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Chlor-alkali plant contamination of Aussa River sediments induced a large Hg-resistant bacterial community

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ABSTRACT

A closed chlor-alkali plant (CAP) discharged Hg for decades into the Aussa River, which flows into Marano Lagoon, resulting in the large-scale pollution of the lagoon. In order to get information on the role of bacteria as mercury detoxifying agents, analyses of anions in the superficial part (0–1 cm) of sediments were conducted at four stations in the Aussa River. In addition, measurements of biopolymeric carbon (BPC) as a sum of the carbon equivalent of proteins (PRT), lipids (LIP), and carbohydrates (CHO) were performed to correlate with bacterial biomass such as the number of aerobic heterotrophic cultivable bacteria and their percentage of Hg-resistant bacteria. All these parameters were used to assess the bioavailable Hg fraction in sediments and the potential detoxification activity of bacteria. In addition, fifteen isolates were characterized by a combination of molecular techniques, which permitted their assignment into six different genera. Four out of fifteen were Gram negative with two strains of *Stenotrophomonas maltophilia*, one *Enterobacter* sp., and one strain of *Brevibacterium frigoritolerans*. The remaining strains (11) were Gram positive belonging to the genera *Bacillus* and *Staphylococcus*. We found *merA* genes in only a few isolates. Mercury volatilization from added $HgCl_2$ and the presence of plasmids with the *merA* gene were also used to confirm Hg reductase activity. We found the highest number of aerobic heterotrophic Hg-resistant bacteria (one order magnitude higher) and the highest number of Hg-resistant species (11 species out of 15) at the confluence of the River Aussa and Banduzzi's channel, which transport Hg from the CAP, suggesting that Hg is strongly detoxified [reduced to Hg(0)] at this location.

1. Introduction

The sediments of Marano lagoon are polluted by mercury (Hg), not only by the Isonzo River that is impacted by former Hg mining in Idrija, Slovenia, but also by the Aussa–Corno River system that enters the lagoon (Piani et al., 2005). The CAP employed the "Solvay Method" that used metallic Hg to separate sodium and chlorine from brine. The industry not only discharged Hg(0) into the river, but also excess brines, ammonia and limestone. In addition, at the same industrial site, cellulose was produced from cane (*Arundo donax* sp), which enriched sediments with organic carbon (Acquavita et al., 2012). In 1984, a modern wastewater treatment system was installed and a significant reduction of Hg in the environment was observed. However, diffuse and periodic cases of similar contamination still occur in several areas (Maserti and Ferrara, 1992; Lodenius, 1998; Biester et al., 2000; Hissler and Probst, 2006).

Covelli et al. (2009) reported that Hg in sediments in this region is mainly in its elemental form, as was expected since it was discharged into this form by the CAP, although a minor fraction was also chelated by organic matter. Therefore, Hg contamination of sediments might induce a selection of Hg-resistant bacterial populations which attenuate Hg toxicity. During the last five decades, scientific aspects of Hg resistance in bacteria, from genes to environmental applications, have been investigated (Barkay et al., 2003)

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even in pristine environments such as in polar regions (Møeller et al., 2010). Hg-resistant bacteria possess the *mer* operon that may be located on a plasmid, transposon, or chromosome. Its genetic components include the gene sequence *merR*, *merT*, *merP*, *merC*, *merD*, *merA*, and *merB* (Nascimento and Chartone-Souza, 2003). The gene *merA* codes for the enzyme mercuric reductase (MerA), which is responsible for Hg^{2+} reduction to Hg(0) and its subsequent volatilization and removal. The passive efflux of Hg(0) from bacterial cells is considered a beneficial adaptation and in the past it was suggested as one of biotechnological process to reduce Hg contamination (Barkay et al., 2003). In this way, Hg-resistant bacteria can survive at high Hg concentration in the environment while contributing to the detoxification of the surrounding habitat.

To better understand Hg toxicity and bioavailability in the Aussa River and lagoon system, the surficial layer of sediments (0–1 cm) was investigated in terms of certain anions and labile organic molecules such as proteins (PRT), carbohydrates (CHO) and lipids (LIP), and their carbon equivalent sum, biopolymeric carbon (BPC). Furthermore, these biochemical molecules are synthesized and/or utilized by microbes under either aerobic or anaerobic conditions in the presence of various nutrients, which are adsorbed to sediment particles and stimulate bacterial growth. Particular attention was dedicated to isolation and identification of strains harboring *merA* genes by using molecular and chemical tests to determine Hg^{2+} reduction activity *in vivo*.

2. Materials and methods

2.1. Sampling

Sediment samples were collected in October 2008. Sampling was performed by boat at four stations (A2, A3, A4 and A6) along the main axis of the Aussa River (Fig. 1). Measurements of temperature, pH, and Eh were conducted *in situ* with conventional tools. Surface sediments (0–1 cm) were sampled using a Kajak light gravity corer. One aliquot of sediments (100 g) was freeze-dried and used for analytical determination of biopolymeric carbon, anions, and for Hg analyses. A second aliquot (50 g) was taken fresh and used the same day for bacterial cell counting, and isolation of total and Hg-resistant bacteria.

