Primers are listed in Table S1 (Supporting Information). Amplification products (~740 bp) were loaded into a double gradient gel of 26–58% denaturant (urea and formamide) and 6–9% (w/v) acrylamide:bisacrylamide (37.5:1) and were run in a DCode System (Bio-Rad) at 160 V for 15 h at 58°C in Tris-acetate-EDTA buffer. Gels were silver stained and digitalized as described above. For cloning, PCR products were pooled, ligated into pGEM-T easy vector (Promega, Madison, Wisconsin, USA) and cloned into *Escherichia coli* JM109 competent cells (Promega) following the manufacturer's instructions. Each clone was PCR-amplified and compared with the sample from which it was extracted by DGGE. When the electrophoretic mobility agreed with the expected band, it was sequenced (Macrogen, Korea) and identified using tblastx and blastx (Altschul *et al.* 1990). Phylogenetic tree was constructed based on the ClustalW alignment of the deduced aminoacid sequences using the maximum likelihood method (Hall 2013). Branch consistence was checked by bootstrap analysis (1000 replicates) with MEGA6 software (Tamura *et al.* 2013). Sequences were submitted to NCBI nucleotide database under the accession numbers KT310224–KT310236 (AC01–AC13).

Detection of IncP plasmids

IncP-1α, β, γ, δ and ϵ were amplified in a multiplex PCR with primers targeting the *trfA* gene of each subgroup as described previously (Bahl *et al.* 2009). IncP-7 *rep* gene was amplified using the P7repA/P7repB primer pair (Izmalkova *et al.* 2005). Plasmids belonging to IncP-9 group were detected by amplification with IncP9-ori69f/IncP9-rep679r pair (Babin *et al.* 2013). For IncP-1 and IncP-9, several probes were used in order to detect all variants belonging to both Inc groups. This avoids false negatives since the variants of each group present in samples are *a priori* not known. Amplification products were detected by Southern blot hybridization with the DIG DNA Labeling Kit (Roche) according to Babin *et al.* (2013). Primers and probes are listed in Tables S1 and S2 (Supporting Information), respectively.

RESULTS AND DISCUSSION

Soil physicochemical properties

Soil samples were taken at five sites along the last ~1500 m from Aconcagua River estuary in November 2011. Sampling sites were covered by water during higher runoffs and tides and therefore these soils are influenced by sedimentological processes. General rough differences can be observed for the five sites along the Aconcagua River estuary (Table 1). Sites I and II belong to the sandbank at the last estuary segment and are under the direct influence of tidal currents entering the estuary (Fig. 1). Low levels of OM (≤0.05%) and pH ~ 8.7 clearly distinguish these alkaline sandy soils from the other three sites. Except sodium, all other measured components were in lower concentrations at sites I and II. Sites IV and V belong to wetland dominated by rushes, bushes and grass. Lower pH and higher values of almost all parameters were observed at these two upstream sites. Site V presented the highest concentrations of almost all components, except nitrogen, TPH and PAH. Concentration of all other components at site V was 1.2–1.8-fold higher than those for site IV, except for sodium (3.8-fold higher), which presented the lowest concentration at site IV. Site III is part of a marsh dominated by rushes. Conductivity, potassium, sodium, nitrogen, boron, TPH and PAH contents were higher at this central site. Manganese presented the same concentration for sites III and IV. The values of the remaining parameters were between the values observed for sites II and IV in a gradient (i.e. II<III<IV). Loamy organic soils at sites III-IV account for the dominant influence of runoffs depositing finer sediments at this estuary level. Particle size characteristics are in agreement with previous sedimentological surveys (Martínez and Cortez 2007). As a general trend, a gradient of almost all parameters could be observed, with higher contents toward upstream sites (Table 1). Concentrations of

