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Microbes and mercury biogeochemical cycle in the Venice lagoon

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INTRODUCTION

The Venice lagoon

Lagoons are shallow, inland bodies of water with limited saltwater inputs characterized by high availability of nutrients, high productivity, and a wide diversity of habitats. As a result they present abundant fauna and flora and are for this reason socio-economically important. Their health is associated to the heterogeneity of environments due to the variability of the bottom floor geomorphology which provides a wide array of habitats and niches supporting a great biodiversity. This bathymetric diversity has been showing an overall decrease in the Venice lagoon causing a decline in the ecosystem health (Sorokin et al. 2002).

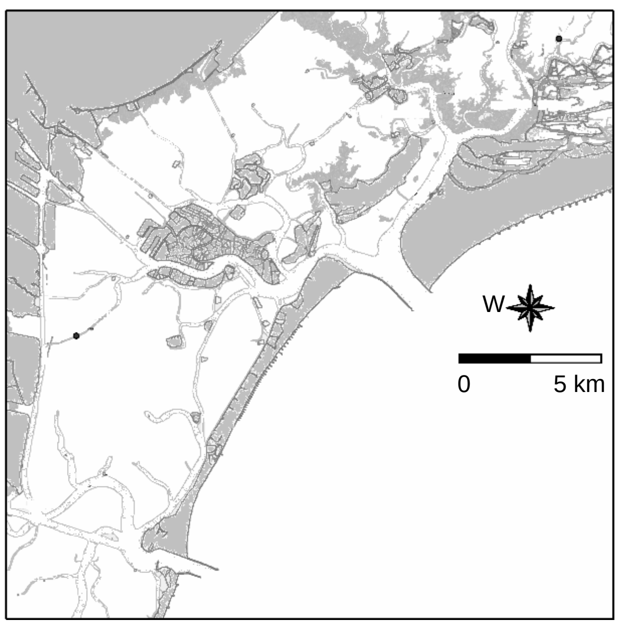
Sediment in these environments is usually highly contaminated because of the extensive human activity and the accumulation of contaminants transported by rivers (Clark 1997). Chemicals like metals and PCBs, or pathogens like microbes and viruses end up in sediment where they accumulate over time and can be more or less available to the local organisms depending on surrounding physical-chemical condition.

Venice Lagoon is the largest lagoon in the Mediterranean (**Fig. 1**) and is characterized by an average depth of 1 m and a surface area of 549 km^2, 40 % of which consists of tidal marshes, islets, and fish farms (Martin et al., 2000).

Three inlets connect the lagoon to the Adriatic Sea (Lido, Malamocco, and Chioggia) and the tidal flow through these inlets determines water exchange with the sea and the internal circulation, which separates the basin north of Malamocco from the southern part.

Because of the presence of Marghera chemical plant, whose wastes were in the past discharged directly in the marine environment, the lagoon’s sediment is highly contaminated with toxic metals like Zn, Pb, Cu, Ni, and Cr (Frignani et al., 1997). Their presence in the ecosystem registered an increase starting from the 1920 s, a maximum between the 1930s and 1970s and a decrease during the recent years due to a diminished input in the lagoon as shown by the analysis of the surface sediment (Frignani et al., 1997). Pollutants adsorbed to particles are scattered in the northern basin because of sediment re-suspension (of anthropogenic origin resulting from dredging activity, clams harvesting, speed boating) and transportation by the tidal flow (Pranovi et al., 2003; Bloom et al., 2004).

Fig.1. Venice lagoon, North Adriatic Sea, Italy. The dots indicate the areas of interest for the SIOSED project: A-Lido inlet; C-Porto Marghera; SS0-shipping channel; S2 and SS1-areas of banks construction.



Mercury in the environment

In the Venice lagoon

Mercury (Hg) is widely present in the Venice lagoon (Bloom et al., 2004; Moretto et al., 2003) with concentrations ranging from 2.2 ng L^{-1} (Bocca di Lido) to 60 ng L^{-1}(Venice canals) with 90– 99% of the total adsorbed to particles. Aqueous monomethylmercury (MMHg) ranged from 0.017 ng L^{-1} (Bocca di Lido) to 0.22 ng L^{-1} (Venice canals) and shows a seasonal pattern with greater methylation during the warmer season (Moretto et al., 2003).

Bloom et al. (2004) showed the Hg detectable in the lagoon is due mainly to suspension of contaminated sediment and only less than 5% derives from new input (industrial, urban, and atmospheric). The same study argued that Venice lagoon appears to be an important source of Hg pollution for the Adriatic Sea (1110 kg yr^{-1}).

Mercury speciation

Mercury is one of the most studied metals since its poisonous potential nevertheless its complex biochemical cycle is not completely understood (**Fig.2**)

Mercury is present in the environment mostly in three forms:

- elemental/metallic Hg^0, considered non toxic
- mercuric ion Hg^{2+}, toxic
- organomercurial forms where the metal is bound to an organic moiety like monomethyl and dimethylmercury (MMHg and DMHg). Among all the organomercurial molecules, methyl-mercury (CH_3Hg) has relevant importance since its neurotoxicity and capability to biomagnify in the food chain (**Fig.3**) (Mason et al., 1994).

Fig.2. Mercury biogeochemical cycle in the marine environment (from Morel FMM., et al., 1998)