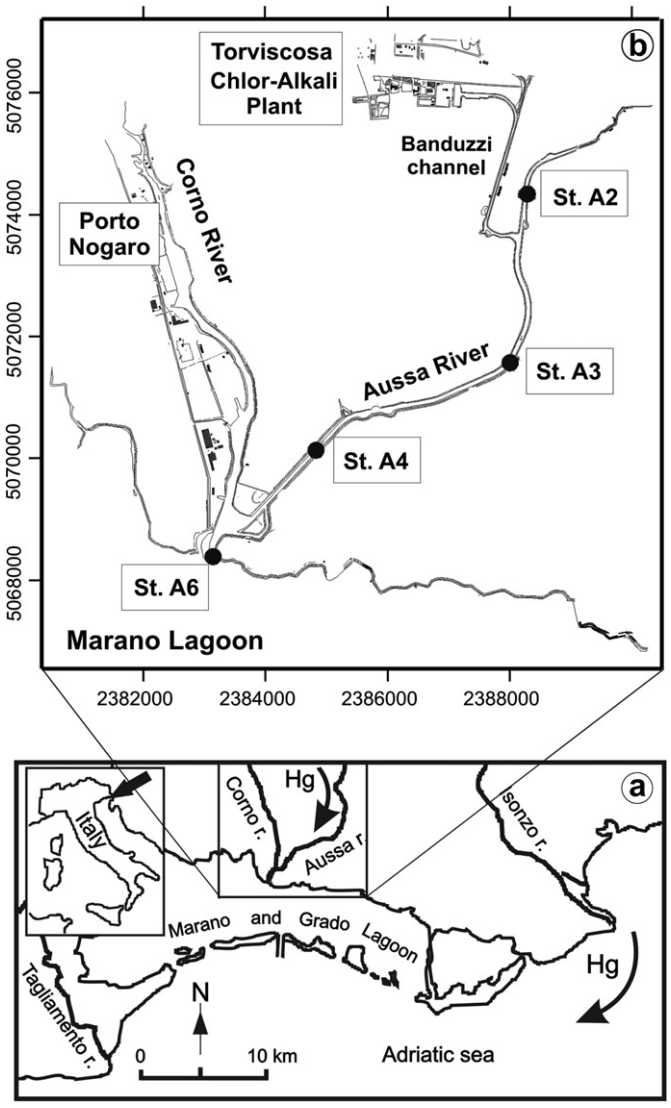

Fig. 1. Sampling sites (A2, A3, A4 and A6) of superficial sediments at Aussa River receiving Torviscosa chlor-alkali plant outflow from Banduzzi's Channel.

2.2. Biochemical composition of sediment polymeric organic matter

The biopolymeric matter is composed of protein (PRT), lipids (LIP) and carbohydrates (CHO), the three fractions of the most labile organic matter that are susceptible to temporal changes (Baldi et al., 2012). The methods to determine PRT, CHO and LIP were reported elsewhere (Baldi et al., 2011). Briefly, PRT content was determined spectrophotometrically using Coomassie Brilliant Blue (Bradford, 1976; Mayer et al., 1986) after extraction of 4 g of freeze-dried sediment with 8 ml 0.5 N NaOH in an ultrasonic bath for 2 h at 40 °C (Nunn and Keil, 2006). After reaction with the dye, absorbance was determined spectrophotometrically at 595 nm. PRT concentrations were reported in Bovine Serum Albumin (BSA, Biorad) equivalents.

CHO content was determined by Dubois et al. (1956) after extraction of 2.5 g sediment with 50 ml 1 N CH_3COOH for 4 h at 20–30 °C in an ultrasonic bath (Mecozzi et al., 2000). CHO concentration was expressed as D(+)-glucose equivalents after reaction with 96% sulfuric acid and 5% phenol followed by spectrophotometric detection at 485 nm.

LIP were extracted overnight from 2 g of dried sediment by direct elution with chloroform and methanol (2:1 v/v) at 4 °C (Bligh and Dyer, 1959) and LIP analysis was carried out by the charring method with H_2SO_4 at 200 °C for 15 min (Marsh and Weistein, 1966). Tripalmitin was used for standard solutions and the absorbance was determined at 375 nm.

Three replicates per each sediment sample were analyzed. For each biochemical class, results are expressed as per gram of sediment dry weight (s.d.w.). For the evaluation of the complexing ability of labile OC on Hg species, the three main biochemical classes, PRT, CHO and LIP were converted into carbon equivalents, assuming their conversion factors of 0.49, 0.40 and 0.75, respectively (Fichez, 1991). The sum of carbon equivalents of constituted biopolymeric carbon (BPC) were expressed as $\mu gC\ g^{-1}$ d.w.

2.3. Water-washed anions

Quantification of anions was carried out to correlate biochemical classes of biopolymeric carbon with anions. Anions were washed from 1 g of frozen sample with 50 ml of milliRo water after 3 min vortexing at 5 °C. The supernatant was filtered through Whatman black ribbon 589/1. An aliquot of 20 µl was injected into a pre-loop filter with a 0.45 µm cartridge ion chromatograph (METROHM 761 Compact IC) with a conductivity detector. The

accuracy of the results was verified with certified standard solutions as reported elsewhere (Baldi et al., 2011).

2.4. Determination of Hg (Hg-T) in sediments

About 50–100 mg of freeze-dried sediment was digested by acid (conc. HNO_3) and measured by the gold amalgamation Cold Vapour Atomic Absorption Spectrophotometer (CV AVS) (Milton Roy). The detection limit, calculated on the basis of three standard deviations of the procedural blanks was 1 ng g^{-1}. Reproducibility of the method was 3–4%. A detailed description of the methods is reported elsewhere (Horvat et al., 1991). The accuracy of the results was verified by the analyses of certified reference materials.

2.5. Determination of methylHg (MeHg) in sediment samples

Approximately 100 mg of freeze-dried sample was processed by a method consisting of acid dissolution/solvent extraction/aqueous phase ethylation/isothermal GC/CV AFS detection. The method is well described (Horvat et al., 1993a, b; Liang et al., 1994). The limit of detection, calculated on the basis of three standard deviations of procedural blanks, was about 50 pg MeHg/g. Repeatability of the method was about 10% (Horvat et al., 1993a, b; Liang et al., 1994). The accuracy of the results was verified by the analyses of certified reference materials.

2.6. Total cell counts

Immediately after sampling, fresh sediments were fixed by the addition of 2% paraformaldehyde and stored at 4 °C in the dark. Before analysis, sediment slurries were prepared by thoroughly shaking the sediments that were diluted 1000-fold in 0.1 M particle-free tetrasodium pyrophosphate dispersing solution. A 1–3 ml aliquot of the suspension were incubated 15 min with 4,6-diamidino-2-phenylindole (DAPI) (final concentration 1 μg ml^{-1}) and filtered through a black polycarbonate membrane (0.2 μm pore size, 25 mm diameter, Isopore, Millipore, Milan, Italy). Cell numbers were determined by counting stained cells on 20 randomly selected fields using a Zeiss Axioplan epifluorescence microscope with excitation/emission filters of 365/420 nm.