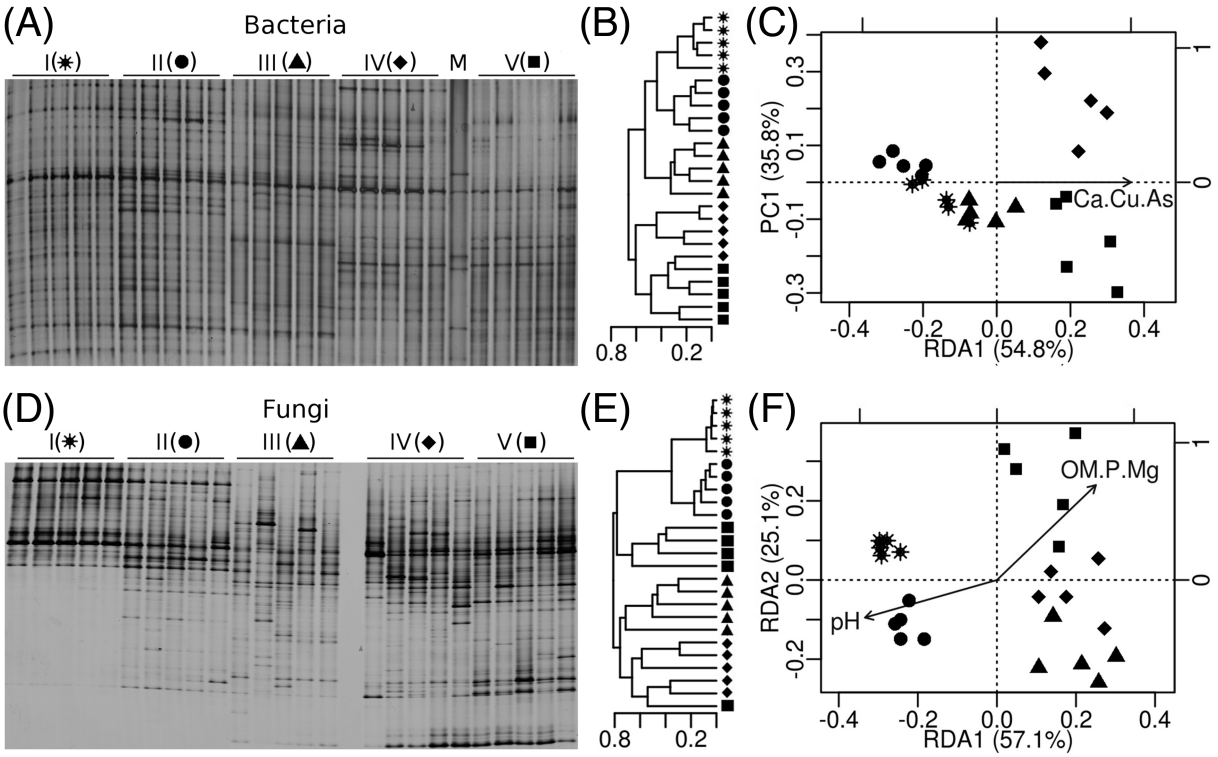

Figure 2. Bacterial and fungal communities and their relationship with the environment. DGGE fingerprints of PCR-amplified bacterial 16S rRNA gene (**A**) and fungal ITS-1 (**D**). Roman numbers indicate sites, with five replicates each, M: marker. UPGMA clustering analysis (**B**, **E**) of sites and replicates, distance is shown in bottom axis. db-RDA biplots (**C**, **F**) of the significant environmental variables ($P < 0.01$) explaining the observed distances among communities.

copper at sites IV-V were ~30–55-fold higher than at sites I and II and ~10–13-fold higher than at site III. Despite the wide range of economic activities along Aconcagua River, only pH values at sites I-II and copper and arsenic levels at sites IV-V exceeded recommended values for industrial soils, according to the Canadian Soil Quality Guidelines for the Protection of Environmental and Human Health (Canadian Council of Ministers of the Environment 2007).

Aconcagua River estuarine soil physicochemical properties were consistent with those observed for other estuarine soils, where positive ions content, particularly metal ions, correlates positively with OM and clay content. Cationic ions are adsorbed to OM by chelation and smaller particle size (i.e. higher clay content) increases the contact surface with water phase, allowing to chelate more metals (Du Laing *et al.* 2009). In contrast, in the present study metals content showed a negative correlation with pH. A similar negative correlation was observed in a study of the Scheldt River estuary in Belgium/Netherlands, where pH fluctuated from 7.2 to 8.7 (Du Laing *et al.* 2007). However, a positive correlation between pH and ion content has been reported (Kemmitt, Wright and Jones 2005; Kemmitt *et al.* 2006; Du Laing *et al.* 2007, 2009; Degryse, Smolders and Parker 2009). It has been proposed that at lower pH, most metals tend to desorb from OM and leach from the sediment/soil. In the present study, soil pH ranged between 7.8 and 8.7. Such a narrow pH range may not be enough to evidence a positive correlation between pH with metal ions content in these well-buffered environments.

Microbial community structure in estuarine soils

Microbial communities of the Aconcagua River estuarine soils were characterized by DGGE community fingerprinting. Permutation test revealed significant differences of DGGE community fingerprints among the five sites ($P < 0.05$) for all taxonomic groups studied (Table S3, Supporting Information). The lowest distance (as depicted by *d*-value) was observed between sites IV and V for *Actinobacteria*, *Alphaproteobacteria*, *Pseudomonas* and *Bacillus* communities (Table S3, Supporting Information). For *Bacteria* and *Betaproteobacteria*, the most similar communities were observed between sites I and II (Table S3, Supporting Information). The lowest distance for *Fungi* was observed between sites III and IV. Overall, a separation of downstream (sites I-II) and upstream (sites IV-V) microbial communities was observed. Community from site III represented a transition between upstream and downstream communities. It shared bands with both downstream and upstream sites, and also exhibited unique bands (Fig. 2–4). In general, only minor community fingerprint variation among replicates within the same site was observed, except for central site III that showed heterogeneity among replicates for *Fungi* (Fig. 2D), *Actinobacteria* (Fig. 3A), *Alphaproteobacteria* (Fig. 3D) and *Bacillus* (Fig. 4D). UPGMA was performed in order to evidence clustering patterns of DGGE profiles. Samples from site III grouped together with upstream or downstream sites depending on the taxonomic group analyzed. Bacterial communities divided into two main clusters, communities from sites I-III and communities from sites IV-V (Fig. 2B). Fungal communities from downstream sites I-II grouped in a tight cluster ($D = 0.35$) separately from the rest of the sites (Fig. 2E). Even when UPGMA located four of the five replicates from site V together with downstream communities, they were clearly distant ($D \sim 0.8$) from downstream sites I-II. UPGMA is useful for visualize grouping. However, permutation test of similarity measures (e.g. pairwise Pearson correlation as in Table S2, Supporting Information) is more suitable as it does not depend on the