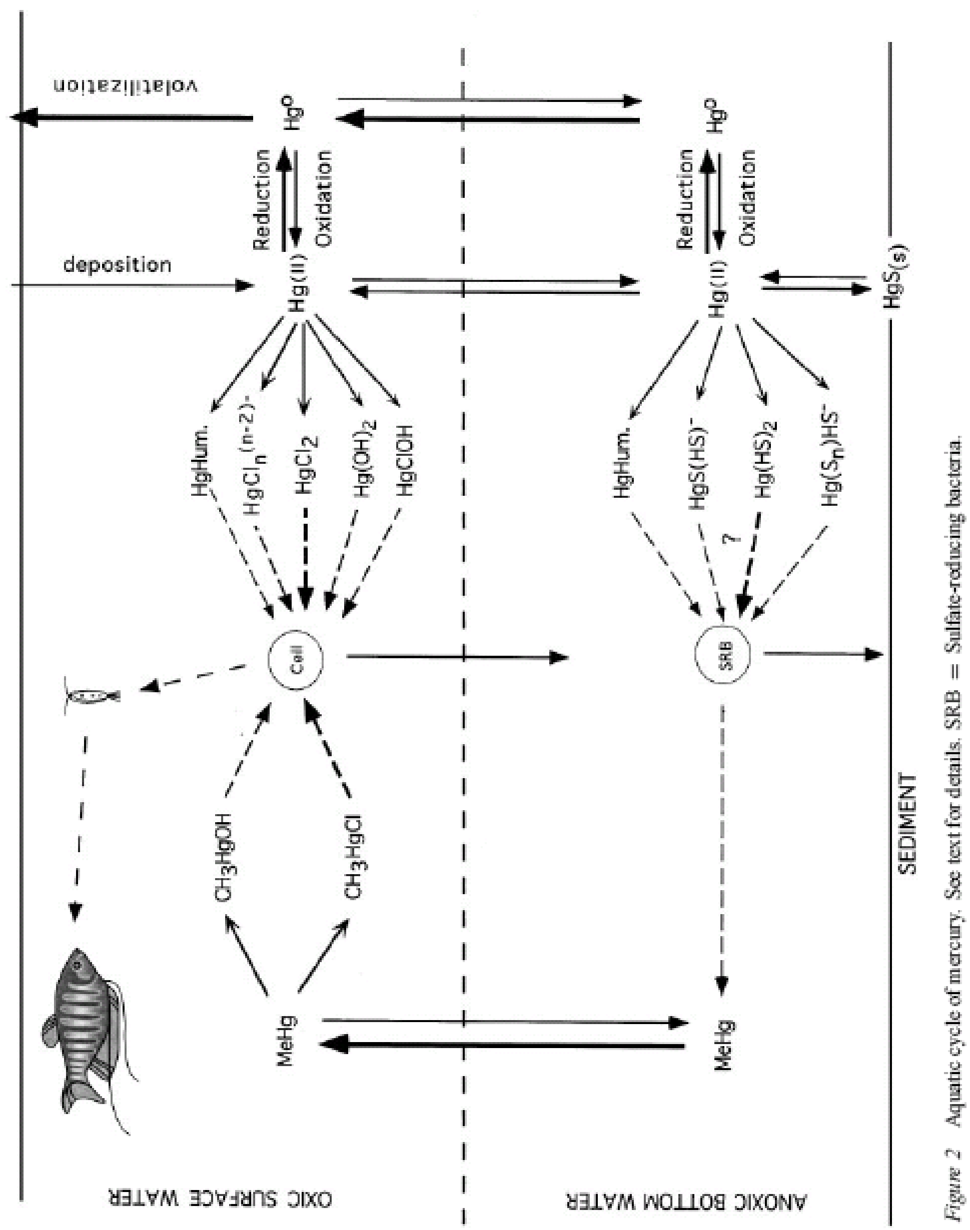
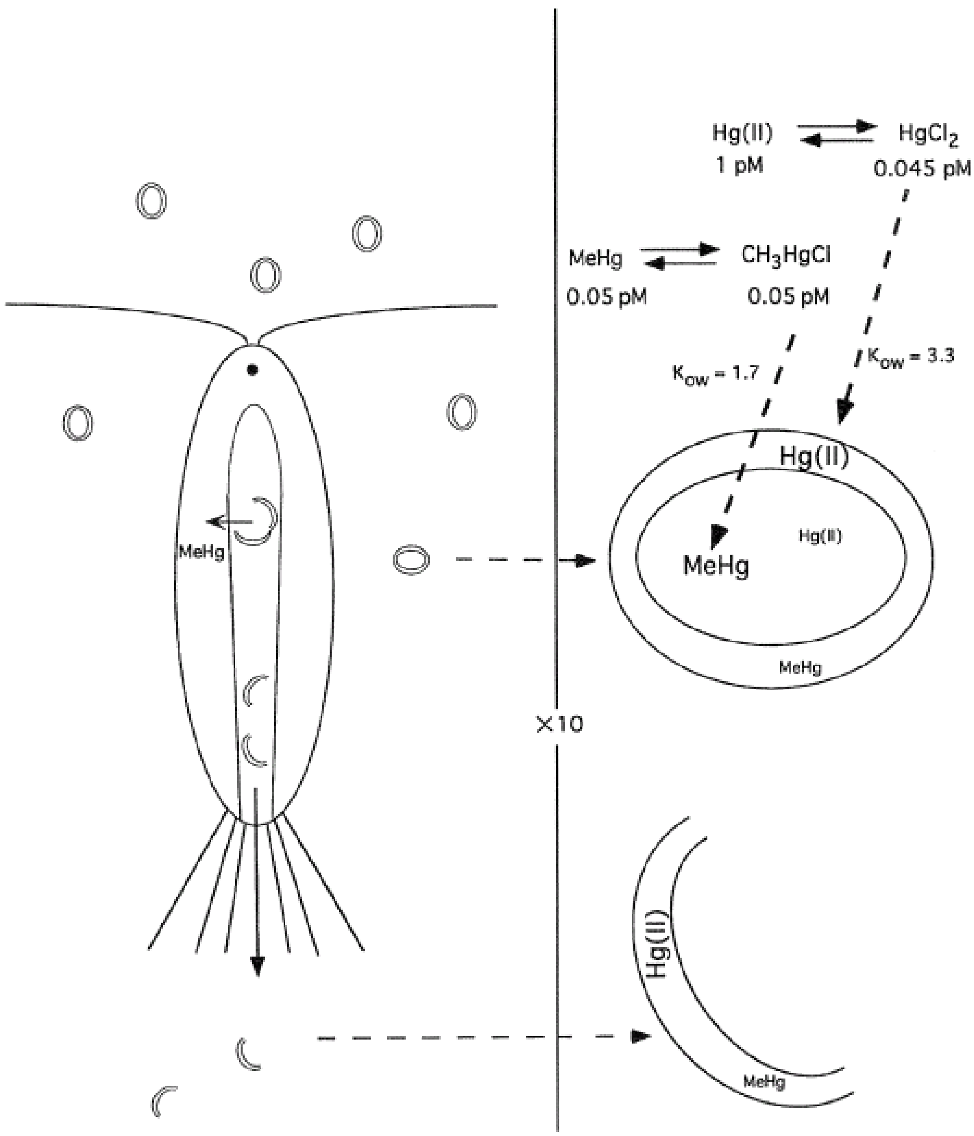


Fig. 3. Bioaccumulation of mercury in the first step of the food chain (from Morel FM et al.1998)



In the atmosphere

In the atmosphere mercury is present in its metallic form Hg^0 which is and Bartha, R. 1984). Natural water is usually supersaturated with Hg^0 compared to the atmosphere and this resul in a flux from the aqueous environment to the air (Maso2002). Here the neutral form is oxidized to Hg^{2+}, primarily by ozone, HClO, HSO_3^- and $OH^{\cdot}$ (Caldwell et al. 2000; Canavan, et al. 2000), and due to its hydrophilic nature it returns on the Earth's surface chiefly via wet precipitation.

Hg^0 resides in the atmosphere for about a year before becoming oxidized to the mercuric form Hg^{2+} (Benoit, 1999), a time sufficient to distribute the element all over the planet making it a global pollutant reaching the most remote areas of the world.