2.7. Counting and isolation of Hg-resistant strains

Total aerobic heterotrophic bacteria and Hg-resistant bacteria were counted and isolated. 2 g of fresh sediments were diluted in 10 ml of sterile water treated for 3 min in an ultrasonic bath to detach most of bacteria from sediment particles. The dry weight of sample was calculated by drying to a constant weight at 50 °C. After 1 min of sediment decantation, the suspension was diluted by ten. A 0.1 ml aliquot of each dilution was spread on solid Nelson medium, at pH 7.5 containing per liter: 2 g D-Glucose, 5 g Casamino acids, 1 g yeast extract, 10 g NaCl, 2.3 g $MgCl_2 \cdot 6H_2O$, 3 g KCl, Bacto-agar (Difco), 15 g, and 5 μg Hg ml^{-1} as $HgCl_2$. Control plates did not receive additional Hg. After 24 h incubation at 30 °C, colony forming units (CFU) were determined. Colonies with different shapes, colors and consistency were isolated and stored at −80 °C in 20% glycerol for further analyses.

2.8. Determination of gaseous Hg in pure cultures

Stored isolated strains were retrieved from −80 °C on liquid Nelson medium amended with 5 μg ml^{-1} $HgCl_2$. For the Hg volatilization test, 2 ml of dense culture (1.0 O.D. at 600 nm) was transferred in 18 ml test tubes sealed with Mininert valve caps (Supelco). Volatile Hg species were collected from the headspace of vials using a gas-tight syringe and injected into a closed double amalgamation system connected to a CV AFS analyzer system. Hg on the sampling gold trap was then released by heating (~500 °C) for 1 min in a flow of Ar into a permanent gold trap, released again (heating for 1 min, ~500 °C) and detected by a CV AFS analyzer (Tekran 2500). The system was calibrated by gas phase Hg [Hg(0)] kept at 15 °C (Tekran, model 2505 Hg vapor calibration unit). An aliquot of 10–20 μl was transferred with a gas-tight syringe into the measurement train through a septum. The amount of Hg injected was calculated from the gas law and a correction for the difference in temperature of the gas phase and the syringe was also applied. The detection limit was 4 pg based on three standard deviations of the procedural blank. The repeatability of the method was 4% (Horvat et al., 2003).

2.9. Analysis of plasmid content

Analytical amounts of plasmid DNA were obtained from 1.5 ml bacterial cultures using either the alkaline lysis method (Sambrook et al., 1989) or the commercial Kit Plasmid Miniprep (Qiagen) method according to the manufacturer's instructions. The presence of plasmid molecules was analyzed by agarose gel (0.8% w/v) electrophoresis in TAE buffer (0.04 M Tris-Acetate, 0.01 M EDTA) containing 0.5 μg/ml (w/v) of ethidium bromide. For preparation of cell lysates, bacterial colonies grown overnight at 28 °C on LB (Luria Broth) plates were resuspended in 20 μl of sterile distilled water, heated to 95 °C for 10 min, and cooled on ice for 5 min. Genomic DNA extraction was carried out as previously described (Giovannetti et al., 1990).

2.10. PCR amplification of 16S rRNA genes from bacterial isolates

Two μl of each cell lysate were used for the amplification via polymerase chain reaction (PCR). Amplification of 16S rRNA genes was performed in a total volume of 50 μl containing 1X Reaction Buffer, 150 μM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 250 μM, and 2.0 U of Polytaq DNA polymerase (all reagents obtained from Polymed, Florence, Italy) and 0.6 μM of each primer [P0 5′ GAGAGTTTGATCCTGGCTCAG, and P6 5′ CTACGGCTACCTTGTTACGA] (Grifoni et al., 1995). A primary denaturation treatment of 90 s at 95 °C was performed and amplification of 16S rRNA genes was carried out for 30 cycles consisting of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, with a final extension of 10 min at 72 °C.

Amplification of *merA* genes was carried out using the following primer sets, set 1 (merAgram-forward CCGTCCAAGATCATGAT merAgram-reverse, GGRTCGGTRAACACCAC) and set 2 (merAgram + forward GGAAGAAMACCRAATAC merAgram + reverse, CCTTCWGHCATTGTTA), for Gram negative (Gram-) and Gram positive (Gram+) bacteria, respectively. The two primer sets were designed as follows: for both Gram and Gram bacteria all the *merA* sequences available in database were retrieved. Then, a multialignment using a reduction set of the retrieved sequences was constructed in order to check for the presence of highly conserved regions that might represent the target sites for *ad hoc* designed primer. Once the conserved regions were identified, two primer sets were designed (one for the Gram and the other for the Gram bacteria) and the presence of the identified target sequence was checked in each sequence of the complete multialignment. The amplification profiles were as follows; for PCR employing the primer set 1 a primary denaturation at 95 °C for 2 min was followed by 30 cycles consisting of 95 °C for 30 s, 51 °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. For PCR employing the primer set 2 a primary denaturation at 95 °C for 2 min was followed by 30 cycles consisting of 95 °C for 30 s, Ta (48, 45, 43) °C for 45 s, and

72 °C for 1 min, with a final extension at 72 °C for 10 min. The expected size of the amplicon was about 930 and 540 bp for Gram− and Gram+ bacteria respectively, corresponding to a *merA* region spanning from bp 451 and 1381 and bp 1330 and 1870 for Gram− and Gram+ bacteria, respectively. Thermal cycling was performed with a MasterCycle Personal Thermal Cycler (Eppendorf); 10 μl of each amplification mixture were analyzed by agarose gel (0.8% w/v) electrophoresis in TAE buffer containing 0.5 μg/ml (w/v) ethidium bromide.