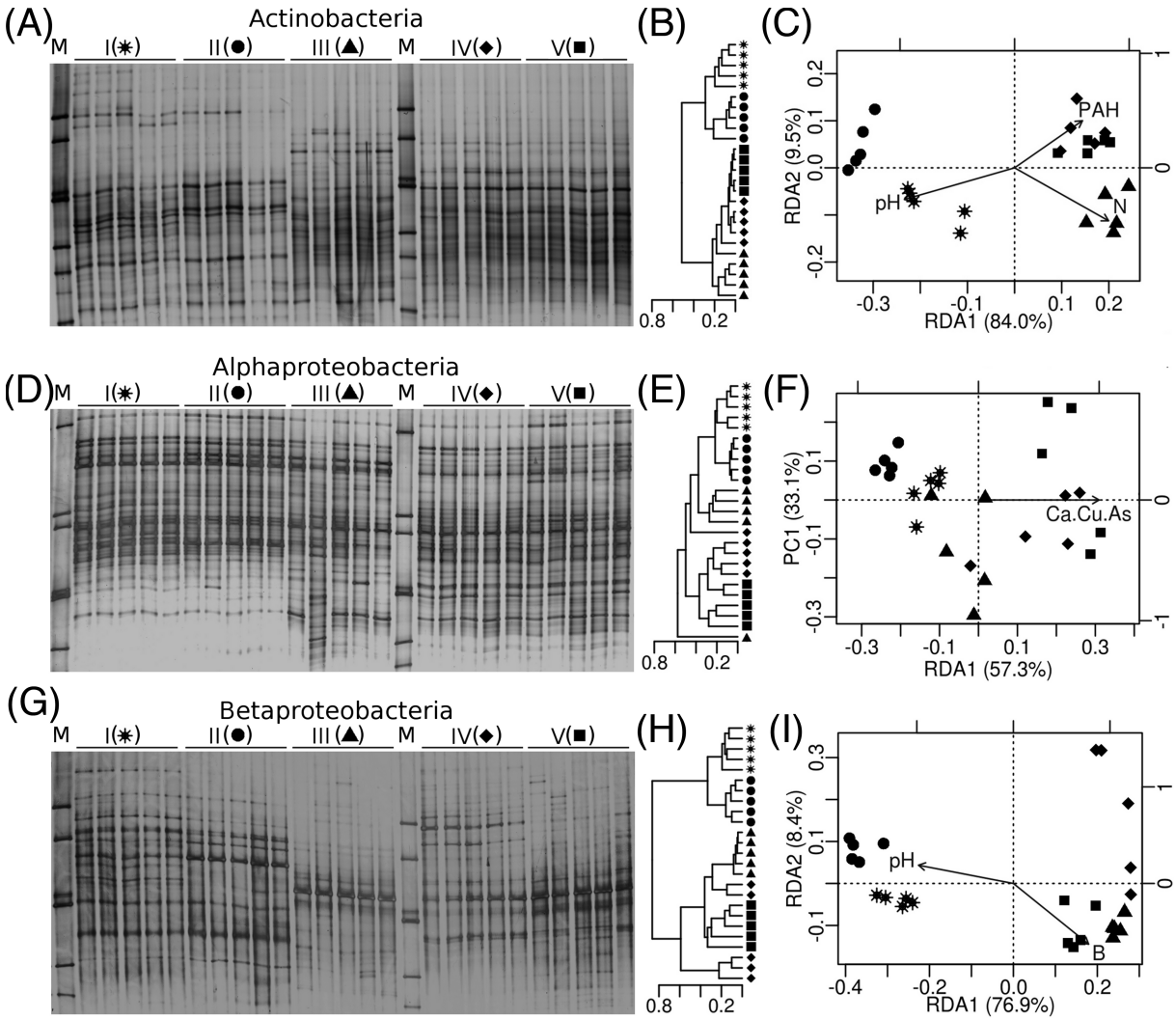

Figure 3. Communities of *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* classes and their relationship with the environment. DGGE fingerprints of PCR-amplified bacterial 16S rRNA gene from *Actinobacteria* (**A**), *Alphaproteobacteria* (**D**) and *Betaproteobacteria* (**G**). Roman numbers indicate sites, with five replicates each, M: marker. UPGMA clustering analysis (**B**, **E**, **H**) of sites and replicates, distance is shown in bottom axis. db-RDA biplots (**C**, **F**, **I**) of the significant environmental variables ($P < 0.01$) explaining the observed distances among communities.