In the ocean, after undergoing a series of transformations, Hg^{2+} is reduced back to Hg^0 and returns for the majority to the atmosphere while a small amount is trapped in the sediment (Boening, 2000). On land, Hg is subjected to a similar faith but in this case a large percentage is buried in the soil and a small amount is released to the atmosphere (Francois et al. 1998). Significant net fluxes of Hg in the air are represented by Hg impacted areas (Bloom and Lasorsa, 1999) which can be of anthropogenic origin as chlor-alkaly plants, metal production plants, coal and wood burning (Lindqvist , et al 1991) or been caused by natural sources as forest fire, volcanic eruption, degassing from water surface (Rasmussen, 1994).

In the marine environment

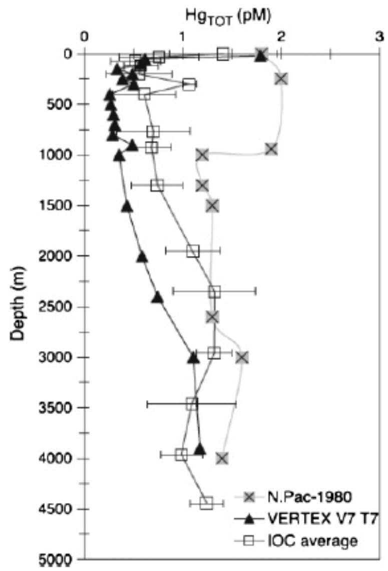
Sources of Hg in the ocean

The atmosphere represents the most important source of Hg for the marine environment (Mason and Sheu, 2002) since the water-air interface is an active exchange surface. Others inputs for the ocean are represented by rivers and subaerial and submarine volcanism (Smith et al. 2005; Bostrom, et al.1969; Barnes and Seward, 1997). In these environments Hg is found primarily as cinnabar, metacinnabar and other sulfide minerals due to its chalcophilic behavior (Huertas-Diaz and Morse, 1992; Belowsky and

Pempkowiak, 2003.) but droplets of liquid metal (Stoffers et al. 1999) and MMHg in solution (Lamborg et al. 2006) have been found in hydrothermal fluids. Total dissolved mercury in the ocean shows a non conservative pattern (Mason and Gill, 2005) like biologically active constituents such as nutrients, gases (O_2 and CO_2), trace metals (Fe, Zn, Cu) and tectonically generated species (Rn). More precisely this element shows a vertical distribution as proved in the North Pacific and in the South Atlantic (Laurier, Mason et al.2004; Mason and Gill 2005; Gill and Bruland 1987; Fitzgerald et al.2004) (**Fig. 4**. In the North Pacific, Mason et al. (1998) report an atmospherically enhanced concentration of 1.8 pM, 0.3 pM in the upper ocean due to the scavenging by particulates (Branfireun, B.A., 1996) and an increase up to 1.2 pM at 4000 m suggesting the presence of remineralization processes.

The cycling and speciation of mercury in the open ocean are similar to those of the coastal and estuarine zones although in the latter the concentration is greater and estimated to range between 1 and 10 pM in filtered water (Heyes et al., 2004; Conaway et al., 2003; Rolfhus and Fitzgerald, 2004). In unfiltered water the concentration is higher, ranging between 2 and 600 pM, and due to the suspended particle load (Heyes et al., 2004; Conaway et al., 2003; Rolfhus and Fitzgerald, 2004; Faganelli, Horvat et al., 2003).

Fig.4 Vertical profiles of total dissolved Hg from the North Pacific Ocean from Laurier, Mason et al. 2007



Mercury speciation and cycling in the water column

In the aquatic environment elemental Hg^0 is found at all depths, representing about the 50% of the total metal load (Mason, Rolfhus and Fitzgerald, 1995), and its cycling is of pronounced importance (Kim and Fitzgerald, 1986; Rolfhus and Fitzgerald, 2004). Hg^0 can be oxidized to Hg^{2+} photochemically or in the dark in presence of DOC (dissolved organic carbon) (Amyot et al., 1997; Amyot et al., 2005). Vice versa, the metallic form is produced by reduction of Hg^{2+} mediated by microorganism like phytoplankton (Ben-Bassat and Mayer, 1977; Jones et al., 1987) and heterotrophic bacteria (Mason et al., 1993; Barkay et al., 1989; Kim, 1987) or photochemically (Amyot et al., 1997; Costa and Liss, 1999). As well, MMHg can be converted to the elemental form throughout demethylation microbially mediated (Barkay et al., 1989).

The majority of Hg^{2+} is present as hydroxide and chloride complexes (Benes, and Havlík, 1979) and is bound to the organic matter in suspension particularly to humic acids and to the molecule's thiol moieties (Branfireun, 1996; Heyes et al., 2004; Conaway et al., 2003).

The oxidized mercury can be reduced to the metallic form and evaporate to the atmosphere or be scavenged by the particulate in suspensions and buried in the sediment while only a small portion is methylated.

In anoxic water and sediment

Since the mercuric ion exhibits high affinity for sulfides (HS^- and S^{2-}), this property controls the metal's chemistry in anoxic environments (Benoit et al., 1999).
Insoluble mercuric sulfides $HgS_{(S)}$ precipitate as meta-cinnabar (black) and cinnabar (red); meta-cinnabar is unstable and spontaneously evolves to cinnabar after few days.
Those two forms have a very low solubility and provide a way of Hg burial in the sediment together with complexation to organic matter, iron oxides and iron and manganese sulfides (Cossa and Gobeil, 2000; Han, Obraztsova et al. in press; Lamborg et al in press; Cossa and Coquery, 2005; Iverfeld, 1998). Anoxic sediment is also the environment where methyl-mercury compounds are produced, mostly due to the metabolism of anaerobic bacteria (King et al., 1999; King et al., 2000; Compeau and Bartha, 1985).

Environmental Hg transformation microbe mediated

Microbial metabolism is crucial in determining mercury transformation in the environment. Of particular importance are the processes leading to:

- Hg^{2+} and MMHg reduction to Hg^0
- Hg sequestration sulfide mediated
- Hg methylation

Hg^{2+} and MMHg reduction to the elemental form is due to the mer operon, a well characterized metal system resistance mechanism broadly present in microbes (Tonomura et al., 1968, Schottel et al., 1974, Clark et al., 1977, Summers and Sugarman, 1974). Some bacteria bearing this plasmid are capable of reducing both organic and inorganic mercury compounds, whereas others have the ability to transform only the inorganic form

(Schottel et al., 1974) depending on the pool of genes present in the operon. MerA is the mercuric reductase capable of reducing Hg^{2+} to Hg^{0} (Summers and Sugarman, 1974) while MerB is a lyase capable of breaking the Hg-carbon bond (Schottel, JL., 1978). Other genes on the plasmid encode for proteins responsible for the Hg^{2+} inward transport (Foster et al., 1979, Jackson and Summer, 1982) or have a regulatory activity (merR). Mer operons present in nature are characterized by a great diversity of gene rearrangement and contents (Barkay, T. et al., 2003)

Mercury methylation and sulfide complexation take place under anoxic condition, the former producing a highly toxic compound, the latter decreasing the metal bioavailability where high sulfide concentration produced by the SRB metabolism dominate Hg^{2+} speciation (Benoit et al., 1999a).