Each sequence was submitted to Genebank; the 16S rRNA gene sequences were assigned the accession number reported in Table 2; the *merA* genes from strains AU3-Mi-01, AU4-01-1098, and AU3-03 were assigned the following accession numbers JQ266282, JQ266280 and JQ266275, respectively.

2.11. Sequencing of 16S rRNA and merA genes

Amplicons corresponding to the 16S rRNA or merA genes (observed under UV, 312 nm) were excised from the gel and purified using the "QIAquick" gel extraction kit (QiAgen, Chatsworth, CA, USA) according to manufacturer's instructions. Direct sequencing was performed on both DNA strands using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and the chemical dye terminator (Sanger et al., 1977). Each sequence was submitted to GenBank and assigned the accession number shown in Table 1.

2.12. Homologs retrieval and phylogenetic analysis

BLAST probing of the DNA databases was performed with the BLASTn option of the BLAST program (Altschul et al., 1997), using default parameters. Nucleotide sequences were retrieved from the GenBank, EMBL, and RDP databases. The ClustalW program (Thompson et al., 1994) was used to align the 16S rRNA gene sequences obtained with the most similar ones retrieved from the databases. Each alignment was checked manually, corrected, and then analyzed using the neighbor-joining method (Saitou and Nei, 1987) according to the model of Kimura 2-parameter distances (Kimura, 1980). The Molecular Evolutionary Genetics Analysis 4 (MEGA4) software (Tamura et al., 2007) was used to construct phylogenetic trees whose robustness was evaluated by 1000 bootstrap resamplings.

2.13. Random amplified polymorhic DNA (RAPD) analysis

Random amplification of DNA fragments from bacterial strains was carried out in 25 μl containing 1X NH_4 Reaction Buffer, 300 μM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 μM, 0.5 U of Biotaq DNA polymerase (all reagents obtained from Bioline, London, United Kingdom) 500 ng of primer 1253 (5′ GTTTCCGCCC 3′) (Mori et al., 1999) and 2 μl of lysate cell suspension prepared as described above. The reaction mixtures were incubated in a thermal cycler MJ Research PTC 100 Peltier Thermal Cycler (CELBIO) at 90 °C for 1 min, and 95 °C for 90 s. They were then subjected to 45 cycles, each consisting of incubation at 95 °C for 30 s, 36 °C for 1 min, and 75 °C for 2 min. Finally, the reactions were incubated at 75 °C for 10 min and then at 60 °C for 10 min, and 5 °C for 10 min. Thermal cycling was performed with a MasterCycle Personal Thermal Cycler (Eppendorf). Reaction products were analyzed by agarose (2% w/v) gel electrophoresis in TAE buffer containing 0.5 μg/ml of ethidium bromide.

3. Results and discussion

3.1. Sediment characterization

The first centimeter of sediments was characterized by determining *in situ* pH, Eh and temperature for each station, and they were mainly anoxic at neutral pH. In addition, biopolymeric carbon, Hg-T, MeHg and anions were also analyzed in freeze-dried samples (Table 1). During sampling in October, the sediment temperature was from 19 to 21 °C due to a mild autumn.

In general, the three biochemical classes, i.e. PRT, CHO and LIP varied, especially at stations A2 and A3, before and after Banduzzi's channel effluent from the CAP (Fig. 2A). At station A3, CHO was the most abundant class. Cellulose residues might explain this finding from the previous activity of cellulose production from cane (*A. donax*). Sediments collected at station A2 were very anoxic (−325 mV) (Table 1) and PRT was the prevailing biochemical class, probably because it is less degradable under these conditions (Baldi et al., 2010). However, all these parameters were decreasing from upstream to downstream (Fig. 2A), which was also true of the water leached anions Cl^-, SO_4^{-2}, NO_3^- (Table 1). Cl^- concentrations were correlated with BPC values ($r^2 = 0.927$, $n = 3$) (Fig. 2B), except at station A2, which exhibited high concentrations of BPC due to a high PRT content. Water leached anion content was very high and Cl^- was very well correlated with NO_3^- ($r^2 = 0.920$, $n = 4$), and SO_4^{-2} ($r^2 = 0.988$, $n = 4$) (Table 1). At station A2 and A3, Cl^- had a concentration of almost 60 mg g^{-1} d.w. (Table 1; Fig. 2B), which is equal to a salinity of 110. Furthermore, salinity at station A3 was 3.5 times higher than in the sediments at station A6 and at Marano Lagoon (Baldi et al., 2012), which suggests an intense discharge of Cl^- typical of brines used in the Solvay process of the CAP. The Solvay process uses limestone, which is a source for carbonates and sulfates, and the brines are sources of chlorine and ammonia, which was partly converted to nitrates, and nitrites. So far, all these parameters tend to decrease from upstream to downstream with the exception of carbonate ions and Hg species (Table 1). Sediments at station A6 are affected by the brackish lagoon ecosystem with a significant content of organic matter of marine origin (Acquavita et al., 2012) and carbonates, which come from calcitic and dolomitic grains (Covelli et al., 2009).

A good correlation of Hg was found with PRT in sediments at Marano and Grado Lagoons (Baldi et al., 2012), whereas in the current study the complete lack of correlation with chemical and biochemical data suggest a different behavior for Hg. However, a good correlation was found between Hg-T and MeHg ($r^2 = 0.987$; $n = 4$) in sediments, but MeHg values were three order of magnitude lower than those of Hg-T. This significant difference was

Table 1
Determination of physical and chemical parameters in sediments of 4 stations in Aussa River.