clustering algorithm (Kropf *et al.* 2004). High similarity was observed for the three upstream *Actinobacteria* communities III-IV-V, whereas sites I and II were grouped in a second cluster. Communities of *Proteobacteria* classes showed different clustering patterns. Clustering of *Alphaproteobacteria* communities was similar to *Bacteria*, with two groups formed by sites I-III and IV-V (Fig. 3E). The separation between downstream and upstream communities was higher for *Betaproteobacteria* (Fig. 3H), similar to the observed for *Actinobacteria*, where a tight cluster formed by communities from sites I-II was clearly separated from the second cluster formed by sites III-V. *Pseudomonas* and *Bacillus* are two bacterial genera commonly isolated from environments impacted by anthropogenic activities due to their metabolic versatility (Hernández *et al.* 2008b; Lin and Cai 2008; Eppinger *et al.* 2011; Silby *et al.* 2011). *Pseudomonas* presented low richness, as few DGGE bands were observed (Fig. 4A). Four *Pseudomonas* bands were present at all sites. Clustering and ordination were not able to evidence clusters or patterns, indicating the homogeneity of this genus along the Aconcagua River estuary. Dendrogram distances were the lowest of all fingerprints analyzed (Fig. 4B). *Bacillus* communities were more diverse (based on the number of bands) than *Pseudomonas*. Similar to *Actinobacteria* and *Betaproteobacteria*, *Bacillus* communities from sites I and II clustered together separated from sites III-V (Fig. 4E). In this case, however, distances inside each cluster were higher.

Relationship of microbial community structures and environmental parameters

Constrained multivariate analysis using microbial communities and environmental data was performed in order to find the best descriptors of microbial communities at estuarine soils. To analyze the relationship between environmental variables and microbial community structure, in a first step highly correlated variables were combined into one, as the effect of each variable could not be analyzed independently. Forward selection allowed identifying the descriptors that significantly contribute to explain the observed community variance (Blanchet, Legendre and Borcard 2008). Although selection is performed automatically, descriptors were further checked by significance, and only the

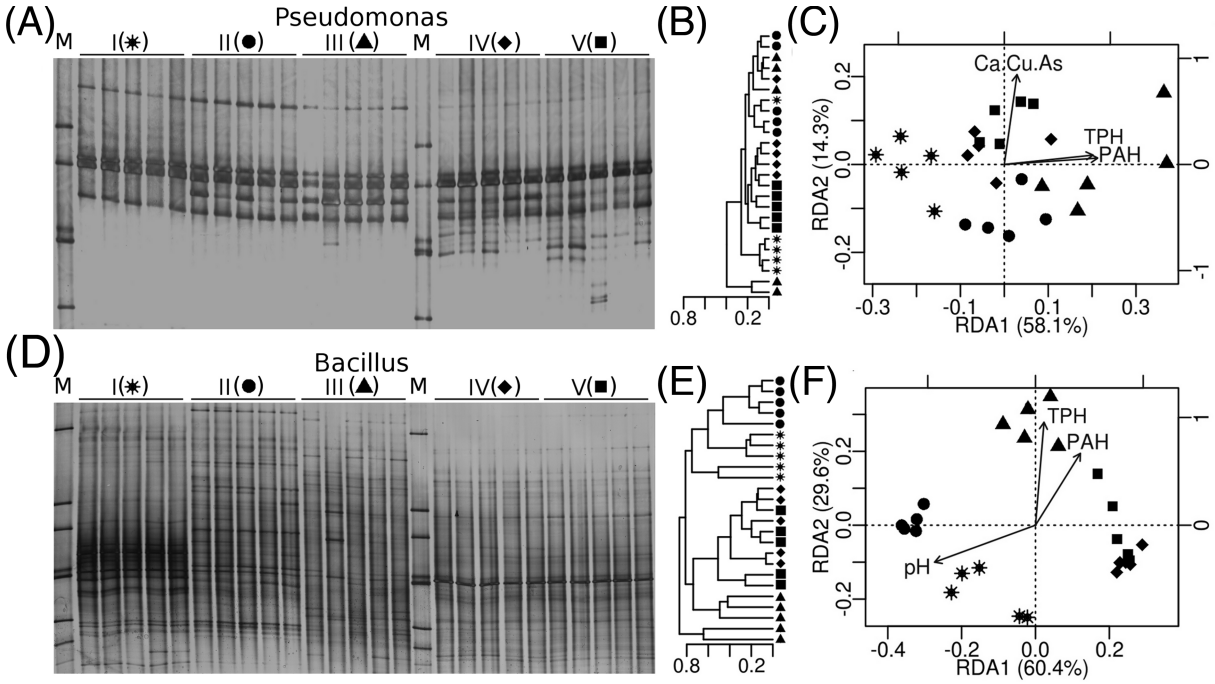

Figure 4. Communities of *Pseudomonas* and *Bacillus* genera and their relationship with the environment. DGGE fingerprints of PCR-amplified bacterial 16S rRNA gene from *Pseudomonas* (**A**) and *Bacillus* (**D**). Roman numbers indicate sites, with five replicates each, M: marker. UPGMA clustering analysis (**B**, **E**) of sites and replicates, distance is shown in bottom axis. db-RDA biplots (**C**, **F**) of the significant environmental variables ($P < 0.01$) explaining the observed distances among communities.