Microbial community related to Hg methylation

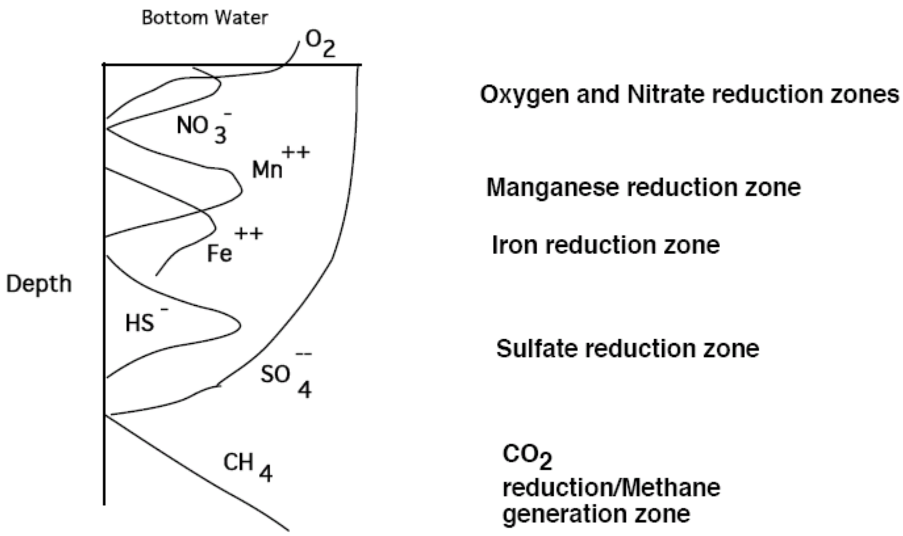
Abiotic MMHg production seems to play a minor role in the environment (Berman and Bartha, 1986), whereas bacteria have a critical role for the methylation. Among microorganisms, sulfate-reducing bacteria (SRB) appear to be of major importance for the process in anaerobic marine sediment (King et al., 1999; King et al., 2000; Compeau and Bartha, 1985; Devereux, Winfrey et al., 1996; Gilmour Henry and Mitchell, 1992) and well recognized Hg-methylators species are *Desulfovibrio desulfuricans, Desulfobulbus propionicus, Desulfococcus multivorans, Desulfobacter sp,* and *Desulfobacterium sp* (King et al., 2000). Dissimilatory sulfate reducers are anaerobic microorganism reducing sulfate to sulfide in a respiratory process producing energy. Their metabolism is sensitive to oxygen which inhibits their activity. Sulfate reducing prokaryotes (SRP) belongs to both bacteria and archaea group (Wagner et al. 2005) and share the anoxic environment with other anaerobes respiring iron, manganese, nitrate and methanogens. Those microbes inhabit the sediment in depth profile following a red-ox gradient (**Fig. 5**).

Mercury is believed to be taken up passively by the cell in the form of neutral HgS^{0} complex (Benoit et al., 1999a; 1999b). The methyl moiety is transferred to Hg via the acetyl-CoA pathway, widely present in microbes, through methylcobalamine (vitamin

B12) and methyltransferase catalytic activity (Choi et al., 1994). However, potential Hg methylation by the sulfate-reducing bacteria lacking the oxidation ability provided by acetyl-CoA pathway was reported (Ekstrom et al., 2003). In addition, other microorganisms seem to have the capability of Hg methylation like *Neurospora crassa* (Landner, L., 1971), *Enterobacter aerogenes* (Hamdy and Noyes, 1975), *Candida albicans* (Annai et al., 1991) and methanogen's cell extract (Wood et al., 1968) although some studies have indicated that archaea are not involved in this process (Mcbride and Edwards, 1977, Compeau and Bartha, 1985, 1987). Lately, two Fe-respiring species were proven to methylate Hg at rates comparable to the ones measured for sulfate-reducing bacteria: *Geobacter sp* (Fleming et al., 2006) and *Desulfuromonas sp* (Kerin et al., 2006) and data retrieved from sediment in situ measurements did not show a strong correlation between sulfate reduction and mercury methylation potential (Macalady, Mack et al., 2000; Mack, 1998).

Due to its toxicity, mercury cycling in the environment has been extensively investigated, nevertheless the biochemical pathway leading to MMHg formation is still not clearly understood and evidence of methylation by microorganisms different than SRB has been reported in literature. For these reasons we were interested in the study of the sediment total microbial community in a marine environment as the Venice lagoon where microbial specie composition related to Hg biogeochemistry was not reported before.

Fig. 5. Tipical redox reactions occuring in organic carbon enriched marine sediment. This redox sequence is bacterially mediated and the reaction sequence is mostly in terms of energy yields of the various reactions. For these reasons the following sequences occurs: O_2 reduction; NO_3 reduction; Mn-oxide and Fe-oxide reductions; SO_4 reduction; CO_2 reduction and methanogenesis.



The SIOSED project

The Venice lagoon's sediments are classified on a pollutant contamination basis in type A, B, and C (**Table 1**). These criteria have been established by the Protocollo d'intesa law enacted on 8/4/93 establishing which sediment can be dredged and re-employed in the lagoon considering the presence and concentration of some metals, PCB, IPA, hydrocarbons and pesticides.

-Type A sediment: not polluted sediment that can be dredged and re-used directly in morphology restoration activity.

-Type B Sediment: slightly polluted sediment suitable for restoration activity of internal islands but must be confined to avoid the release of contaminants in the ecosystem.

-Type C sediment: polluted sediment suitable for level raising activity in emerged island that must be permanently confined to hamper the contact with the surrounding water.

Sediments characterized by higher toxicity level must be disposed outside the lagoon's area.

Since type B sediment is the most widespread in the lagoon and bottom floor restoration is needed, there is the necessity to understand if the dredging and transplanting can be harmful for the environment and the people. Furthermore the cleaning of the shipping channels and the mobile gates building continuously create the need to find areas suitable for a permanent translocation. In order to find a solution for bottom floor restoration and sediment transplantation, a scientific approach has being adopted.

SIOSED was a multidisciplinary project commissioned to Scripps Institution of Oceanography (La Jolla, CA) by the Consorzio Venezia Nuova, a government department operating in Venice for the Italian water authority (MAV). The full plan comprehended an ecosystem background assessment, the building of sub-tidal banks employing type B sediment and the monitoring over a period of two years after the banks construction. The areas of expertise involved comprise various aspects of ecology, chemistry, eco-toxicology, microbiology, physical oceanography. My area of study, part of line D of the SIOSED project, focused on microbial community categorization and its involvement in mercury biogeochemical cycle with particular interest on methyl-Hg production.