Stations	*T*	pH	Eh	Cl^-	NO_3^-	NO_2^-	SO_4^{-2}	HCCV	Hg-total	SD	MeHg	SD
	°C	pH	mV	mg g^{-1}					μg g^{-1}		ng g^{-1}	
A2	19.1	7.3	−320	60.2	0.119	0.065	2.34	54.5	0.715	0.024	0.858	0.023
A3	19.1	7.3	−131	60.8	0.137	0.067	2.16	46.5	1.841	0.070	1.637	0.064
A4	19.1	7.25	−140	44.8	0.115	n.d.	1.62	42.8	4.019	0.145	2.690	0.097
A6	21.6	7.3	6	17.1	0.070	n.d.	0.63	47.4	5.264	0.190	3.796	0.137

Table 2
16S rRNA gene sequence affiliation, with their closest phylogenetic neighbors, of sediments Aussa River bacterial isolates with ionic Hg reduction activity by volatilization of Hg(0) [Hg(0)vol].

Strain	Accession number	Hg(0)-vol	Most closely related entry n GeneBank			Family
			Accession number	Organism	Sequence identity (%)	
A3-1(2)	JQ266283	+	GU252110	*Bacillus* sp.	100	Bacillaceae
AU3-01	JQ266272	+	GU968177	*Staphylococcus* sp.	100	Staphylococcaceae
AU3-02	JQ266273	+	HQ432811	*Bacillus altitudinis*	100	Bacillaceae
AU3-03	JQ266274	+	AB681171	*Bacillus* sp. NBRC 100445	99	Bacillaceae
AU3-04	JQ266275	–	FJ267538	*Arthrobacter* sp.	100	Micrococcaceae
AU3-05	JQ266276	–	AM910303	*Bacillus* sp.	99	Bacillaceae
AU3-06	JQ266277	–	GU726864	*Enterobacter* sp.	99	Enterbacteriaceae
AU3-08	JQ266278	–	AM237368	*Staphylococcus xylosus*	100	Staphylococcaceae
AU3-09	JQ266279	+	GU384265	*Stenotrophomonas maltophilia*	99	Xanthomonadaceae
AU3-01-1098	JQ266271	+	HQ259723	*Staphylococcus pasteuri*	100	Staphylococcaceae
AU3-Mi-01	JQ266282	+	GU384265	*Stenotrophomonas maltophilia*	99	Xanthomonadaceae
AU4-02	JQ266281	+	HM566978	*Bacillus* sp.	99	Bacillaceae
A2-1(5)	JQ266269	+	HM565997	*Staphylococcus* sp.	99	Staphylococcaceae
AU4-01-1098	JQ266280	+	FN645734	*Stenotrophomonas maltophilia*	99	Xanthomonadaceae
A6-2(7)	JQ266270	+	HQ259723	*Staphylococcus pasteuri*	100	Staphylococcaceae

probably due to a strong demethylation activity carried out by Hg-resistant bacteria, as has been reported in more detail for sediments at Marano and Grado lagoons in autumn (Baldi et al., 2012; Hines et al., 2012).

In 2004, Covelli et al. (2009) found the highest Hg-T concentrations at station A3, situated at the confluence of the Banduzzi's channel and Aussa River (Fig. 3), and 90.6% of the Hg-T was in the form of Hg(0). Since then, the complete shutdown of the CPA probably caused a significant decrease in Hg-T at station A3 (river-channel confluence), and Hg-T was probably transported downstream to the river mouth (station A6) from the water column adsorbed to particulates. However, in four years an important Hg removal from superficial sediments occurred at station A3 (Fig. 3), the former most Hg polluted station. Therefore, we wondered whether Hg-reducing bacteria have a role in removing and detoxifying Hg from this sediment. For this reason a survey of these specific aerobic heterotrophic bacteria was performed in sediments from the Aussa River.

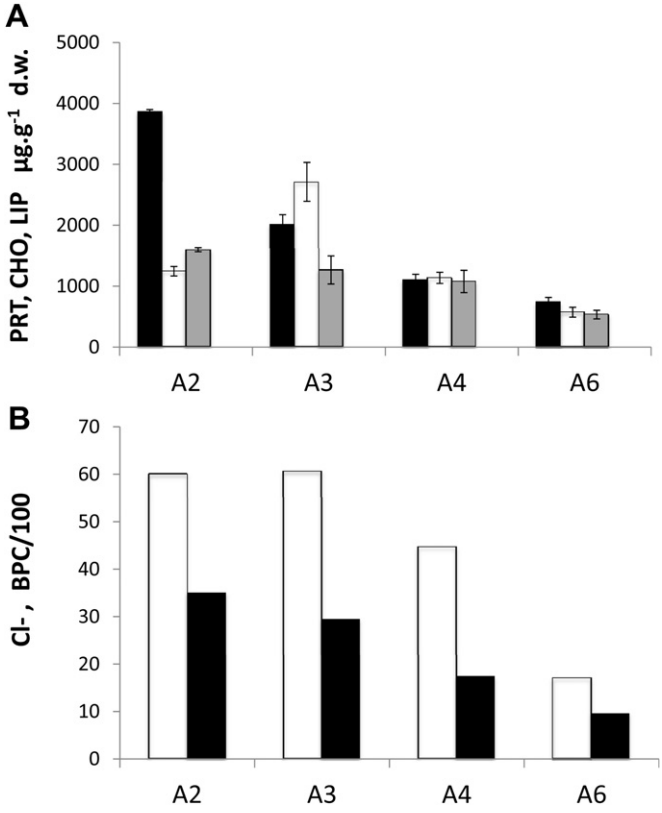

Fig. 2. A) Distribution of proteins (PRT – black histograms), carbohydrates (CHO – empty histrograms) and lipids (LIP – gray histrograms) along the sediments of Aussa river at the four stations; bars are standards deviations of duplicate analyses. B) Distribution of Cl^- anion (empty histograms – µg g^{-1} d.w.) and biopolimeric carbon equivalents (BPC – black histrograms – values expressed in µg g^{-1} d.w. in *Y* axis are divided by 100).

3.2. Bacterial activity, distribution and identification

In order to have more information and to try to explain the recent Hg distribution in Aussa River sediments, we isolated Hg-resistant bacteria, which are indicators of Hg pollution (Baldi et al., 1995; Pepi et al., 2006) and are involved in detoxifying Hg. We focused on the Hg-resistant bacterial community by counting aerobic heterotrophic Hg-resistant bacteria, and by isolating and identifying strains harboring *mer* genes.