variables with $P < 0.01$ confidence were kept for db-RDA. Variance of *Bacteria* communities could be mainly explained by the variable composed by calcium, copper and arsenic (Ca.Cu.As) (Fig. 2C). This only significant descriptor accounted for 54.8% of bacterial variance. As db-RDA produced only one RDA axis, the first residual principal coordinate axis was included for plotting purposes. Differences in *Fungi* communities were explained mainly by pH and the variable composed by organic matter, phosphorus and magnesium (OM.P.Mg) (Fig. 2F). These two descriptors explained 82.2% of community variance, where the higher pH values at downstream sites (Fig. 2F and Table 1) are relevant explaining the tight cluster of samples from sites I and II observed by UPGMA (Fig. 2E). In the same way, the dispersion of samples from sites III, IV and V is mainly explained by the increasing amounts of the variable composed by OM, phosphorus and magnesium (III<IV<V) (Table 1). Environmental variables correlated with *Actinobacteria* community structure are shown in Fig. 3C. The higher pH at downstream sites I and II, the higher PAH concentration at upstream sites III, IV and V and the slightly higher nitrogen concentration at site III (Table 1) account for the 93.5% variance in community data. db-RDA of *Alphaproteobacteria* communities yielded plots similar to *Bacteria* domain, where only Ca.Cu.As resulted significant and explained 57.3% of community variance (Fig. 3F). Differences in *Betaproteobacteria* communities (85.3% variance) were mainly explained by the lower pH at downstream sites and higher amounts of boron at sites III-V (Fig. 3I). At the bacterial genera level, *Pseudomonas* structure was explained by Ca.Cu.As, TPH and PAH variables, accounting for the 72.4% of community variance (Fig 4C). However, ordination of samples in 2D space was less clear, as observed by UPGMA (Fig. 4B). This is probably due to the homogeneity observed in DGGE patterns. *Bacillus* community structure was explained (90.0% variance) by pH, TPH and PAH. The clustering of sites I-II was explained by the higher pH values, which was also observed for *Fungi*, *Actinobacteria* and *Betaproteobacteria*. Additionally, the higher concentrations of petroleum fractions could explain the dispersion observed in sites III, IV and V (Fig. 4F).

The narrow soil pH range along the estuary did not reflect the expected relationship with the metal ions content. Soil pH was a major variable explaining microbial community structure by db-RDA. In this study, pH was a significant ($P < 0.01$) descriptor for *Fungi* kingdom, *Actinobacteria* and *Betaproteobacteria* classes, and *Bacillus* genus communities at Aconcagua River estuarine soils. Soil pH is a major environmental factor influencing microbial communities, including *Bacteria*, *Archaea*, *Fungi* and *Algae* from a wide range of environments (González-Toril *et al.* 2003; Rousk, Brookes and Bååth 2009; Baffico 2010; Rousk *et al.* 2010; Fernández-Calviño *et al.* 2011; Vasileiadis *et al.* 2013). The pH value is a major predictor of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Nitrospira* and *Proteobacteria* abundance (Lauber *et al.* 2009; Rousk *et al.* 2010). In several American soils (pH 3.5–8.9), pH was the only environmental factor and significant predictor of bacterial diversity (Lauber *et al.* 2009). Higher diversity scores were observed toward neutral pH values, the same range where bacterial growth and activity are higher in soil (Fernández-Calviño *et al.* 2011). However, the effect of pH is different for each taxon. For instance, different *Acidobacteria* groups are affected positively or negatively by soil pH (Jones *et al.* 2009; Lauber *et al.* 2009), and the effect on *Proteobacteria* is apparently major on γ and δ classes (Rousk *et al.* 2010). A study carried out on microbial communities from the Italian Po River (pH 6.4–7.2) reflected the relevance of pH as a major environmental driver of the structure of *Bacteria* and *Archaea* communities (Vasileiadis *et al.* 2013). In contrast, the effect of pH on *Fungi* abundance is apparently slighter than for *Bacteria* (Rousk *et al.* 2010). The present study did not show a pH-community relationship for the *Bacteria* domain. However, this correlation was observed for lower bacterial taxa. Narrow neutral-alkaline pH range at the Aconcagua River estuary soils might not be enough to explain such relationship. Additionally, both studies mentioned above analyzed

communities by massive sequencing of 16S rRNA gene, resulting in deeper analyses (Rousk *et al.* 2010; Vasileiadis *et al.* 2013). As DGGE fingerprints for *Bacteria* include all lower taxa, subtle differences might be less evident. The Po River study also used forward selection and db-RDA, depicting the usefulness of this statistical approach regardless of the culture-independent technique used to generate the community data (Legendre and Anderson 1999; Blanchet, Legendre and Borcard 2008; Vasileiadis *et al.* 2013). Overall, pH was a major environmental factor influencing microbial communities at Aconcagua River estuarine soils.