Table 1. Marine sediment classification for the Venice lagoon as established by the Protocollo d'intesa (8/4/1993)

Elementi e composti *	**Unità di misura**	**Classe A**	**Classe B**	**Classe C**
Hg	mg/kg ss	0,5	2	10
Cd	mg/kg ss	1	5	20
Pb	mg/kg ss	45	100	500
As	mg/kg ss	15	25	50
Cr	mg/kg ss	20	100	500
Cu	mg/kg ss	40	50	400
Ni	mg/kg ss	45	50	150
Zn	mg/kg ss	200	400	3.000
Idrocarburi totali	mg/kg ss	30	500	4.000
IPA totali	mg/kg ss	1	10	20
PCB totali	mg/kg ss	0.01	0.2	2
POC totali	mg/kg ss	0.001	0.02	0.5

Areas of study

The study areas of interest for this microbiological analysis consist of six sites (**Fig. 1**) named after the category of sediment (A, B, and C) present at each site, or based on their location respect to the dredged channel which corresponds to SS0. The sites in the present study are Site A (Lido Inlet, the cleanest area), Site C (Porto Marghera, the most polluted area), Site SS0 (dredged channel near Malamocco Inlet), Site SS1 (Malamocco Inlet close to SS0), Site S2 southern lagoon away from SS0. Site SS0 sediments were used to construct sub-tidal banks in Sites SS1 (bank V1) and S2 (bank V2). These sites span a range of sediment types containing different amount of sand, clay and organics dependent on the distance from the Adriatic Sea and Porto Marghera.

SIOSED Line D: microbes and Hg biogeochemistry

One of the important questions addressed under line D was the microbial species composition in the sediment sampled in the different areas of interest for the project and its relationship with Hg speciation. We were interested in both total and sulfate-reducing bacterial communities since sulfate-reducing bacteria are the major Hg-methylators but the acetyl-CoA pathway is not a prerogative of sulfate-reducing bacteria and a range of other microorganisms have been reported to methylate Hg.

We focused on the comparison among communities dwelling in the different areas being characterized by different composition of sand, organics and pollutants (especially mercury). For the background campaign we aimed to explore the dissimilarities among:

- Site A (classified type A, rich in sand coming from Lido beach)
- Site C (classified type C, black mud rich in organics sampled in the highly polluted area of Porto Marghera)
- Site SS0 (classified type A but composed by black mud rich in pollutants coming from the shipping canal)
- Site SS1 (classified type B, high percentage in sand)
- Site S2 (classified type A, high percentage in sand)

After the banks construction, the monitoring concentrated the banks V1 and V2, the surrounding areas (SS1 and S2) and SS0.

The microbial composition was explored through TRFLP (Terminal Restriction Fragment Length Polymorphism) targeting the bacterial 16S gene to disclose the total community and the DSR gene to focus only on SRB. Clone libraries for the 16S and DSR genes were sequenced. Archaea presence was explored only for site V2 in November 2005. Enrichments for anaerobic bacteria were set up to study the microbial physiology influencing Hg methylation and to assess SRB cell count.

MATERIALS AND METHODS

Sample collection

Sediment collection was conducted with logistic support of Thetis Spa. Twenty cm sediment cores have been collected in June (A, C, and S2) and in August 2005 (SS1 and SS0) for the background assessment of Hg speciation and microbial community. Constructions of experimental banks, V1 (built in site SS1) and V2 (built in site S2), were completed in October 25 – 29 (V1) and November 2 – 16, 2005 (V2). Shortly after the completion of banks, the surface 2.5 cm sediments were collected in mid- and late-November 2005, February, June, July 2006, and February 2007. Long cores (20 cm) were collected at longer intervals in December 2005, May, September and November 2006, and February 2007. Acid-cleaned polypropylene cores (10 × 30 cm) were used to collect sediment samples. Collected cores were extruded and sectioned within 24 h in a N_2-filled glove box at intervals of 2.5 cm for the 0 to 10 cm layer, and 5 cm for the 10 to 20 cm layer. Sediment was collected with different techniques depending on the type measurement it had to serve for. Mercury methylation and sulfate reduction experiments: approximately 20 g of the sediment slices were sealed in amber glass vials under N_2 saturated condition and transported to the laboratory (Scripps Institution of Oceanography, San Diego, CA, USA) in a portable electric cooler (~ 4°C). Mercury methylation and sulfate reduction experiments were carried out within two weeks from the sampling date under appropriate temperatures (field temperature ± 2 °C). Those analyses were performed by Dr. Seunghee Han and Dr. Anna Obrastzova.

Total Hg, dissolved sulfide, sulfate and Fe concentration: after extrusion and sectioning of core in the glove box, pore waters were extracted by means of centrifugation at

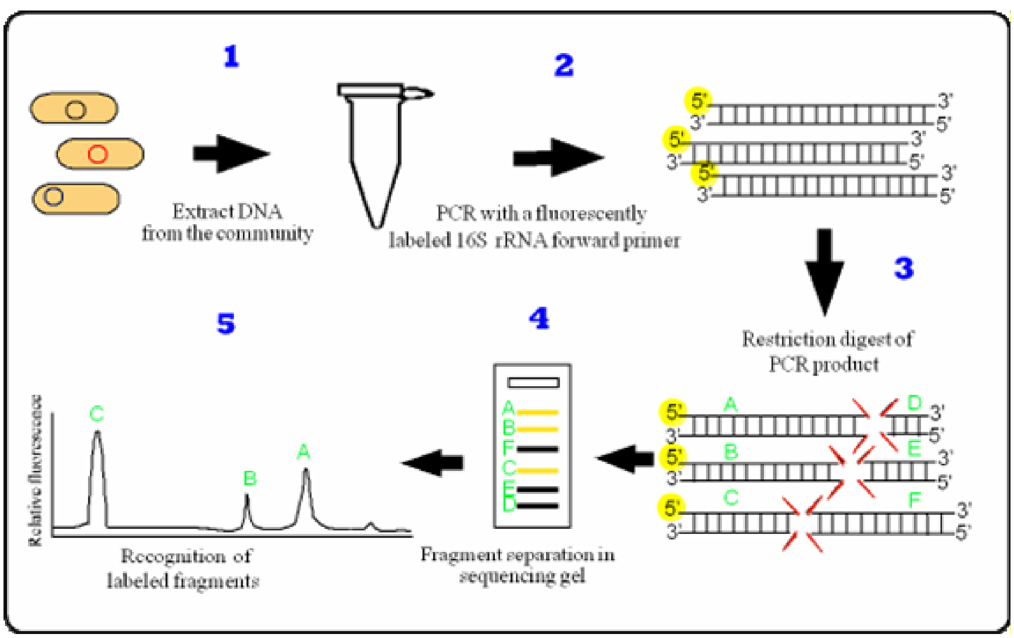
approximately 5,000 rpm. After pore water filtrations (0.45 μm) under anaerobic conditions, approximately 10 – 20 cm^3 of the filtered pore water sample was acidified for analysis of total Hg and approximately 5 cm^3 was used for measurement of dissolved sulfide, sulfate and Fe concentrations. Measurement of dissolved sulfide, sulfate, and Fe was kindly provided by Dr. Gieskes and total Hg concentration by Dr. Seunghee Han.
Microbial community analysis: about 10 g of sediment were placed in sterile plastic vials and stored at -20°C. The leftover sediment slices were stored frozen for analyses of sedimentary Hg and MMHg. The amounts of pore waters collected from cores A and SS1 were not sufficient for chemical analyses in June 2005 and September 2006, respectively, due to high ratio of sand in sediments.