The counts of total bacteria (cells g^{-1}) determined by DAPI staining and microscopy, and heterotrophic aerobic bacteria (CFU g^{-1}, d.w.) determined by plate-counting, were well correlated with

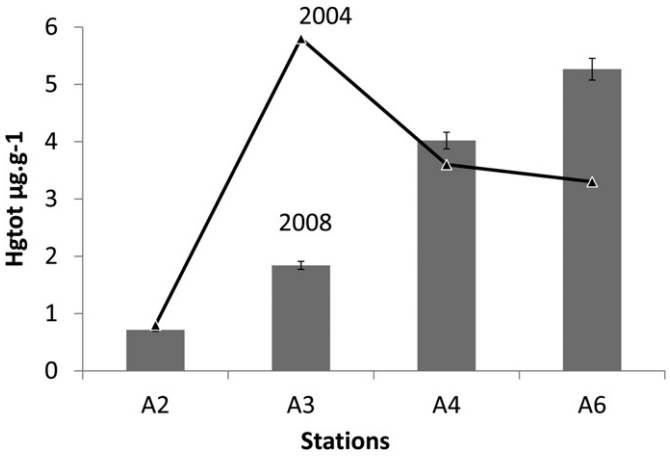

Fig. 3. Comparison of Hg-T in this study (gray histograms), sampling period 2008; bars indicate standard deviation of triplicate analyses. Hg-T determined in sediments of same stations in 2004 (continuous line), data from Covelli et al. (2009).

BPC in sediments except at station A2 (Fig. 4A). Station A2 is situated upstream of the Banduzzi channel outflow and aerobic heterotrophic bacteria at that site were probably inhibited by the very low redox potential (−320 mV).

The aerobic heterotrophic Hg-resistant bacteria (CFU g^{-1}, d.w.) were well correlated ($r^2 = 0.957$, $n = 4$) with total aerobic heterotrophic bacteria (Fig. 4B) and the viable counts of Hg-resistant bacteria were significantly higher ($r^2 = 0.961$; $n = 7$) than in the Marano Lagoon (broken line) (Baldi et al., 2012). This finding suggests that sediments of the Aussa River contain more bioavailable Hg-T than in Marano and Grado Lagoon. In addition, the highest abundance of Hg-resistant bacteria was found at station A3 downstream of the CAP, which underscores the importance of the CAP in stimulating the growth of Hg-resistant bacteria. On the other hand, the highest percentage (75%) of Hg-resistant bacteria was found at station A4, indicating that the effect of the CAP continues downstream. These types of parameters have been used previously as an indication of biologically available Hg in other habitats (Baldi et al., 1995).

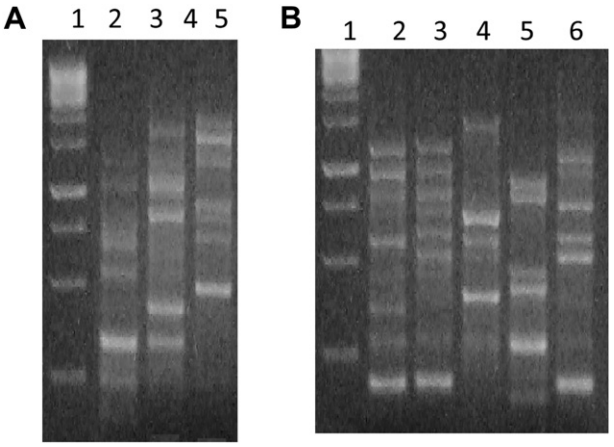

Fig. 5. Agarose gel electrophoresis of RAPD profiles obtained from *Staphylococcus* (A) and *Bacillus* (B) strains. (A) Lines: 1) marker, 2) AU3-01, 3) AU3-09, 4) A6-2(7), 5) AU3-01-1098. (B) Lines: 1) marker, 2) A3-1(2), 3) Au3-03, 4) AU3-02, 5) AU3-05, 6)AU4-02