A set of environmental variables, most of them directly associated with anthropogenic activities (i.e. copper, arsenic, TPH and PAH) were measured. Mining of copper ores and the use of copper-based pesticides in agriculture are two common copper sources for the Aconcagua River. Arsenic occurs in several chemical forms, mainly as As_2O_3, and is usually coextracted from copper ores. Calcium, as $CaCO_3$, is a major limestone component and commonly used in the cement industry that is also located close to the Aconcagua River. Calcium, copper and arsenic in the area studied showed a high correlation ($R^2 > 0.98$) and therefore were grouped together as one variable. According to the Canadian Guidelines for the Protection of Environmental and Human Health, maximum copper concentration levels in soils should be 63 and 91 mg kg^{-1} for domestic/agricultural and commercial/industrial land use, respectively (Canadian Council of Ministers of the Environment 2007). Arsenic levels should not be higher than 12 mg kg^{-1} soil, independent of the land use. Copper and arsenic were above these concentration levels at sites IV and V, with 108 and 147 mg kg^{-1} of copper and 26 and 34 mg kg^{-1} of arsenic, respectively (Table 1). Thus, copper and arsenic could be the factors influencing microbial community structures, as it is well known that copper and arsenic, but not calcium, have impairing biological effects (Canadian Council of Ministers of the Environment 2007). Ca.Cu.As resulted to be the only significant explanatory variable for *Bacteria* and *Alphaproteobacteria* communities, and a significant descriptor for *Pseudomonas* community. However, separate contribution from each element was unfeasible in the present study due to the high correlation ($R^2 > 0.98$) among them. These results are in agreement with a previous study of copper-impacted soils located at upstream areas from Aconcagua valley, where copper affected bacterial communities (Altimira *et al.* 2012). Several *Sphingomonas* (*Alphaproteobacteria*) strains isolated from these copper-polluted soils showed high copper tolerance and harbored the multicopper oxidase *copA* gene (Altimira *et al.* 2012). Metal resistance determinants have been described in *Proteobacteria* and *Firmicutes*, including *Alcaligenes*, *Ralstonia*, *Cupriavidus*, *Pseudomonas*, *Sulfurospirillum*, *Shewanella*, *Enterococcus*, *Staphylococcus*, *Bacillus* and *Desulfitobacterium* genera (Nies 1999; Oremland and Stolz 2005). The metal-resistant model *Cupriavidus metallidurans* CH34 is a Betaproteobacterium with metal resistance determinants for copper, mercury, lead, cadmium, zinc, cobalt, nickel and arsenic (Mergeay *et al.* 2003). In soil, copper may affect negatively *Acidobacteria* and positively *Actinobacteria* and *Firmicutes* (Wakelin *et al.* 2010). Copper adaptation has been related to the OM composition (Lejon, Pascault and Ranjard 2010). *Firmicutes* (mainly *Bacillus* species) and *Gammaproteobacteria* (mainly *Pseudomonas* species) seem to be the main responders toward arsenic pollution in soil (Valverde *et al.* 2011). Overall, response, resistance and resilience of microbial communities toward pollution are highly dependent on the disturbance nature, soil history and soil type (Griffiths and Philippot 2013).

TPH and PAH are two major petroleum fractions which could be associated with the crude oil refinery activity close to the Aconcagua River estuary (Fig. 1). High levels of PAH on the seacoast close to the Aconcagua River mouth have been reported (Palma-Fleming *et al.* 2008). In Aconcagua River estuarine soils, both petroleum fractions were present in low amounts (TPH <2 mg kg^{-1} and PAH $<25\,\mu$g kg^{-1}), indicating that a decade after the last massive crude oil spill, hydrocarbons may have decayed to harmless levels. However, hydrocarbon levels were significantly explaining the bacterial structures of *Actinobacteria* (PAH), and *Pseudomonas* and *Bacillus* genera (TPH and PAH). Several members belonging to *Actinobacteria*, *Proteobacteria* and *Firmicutes* phyla have successfully evolved toward the use of hydrocarbons as carbon source for growth. Aerobic *n*-alkane degradation genes have been reported in *Burkholderia*, *Acinetobacter*, *Pseudomonas*, *Alcanivorax*, *Oleiphilus*, *Mycobacterium*, *Rhodococcus*, *Bacillus*, *Nocardia* and *Prauserella* genera (van Beilen *et al.* 2003; Fuentes *et al.* 2014). The initial step in aerobic degradation of alkanes is oxidation via an alkane oxygenase, carried out by either a multimeric monooxygenase or by a cytochrome P450 monooxygenase (Fuentes *et al.* 2014). Multimeric monooxygenases vary in substrate range and specificity, but structural and catalytic motifs are conserved among them (van Beilen *et al.* 2003). Hydroxylated products are subsequently transformed via oxidation into fatty acid before funneling into β-oxidation and Krebs cycle (van Beilen and Funhoff 2007; Peng *et al.* 2008; Fuentes *et al.* 2014). Aerobic microbial metabolism of PAH involves an initial dioxygenation reaction by a RHD to yield the corresponding *cis*-diol product, which is rearomatized by a dihydrodiol dehydrogenase (Peng *et al.* 2008; Pieper and Seeger 2008; Seeger and Pieper 2010). Products are funneled into central aromatic catabolic pathways (Phale *et al.* 2007; Fuentes *et al.* 2014). Culture-independent surveys also suggest that these phyla primarily respond to oil spills, discarding a culture bias toward these groups (Uhlik *et al.* 2012; Yergeau *et al.* 2012). *Bacillus* and *Pseudomonas* are two ubiquitous genera presenting outstanding metabolic versatility (Lin and Cai 2008; Eppinger *et al.* 2011; Silby *et al.* 2011) and are well adapted for hydrocarbon degradation and heavy metal resistance. However, Ca.Cu.As was a significant descriptor only for *Pseudomonas*, whereas petroleum fractions consistently explained both bacterial communities. Other groups such as methanotrophic *Alpha-* and *Gammaproteobacteria* or anaerobic sulfate-reducing alkane-degrading *Deltaproteobacteria* were not analyzed. Further analyses by next generation sequencing should be necessary to unveil the relationships between these groups and environmental variables.