Microbial community investigation through TRFLP.

TRFLP is a technique employed in molecular ecology to enlighten the microbial composition. Briefly the bulk DNA is extracted from the sediment and used as template for the amplification of target genes (16S and DSR genes were the ones chosen for our purpose). One of the primers employed is fluorescently labeled leading to a mixture of fluorescent amplificates further enzymatically digested. The obtained fragments are separated on an electrophoresis gel, depending on their length, in an automated sequencing machine able to measure the fluorescent light. The visualized fragments start with the fluorescent primer for a length ending at the first enzyme cutting site. The final result is a plot showing peaks having on abscises the fragment's length and on ordinate the signal intensity (**Fig. 6**).
Genomic DNA from the sediment was isolated using the Power Soil kit (Mobio), purified from contaminants through filtration with Microcon YM-50 column (Millipore) and suspended in 50 μl EB buffer (10 mM Tris pH 8 in deionized water). Three different sets of universal primer were used to amplify the eubacterial 16S (27F, 1492R), the archaean 16S (21f, 958 r) and the DSR gene (DSR1, DSR4). 27F(5'-AGAGTTTGATC[A/C]TGGCTCAG-3'); 1492R(5'-ACGG[C/T]TACCTTGTTACGACTT-3'); DSR1(5'-AC[C/G]CACTGGAAGCACG-3'); DSR4(5'-GTGTAGCAGTTACCGCA-3'). Primers for Archaea were 21f (5'-

TTCCGGTTGATCCYGCCGGA-3') and 958R (5'-YCCGGCGTTGAMTCCAATT-3'). 16S Primers 27F, 21F and DSR1 were labeled at the 5' end with the cromophore 56-FAM (IDT, Inc). Each reaction contained 20-50 ng of template DNA, 20 pmol of each primer, 0.4 µg/µL BSA (Sigma-Aldrich) and the amplification conditions consisted in 1 cycle of 94 C for 3 min, followed by 25 cycles of 94 °C for 30 sec, 55°C for 30 sec and 72 °C for 1.5 min, with a final extension step of 72 °C for 5 min. For each sample, triplicate PCR amplifications were performed, combined and cleaned with the kit Qiaquick PCR purification (Qiagen). The final DNA was digested separately with 3 different enzymes HhaI, HaeIII, RSAI (New England Biolab), cleaned with Sephadex-75 (Sigma-Aldrich), dried and resuspended in 15 µL deionized formammide and 0.4 µL of GeneScan 500 Rox size standard (Applied Biosystem). Fluorescently labeled TRF's were separated by electrophoresis in an ABI 310 genetic analyzer (Applied Biosystem) and the data processed with the software Gene Mapper (Applied Biosystem).

Fig.6. Schematic representation of the TRFLP protocol



16S cloning, sequencing and phylogeny analysis

Bacteria and Archaea16S PCR products were ligated into pCR 2.1-TOPO and transformed in TOP 10 E coli competent cells following the manufacturer's indications (TOPO TA cloning system, Invitrogen). Final transformed cells reaction (50 µL and 200 µL) were plated in LB agar medium amended with kanamycin and x-gal reactive following the manufacturer's indication (TOPO TA cloning system, Invitrogen). After overnight incubation at 37°C, white colonies were picked and growth independently in 2ml liquid LB media added with 50 mM kanamycin for 14 hours under vigorous shaking at 37°C. Plasmids were purified with the column Qiaquick plasmid prep (Qiagen) and enzymatic digestion with the enzymes Eco RI and HhaI was performed in order to screen for the inserts with the same RFLP pattern. Sequences were obtained with the M13 forward and reverse primers after electrophoresis run in an ABI 3100 genetic analyzer (Applied Biosystem). After the Chimera check with Bellerophon and RDP chimera detection programs, a dendrogram was produced with the software Mega 3.1. Matrices of evolutionary distance were constructed by the maximum parsimony method and bootstrap values determined from 1,000 iterations to estimate the confidence of the tree topology.

Enrichment cultures

Enrichment cultures were obtained adding 0.1 g of sediment stored at 4°C into 10 ml sterile, anoxic sea water Widdel's medium (Widdel, and Bak, 1992) prepared without the addition of sulfate and following the Hungate technique. Electron donors were added in 20 mM concentration as formate, acetate and lactate. Electron acceptors were added in 20 mM concentration as Na_2SO_4, $NaNO_3$, $FePO_4$-citrate, amorphous Fe oxides, amorphous Mn oxides. After about a week of incubation at room temperature, each culture was transferred into 10 ml fresh anoxic medium in 10 fold dilution up to 10^{-5}. Negative controls were prepared inoculating fresh medium without electron acceptors. The last dilution showing bacterial growth on electron acceptors were transferred into fresh medium and kept as stabile cultures suitable for the following experiments of DNA extraction and Hg methylation capability. Growth of bacteria was tested as following:

Mn-reducing bacteria by the change in color from brown precipitate of Mn(IV) oxides to white precipitate of Mn(II) insoluble salt (**Fig.7**), Fe-reducing bacteria by the chance in color of green soluble $FePO_4$-citrate to white insoluble precipitate of Fe SO_4,or by the change in color of bright orange precipitate of Fe(III) oxides to brown precipitate of Fe(II) oxide, Sulfate-reducing bacteria by the formation of a brown precipitate of CuS when a drop of culture containing sulfide was added to a 1M solution of $CuSO_4$ and nitrate reducing bacteria by the presence of a dense bacterial growth in contrast to the absence of growth of an inoculums without the addition of nitrate. Aerobic heterotrophic bacteria were obtained using Widdel's sea water sulfate free medium (Widdel, and Bak, 1992) with 0.5% yeast extract.

Fig.7 Anaerobic enrichments for Mn and Fe reducing bacteria. Serum bottles without inoculums on the left, grown bacteria consortium on the right.

Mn oxides

Insoluble Fe oxides

Soluble $FePO_4$

Mercury methylation by anaerobic enrichments

Experiment was set up in 100 ml of serum bottles containing 50 ml of fresh anoxic medium. Appropriate electron donor and acceptor at concentration of 20 mM were added. Each bottle was inoculated with 0.5 ml of fresh established enrichment culture of sulfate, iron, manganese or nitrate reducing bacteria and gently shacked at room temperature. After 48 h (T_0), 10 ml of culture was removed with a sterile syringe and needle, acidified with HCl 0.06 M final concentration and stored at -20°C in a Teflon vial. $HgCl_2$ in a 0.06 M HCl solution was amended to the remaining culture at final concentration of 50 ng/L and incubated at room temperature in the dark. Two 10 ml aliquots were removed from the serum bottle after 6 (T_1) and 24 h (T_2), acidified and stored as previously described. MMHg concentration was measured by cold vapor atomic fluorescence spectrometry (Choe et al., 2004) by Dr Seunghee Han.