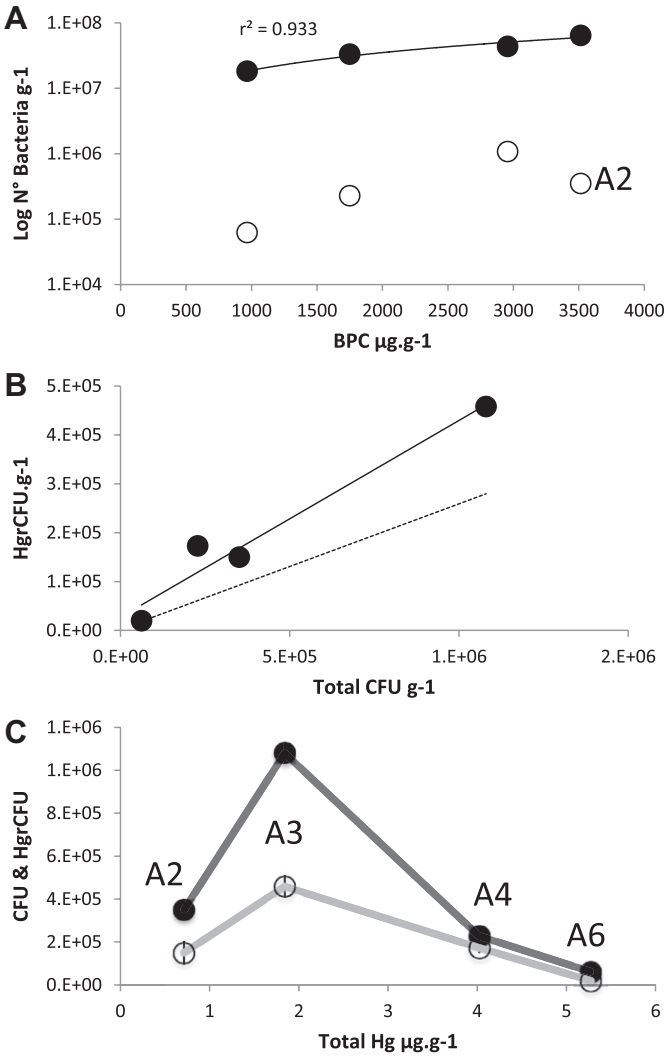

Fig. 4. A) Determination of total bacteria (black dots) in sediments by DAPI staining with epifluorescence microscope and total bacteria determined by plate-count method (empty dots) in correlation with biopolimeric carbon (BPC) in sediments. B) Correlation between Hg-resistant bacteria (CFU g^{-1}, d.w.) and total bacteria (black dots) ($r^2 = 0.957$; $n = 4$) determined by plate-count in respect to the correlation ($r^2 = 0.961$; $n = 7$) (broken line) found in Marano e Grado lagoons (Baldi et al., 2012). C) Distribution of Hg-resistant bacteria (Hg-CFU g^{-1}, d.w.) (empty dots) and total bacteria (CFU g^{-1}, d.w.) (black dots) determined by plate-counting along superficial sediments of Aussa River.

Bacterial abundance tends to decrease at high concentrations of Hg-T and MeHg except at station A3, which directly receives Banduzzi's Channel outflow from the CAP (Fig. 4C). Therefore, at this station the highest number of total heterotrophic bacteria and Hg-resistant bacteria were found and isolated. Seven Hg-resistant strains out of 11 were able to reduce Hg^{2+} from $HgCl_2$ additions and the resulting Hg(0) was partitioned into the headspace of vials by its physical properties (Table 2). Four other strains, isolated from stations A4 and A6, were also capable of volatilizing Hg(0) (Table 2). In total, 15 Hg-resistant strains were isolated from the 3 stations (A3, A4 and A6) located downstream of Banduzzi's channel and the CAP effluent. However, no Hg-resistant strains was isolated from sediments located upstream at the uncontaminated station A2 station, which again illustrates the strong effect of the CAP effluent on the presence and activity of Hg-resistant bacteria.

In order to connect each bacterial isolate to a given taxon, the nucleotide sequence of the 16S rRNA gene was determined. To this purpose, the 16S rRNA genes were amplified via PCR from the fifteen isolates as described above. An amplicon of the expected size was obtained from each strain (Fig. 5). Each amplicon was purified from an agarose gel and the nucleotide sequence was then determined. Each of the 15 sequences obtained was used as query to probe the nucleotide databases using the BLASTn option of the BLAST program (Altschul et al., 1997). Data obtained revealed that the fifteen isolates were representative of five bacterial genera, three Gram+ (*Staphylococcus*, *Arthrobacter* and *Bacillus*) and two Gram− (*Stenotrophomonas* and *Enterobacter*) (Table 2). The most similar sequences were then retrieved and aligned using the Muscle (Multiple sequence alignment) (Edgar, 2004) program and the alignment was then used to construct phylogenetic trees (not shown), which confirmed the assignment obtained through BLAST probing.

Since isolates AU3-01 and A6-2(7) clustered together within the *S. pasteuri* strains, and exhibited identical 16S rRNA gene sequences, a RAPD (Welsh and McClelland, 1990; Williams et al., 1990) analysis using the primer 1253 was carried out on the DNA of these two strains, which showed that the two isolates exhibited different RAPD profiles (not shown) that indicated that they represent two different *Staphylococcus pasteuri* strains.