Nitrogen, phosphorus and potassium are widely used as fertilizers in agriculture. The relationship between these three elements and microbial communities at the Aconcagua River estuarine soils was ambiguous. Phosphorus showed high correlation ($R^2 > 0.98$) with magnesium and OM; therefore, they were treated as one combined variable. OM was also correlated with potassium, calcium, zinc, manganese, iron, copper and arsenic ($0.90 < R^2 < 0.98$), suggesting the chelation and adsorption of cationic ions to OM (Du Laing *et al.* 2007). However, correlations among OM and these elements were lower and too inconsistent to allow grouping all in one variable. OM.P.Mg variable was a significant descriptor only for *Fungi* (Fig. 2F). On the other hand, nitrogen was significant for *Actinobacteria* (Fig. 3C). In order to test the robustness of these results, db-RDA was also performed using different criteria, as constrained techniques outputs can be influenced by initial set of constraints (Legendre and Legendre 2012). Thus, db-RDA was performed with a reduced set of anthropogenic constraints as the initial environmental matrix,

i.e. OM.P.Mg, N and K (fertilizers), Ca.Cu.As (mining), TPH and PAH (petroleum refinery). Soil pH was also included for being extensively reported as a global descriptor (Lauber *et al.* 2009; Vasileiadis *et al.* 2013). Conductivity, which accounts for global ion concentration, was not included because it was not a significant descriptor in previous analyses (db-RDA biplots, Fig. 2–4). Results using these criteria for db-RDA were highly consistent, with only three exceptions. First, for *Bacteria*, potassium (K) resulted a significant ($P > 0.01$) descriptor in addition to Ca.Cu.As. Output included a second RDA axis explaining an additional 26.7% of community variance. Second, for *Betaproteobacteria*, K descriptor was significant replacing boron variable, with a subtle drop in the overall explained variance (83.6%). Third, PAH descriptor was not significant for *Pseudomonas* community, with pH and TPH explaining only 57.9% of variance (Fig. S1, Supporting Information). Despite these three exceptions, these additional db-RDA outputs support the results obtained with the initial criteria, i.e. taking into account all measured variables. Overall, anthropogenic variables together with pH were for far the most consistent explaining the observed variance in community structure independent of the original set of constraints. Additionally, stopping criteria used during forward selection minimize the type I error, avoiding the inclusion of false positives into the RDA model (Blanchet, Legendre and Borcard 2008; Oksanen *et al.* 2013).

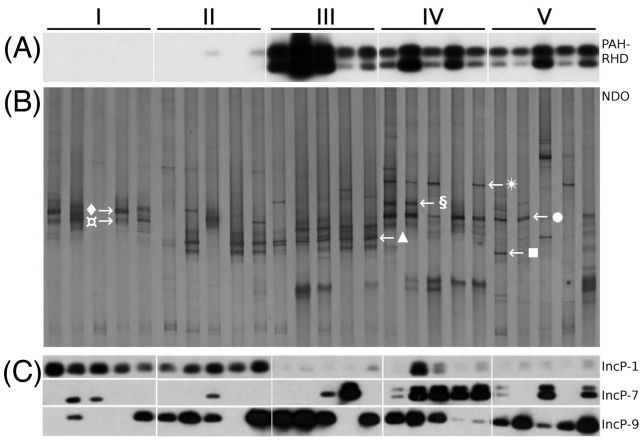

Figure 5. Distribution of PAH-RHD and *ndo* genes and IncP plasmids along Aconcagua river estuarine soils. PAH-RHD gene content was determined by Southern blot hybridization of PCR products and revealed by chemiluminescence (**A**) and PCR-DGGE fingerprints of *ndo* genes (**B**). Distribution of IncP-1, IncP-7 and IncP-9 plasmids was detected by Southern blot hybridization of PCR-amplified *trfA* (IncP-1, upper panel), *rep* (IncP-7, middle panel) and *ori-rep* (IncP-9, lower panel) genes, and revealed by chemiluminescence (**C**). Roman numbers indicate the same sampling sites analyzed by DGGE. Symbols in panel B indicate the cloned and sequenced bands: section symbol, AC01-AC03; asterisk, AC04-AC05; filled circle, AC06; filled triangle, AC07-AC09; filled square, AC10; filled diamond, AC11; currency sign, AC12-AC13.