SRB cell count by MPN

Viable SRB were enumerated using an MPN technique (Rowe, Todd and Waide, 1976). Briefly, inside an anaerobic chamber, anoxic sea water medium for SRB (Widdel and Back 1992) amended with $FeSO_4$, was added with a pipette to a microtiter 48 well plate in 1.8 ml aliquots. Carbon sources were formate, acetate, lactate added at final concentration of 20 mM. In anoxic condition, 1 g of sediment from each site and depth sampled during the June and July 2005 campaign, was added to 10 ml of anoxic sea water medium in a falcon tube, shacked vigorously and 0.2 ml of this solution were added to each well column. The slurries were diluted in steps 1:10 by transferring aliquots to the wells in the same plate's row. Two replicates were prepared for the same carbon source. Growth was determined visually by the presence of FeS black precipitate.

Oxygen micro profile in the sediment

The core sediment was taken with a Plexiglas cylinder (10 cm diameter, 1m length) and the two openings were closed with a butyl stopper at the bottom and a plastic lead on the top. The sediment was kept in a cooler in dark condition on the boat and was transported to the analytical facility avoiding shaking. The measurements were taken within one hour up on the arrival to the laboratory. The presence of oxygen in the top layer was measured with an oxygen microelectrode connected to a microamperometer (Unisense, DE) and the software Profix (Unisense, DE). The microsensor was left on over night for the signal to stabilize and calibrated using marine water purged with nitrogen as minimum oxygen saturation condition and marine water inflated with air as maximum oxygen saturation point.

RESULT AND DISCUSSION

Bacterial community analysis

About 120 samples of bulk sediment coming from the different areas of interest, over a period of almost two years and in depth profile (up to 10 cm) were analyzed for the bacterial community employing TRFLP with three digestion enzymes. The resulted profiles, in the range of 50-500 bp, were characterized by a large number of distinct peaks indicating a multitude of dominant ribotype groups, a typical complexity observed for bulk sediment and soil patterns (Costa et al., 2006; Ravenschlag et al., 1999; Torsvik et al., 2008).

High profile reproducibility was found for presence and relative abundance of T-RFs (terminal restriction fragments) and was relative to the same sediment sample and for the different field replicates. Relative peak signal intensity, although reproducible, was not considered as a measure of relative species abundance because of the amplification bias occurring when employing PCR-based techniques mostly due to the use of degenerated universal primers and effects of genome size and rrn gene copy number (Suzuki and Giovannoni, 1996; Farrelly et al., 1995; Poltz et al.,1998; Tillman et al., 2002).Variations in the presence of T-RFs among the replicates were restricted to small peaks and could be caused partially by Taq polymerase poisoning since the high presence of contaminants and pollutants typical of coastal, human impacted sediment (Tebbe and Vahjen, 1993; Kreader, 1996). Small peaks were absent indeed in fingerprinting with a relatively low total intensity (data not shown).

The bacterial community shows similar species composition in areas of interest for the SIOSED project before (**Fig. 8**) and after the construction of the banks (**Fig.9**) even if the organic carbon and Hg contents are different (data not shown). Resembling data for the Venice lagoon sediment have been obtained performing DGGE analysis 16S gene based (Malfatti F. and Azam F., personal communication).

Fig. 8. TRFLP of bacterial community in the different sites before the banks construction in June and August 2005 (months 4 and 6, enzyme Hae III).

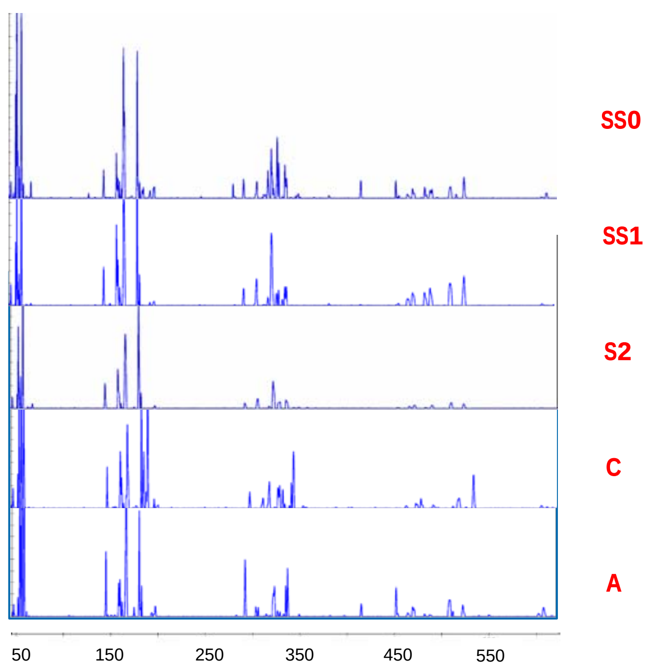
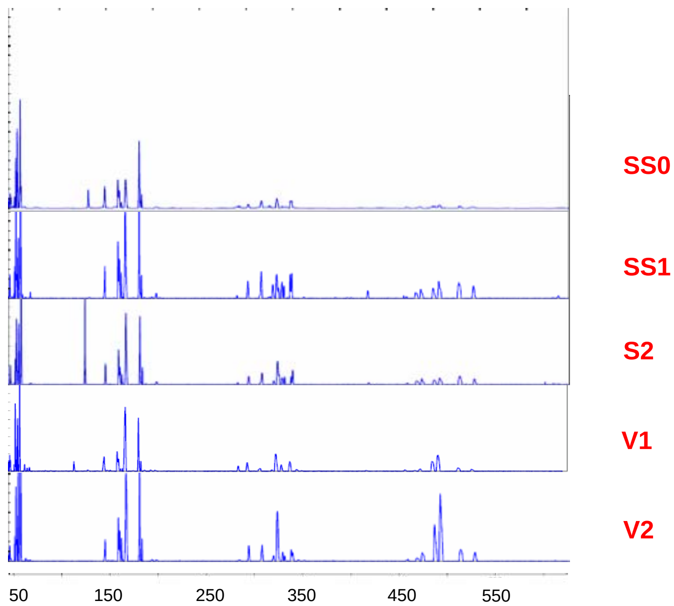


Fig. 9. Bacterial community comparison between the banks and the surrounding areas in November 2005 (month 9, enzyme HhaIII).



Novitsky (1990) observed a microbial community on the surface sediment layer highly similar to the one on sedimenting particles and suggested the community inhabiting the sediment take origin from the sinking particulates. An internal water circulation pattern transporting particulates and associated microbes from the inlets to all over the basin (Elwani, H, personal communication) characterizing the Venice lagoon could be a major reason for a homogeneous bacterial population inhabiting the whole area. Furthermore, a considerable sediment resuspention has occurred after dredging activities at the Malomocco Inlet before the beginning of the SIOSED project (Nasci C and Deheyn D, personal communication) and evidence of particle transportation throughout the lagoon mediated by the water circulation were disclosed by granulometry data (Chiarlo R, personal communication).