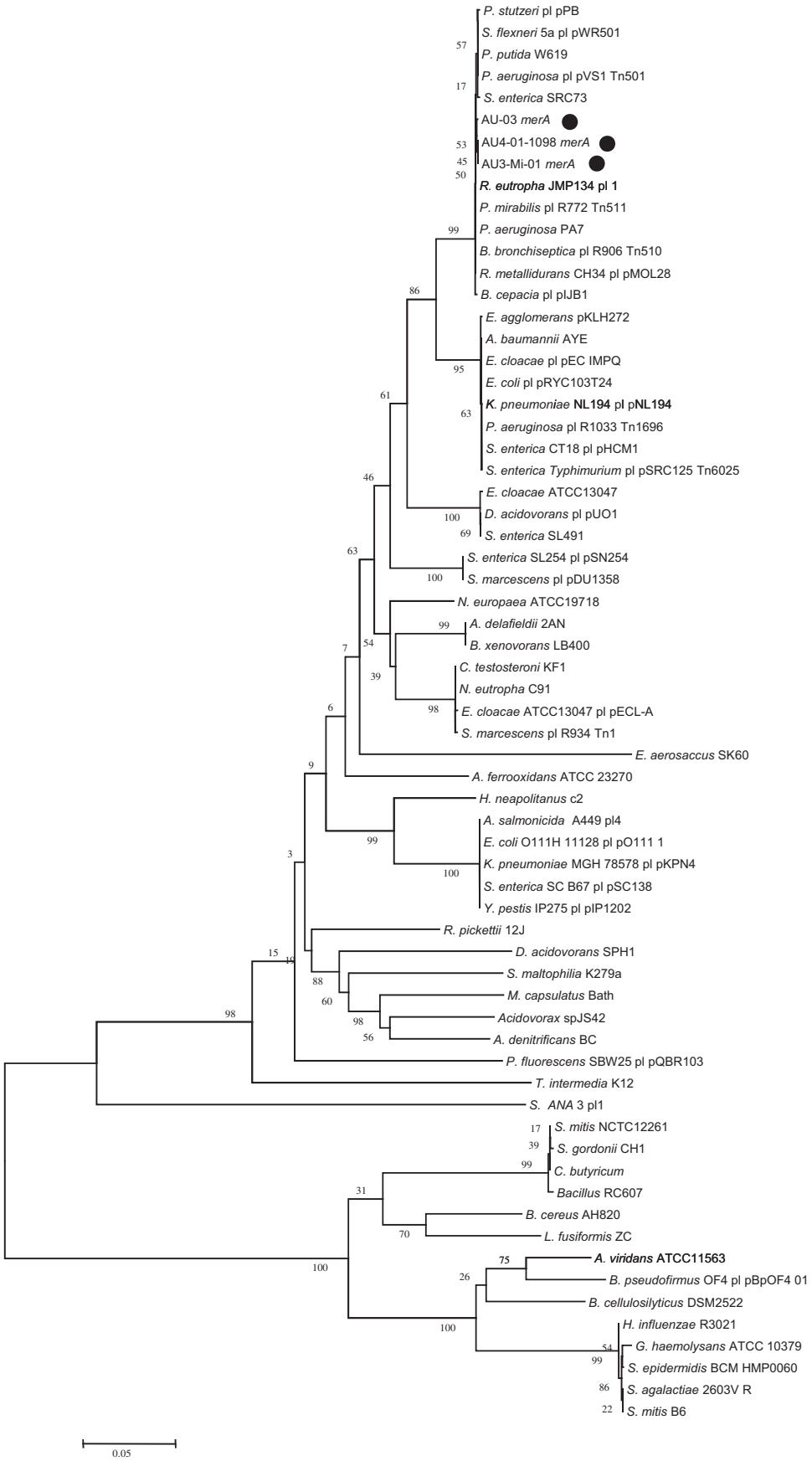

Fig. 6. Phylogenic three constructed using *merA* gene sequences; the three sequences determined in this work from the three strains AU-03, AU3-Mi-01 and AU4-01-1098 are indicated with a black dot.

The evaluation of the presence of plasmid molecules was carried out on each of the fifteen bacterial isolates. Data obtained are shown in Fig. 6 A, B and revealed that eight (AU3-01, AU3-02, AU3-04, AU3-06, AU3-08, A2-1(5), A6-2(7) and AU4-01–1098) out of the fifteen bacterial isolates harbored plasmid molecules. The plasmid size ranged between about 2 kb and 25 kb. Some strains exhibited multiple plasmid molecules of different sizes, The absence of (visible) plasmids of higher size did not per se imply the absence of such molecules; indeed, the plasmid extraction procedure used in this work did not permit the isolation of plasmids whose size is larger than 40 kb. Thus, we cannot *a priori* exclude the possibility that (very) large plasmids (with a sizes exceeding 40 kb) might be present in the bacterial community analyzed in this work.

In order to check for the presence of *merA* (encoding the mercuric reductase) in the genome of the fifteen strains, a PCR amplification of a *merA* region was carried out using the primer sets and the amplification conditions described in the Materials and Methods section. We checked the primer set1 (designed for Gram− bacteria) on the DNA of all the strains and the primer set 2 (designed for Gram+ bacteria) on the DNA of strains AU3-03. Data obtained (not shown) revealed an amplification product of the expected size (about 900 bp) from the *Bacillus* strain AU3-03 (using the primer set 1), and for AU3-Mi-01 and AU4-01-1098 members of the genus *Stenotrophomonas*. In order to verify whether the amplicons actually corresponded to a region of the *merA* gene, each amplicon was purified from agarose gel and its nucleotide sequence determined. The sequence obtained was used as seed to probe the public databases using the BLASTn and BLASTp options of the BLAST program (Altschul et al., 1997). The query sequences retrieved *merA* sequences belonging to different bacteria, demonstrating that they actually corresponded to fragments of the mercuric reductase-encoding gene. The whole body of data obtained suggested that strains AU3-03, AU3Mi-01 and AU3-01-1098 might possess the genetic determinants responsible for mercuric degradation, a finding that, in turn, suggested that these strains might be resistant to Hg. We also performed a phylogenetic analysis of the merA gene. The most similar sequences retrieved from the BLASTn analysis were aligned to the merA gene sequence obtained in this work and the alignment was then used to construct the phylogenetic tree (Fig. 6), whose analysis revealed that the AU3-03, AU3Mi-01 and AU3-01-1098 sequences joined a cluster containing orthologous sequences coming from different Gram negative bacteria, most of which affiliated to Pseudomonadaceae. Most of these sequences were plasmid-borne. The finding that the three sequences joined the same cluster even though they belong to phylogenetically distant genera (*Stenothrophomonas* and *Bacillus*) strongly suggested that they might have been horizontally transferred. This finding is in agreement with previous data showing that Hg resistance genes are frequently exchanged between strains belonging to the same and/or different species of the same or different genera (Fondi et al., 2010 and references therein). The exchange of such genes might be positively favored in an environment under a strong selective pressure, i.e. in the presence of high Hg concentrations like those seen in the Aussa River. Fondi et al. (2010) also found that the flux of *mer* genes between strains belonging to different *Acinetobacter* species is still ongoing and is in many cases mediated by plasmid molecules of different length.

4. Conclusions

Processing and seepage wastewaters containing elemental Hg(0), which were historically discharged by a CAP into the Aussa River system and therefore into the Marano Lagoon, have been significantly reduced since 1984 due to the construction of wastewater treatment facilities (Piani et al., 2005). Lately CAP activity was suspended and some Hg variations in the first cm of sediment were already observed in this study in respect to the last survey (Covelli et al., 2009). In station A3 at the confluence of Banduzzi's channel and the Aussa River a significant loss (from 5.8 in 2004 to 1.84 $\mu g\ g^{-1}$ in 2008) of Hg-T was observed from the first centimeter of sediments and this event was connected with a large population of Hg-resistant bacteria at this site. 11 species out of 15 were isolated from A3 station and the strains belonging to Gram (+) and Gram (−) bacteria were identified by 16S rRNA sequence. The presence of *mer* genes in most of the isolates and the large number (4.85×10^5 CFU g^{-1}, d.w.) of cultivable heterotrophic aerobic Hg-resistant strains were higher than one order of magnitude compared with the other sediments of three stations. Therefore, these bacteria probably, together with other chemical physical factors, play an important role in sediment Hg detoxification by removing Hg from sediments by reducing ionic Hg to Hg(0) and preventing Hg(0) re-oxidation into more toxic forms of Hg.

Acknowledgments

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