Functional characterization of microbial communities

In order to explore genetic determinants related to the degradation of aromatic compounds, PAH-RHD genes were studied. RHD gene content usually becomes enriched after exposition of soil to PAHs and thus gene shifts can be an indicator of PAH exposure (Ní Chadhain *et al.* 2006; Ding *et al.* 2010). RHDs catalyze the first step of bacterial aerobic PAH degradation (Fuentes *et al.* 2014). Highly degenerated primers targeting the RHD α-subunit genes from several Gram-negative and Gram-positive bacteria were used (Ding *et al.* 2010). In general, RHD genes were in low abundance in all sites, as no RHD gene amplicon could be observed in agarose gel electrophoresis (data not shown). Nonetheless, Southern blot hybridization revealed the presence of RHD genes only at upstream sites III, IV and V (Fig. 5A). This suggests that RHD gene content could not be related to PAH content but with other physicochemical variables such as OM content, pH or soil texture (Table 1). Among PAH-RHD enzymes, the NDO family is usually regarded as the dominant group of enzymes catalyzing the initial dioxygenation step of low-molecular-weight PAHs such as naphthalene (Gomes *et al.* 2007). PCR-DGGE analysis revealed low richness and a heterogeneous distribution of *ndo* genes along the Aconcagua River estuary. Sites III and IV showed a higher richness of bands compared to sites II and V, whereas site I showed the lowest *ndo* gene diversity (Fig. 5B). Sequencing of 13 *ndo* genes clones revealed that they belong to two main bacterial groups. Seven were related to *ndo* genes from *Pseudomonas* (*Gammaproteobacteria*), whereas the other six were closely related to Betaproteobacterial *ndo* genes from *Ralstonia*, *Comamonas* and *Polaromonas* strains (Fig. S2, Supporting Information). Dominance of *Pseudomonas ndo* genes is not surprising since this genus is involved in PAH degradation and is usually enriched facing hydrocarbon pollution (Ruberto, Vazquez and Mac Cormack 2003; Ma, Wang and Shao 2006). *Pseudomonas* was the most homogeneous community along the estuary (Fig. 4A). However, *Pseudomonas*-related *ndo* genes should not necessarily be homogeneous as well, due to the flexible genetic pool located in mobile genetic elements. Here, the heterogeneous distribution of *ndo* genes contrasted with the homogeneous distribution of *Pseudomonas* community. However, in this study the genes were cloned as a pool and therefore it was not possible to determine to which site belonged each *ndo* gene. The petroleum aliphatic fraction is less recalcitrant than the PAH fraction and is usually more easily degraded. Genes coding for alkane monooxygenation systems, such as AlkB-like or cytochrome P450, were not analyzed as they are not necessarily related to petroleum pollution. These alkane monooxygenation systems can use substrates other than alkanes (e.g. fatty acids) (van Beilen and Funhoff 2007), contrasting with PAH-RHD that are only involved in degradation of PAH and derived xenobiotics (Pieper and Seeger 2008; Kweon *et al.* 2010).

Hydrocarbon and xenobiotic degradation and heavy metal resistance genes can be located in mobile genetic elements, decoupling function and structure of microbial communities (Peng *et al.* 2008; Pieper and Seeger 2008; Seeger and Pieper 2010; Shintani *et al.* 2010; Boon *et al.* 2014). As several PAH catabolic routes and heavy metal resistance genes are encoded in IncP plasmids (Izmalkova *et al.* 2005; Sevastsyanovich *et al.* 2008; Shintani *et al.* 2010), which might contribute to bacterial adaption to polluted environments, the presence of IncP plasmids in the five Aconcagua River estuarine soils was analyzed (Fig. 5C). Promiscuous IncP-1 plasmids can be stably maintained in almost all Gram-negative bacteria, and have been transferred to Gram-positive bacteria, yeasts and eukaryotic cell lines (Adamczyk and Jagura-Burdzy 2003). On the other hand, IncP-7 and IncP-9 have a narrow host range and are found preferentially in *Gammaproteobacteria* (mainly *Pseudomonas*) (Shintani *et al.* 2010). IncP-1 plasmid levels were found predominantly at downstream sites I and II (Fig. 5C). IncP-1 plasmid distribution contrasted with the distribution of PAH-RHD genes (Fig. 5A). On the other hand, IncP-7 plasmids were found predominantly at site IV and, to a lesser extent, at sites V and III (Fig. 5C). Except site IV, the other estuarine sites showed heterogeneous distribution of IncP-7 plasmids. IncP-9 plasmids showed to be more widely distributed

along the estuary (Fig. 5C). Heterogeneous signals among replicates and sites, especially for IncP-7 and IncP-9, are indicative of a heterogeneous rather than even distribution of plasmids along the estuary (Fig. 5C). IncP-1 plasmids are the most promiscuous replicons (Adamczyk and Jagura-Burdzy 2003), but they were detected only at specific sites. IncP-1 plasmids can carry genes related to heavy metal and antibiotic tolerance/resistance, as well as xenobiotic degradation. The IncP-1 content at sites with lower amounts of heavy metals suggests that they could be related to other factors than metal resistance. IncP-7 distribution was similar to PAH-RHD genes, with more positive signals toward upstream sites. Even when IncP-7 and IncP-9 plasmids are of narrower host range, both were more widely distributed along estuary soils than IncP-1 plasmids and could be indicative of bacterial adaptation toward xenobiotic (e.g. PAH) degradation.

In conclusion, the present study showed a high degree of variability in microbial community structures at five river sites in a reduced area of ~1500 m from the Aconcagua River estuary. A close relationship between specific soil parameters (i.e. pH), anthropogenic activities (i.e. copper mining, crude-oil refinery and agriculture) and microbial community structures along the Aconcagua River estuary was observed. The microbial resistance and resilience are generally diminished by pollution and other disturbances (Griffiths and Philippot 2013). Therefore, the effects of anthropogenic activities on microbial communities in industrial regions should be studied toward the protection of the ecosystems for a sustainable development. The present study revealed the relationship among environmental factors (either anthropogenic or natural) such as pH, copper, arsenic, hydrocarbons, organic matter, phosphorous and nitrogen on the soil microbial community structure and function along the Aconcagua River estuary.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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