Homogeneous species composition was also revealed among the newly built banks and the surrounding environments (**Fig. 9**). Assuming changes in species composition occurred during the banks construction due to different microbes inhabiting in the dredged SS0 sediment in depth, the homogeneous community on the surface have been re-established in short time period. No considerable changes are visible either in depth profiles (**Fig.10**) or over time (**Fig. 11**). If the microbial population bound to the particles is deposited (Novitsky, 1990) and become buried in the sediment a homogeneous microbial community in the first depth profile can take place (Urakawa et al. 2000). Bioturbation caused by benthic invertebrates, as found during the sampling campaigns, is another reason supporting the above mentioned finding (Lisa Levin, personal communication) as well as sediment re-suspension due to boat activity and tidal flushing.

In contrast to a homogeneous population, total Hg and MMHg concentrations show a large range of variation (Fig.36 from Han et al. 2007). In a polluted environment like the Venice lagoon the living microbes must be adapted to the different contaminants, especially in the area adjacent to the Porto Marghera chemical plant. De Lipthay, Rasmussen et al. (2008) pointed out that populations pre-exposed to high level of toxic substances (Hg in particular) develop a tolerance which is maintained even if the concentration becomes very low. In other words the prokaryotes found in site A may have been previously selected for their tolerance to Hg and this explain their similarity in terms of type of species to the ones in site C.

A limitation in disclosing the microbial diversity is represented also by the employed technique. Since TRFLP provides information only on the presence or absence of different species and the relative peak abundance has not been considered as a quantitative data, the technique does not give information on the microbial activity. The bacteria can be metabolically inactive or dead due to the unfavorable temperatures (seasonal changes), sediment compositions (nutrients availability, presence of inhibitors), and microbial competitions even though their 16S gene T-RFs is present. For the same reason it could be hypothesized the coexistence of physiologically distinct groups metabolically active in dependence of the reduction-oxidation potential in the sediment depth profile (**Fig.12**) with species present as not active living cells.

Little information on the species typology can be provided by the 16S gene sequencing results since after the screening of 120 clones 30 species/strains have been recognized all belonging to uncultured organisms (**Fig. 13**).

Fig.10. Bacteria population in depth profile for site A in June 2005 (month 4, enzyme HaeIII).

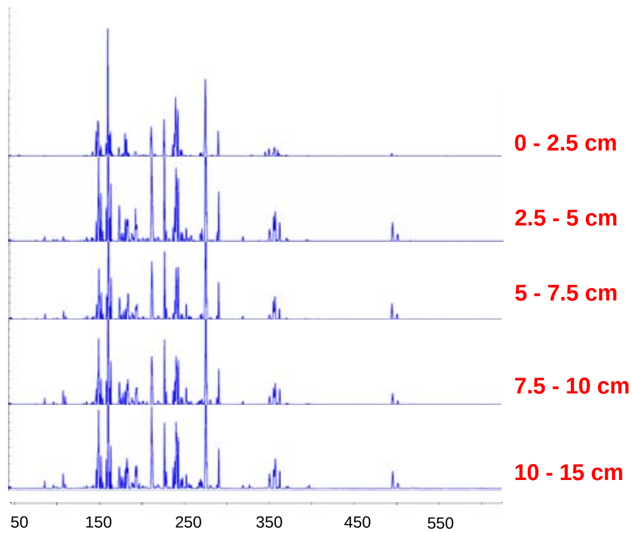


Fig.11. Bacteria population in depth profile for site V2 in December 2005 (month 9, enzyme HaeIII)

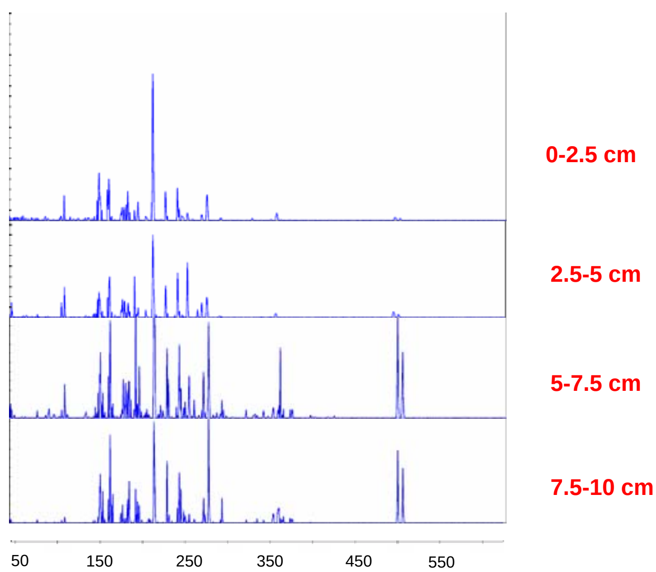


Fig. 12. Microbial community over time for site SS0, surface layer (0 - 2.5 cm, enzyme HhaI).

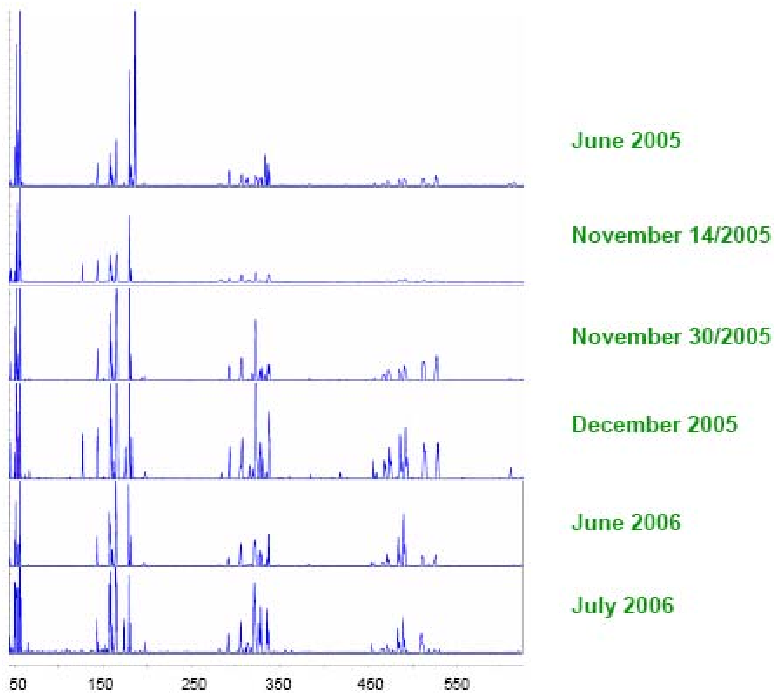
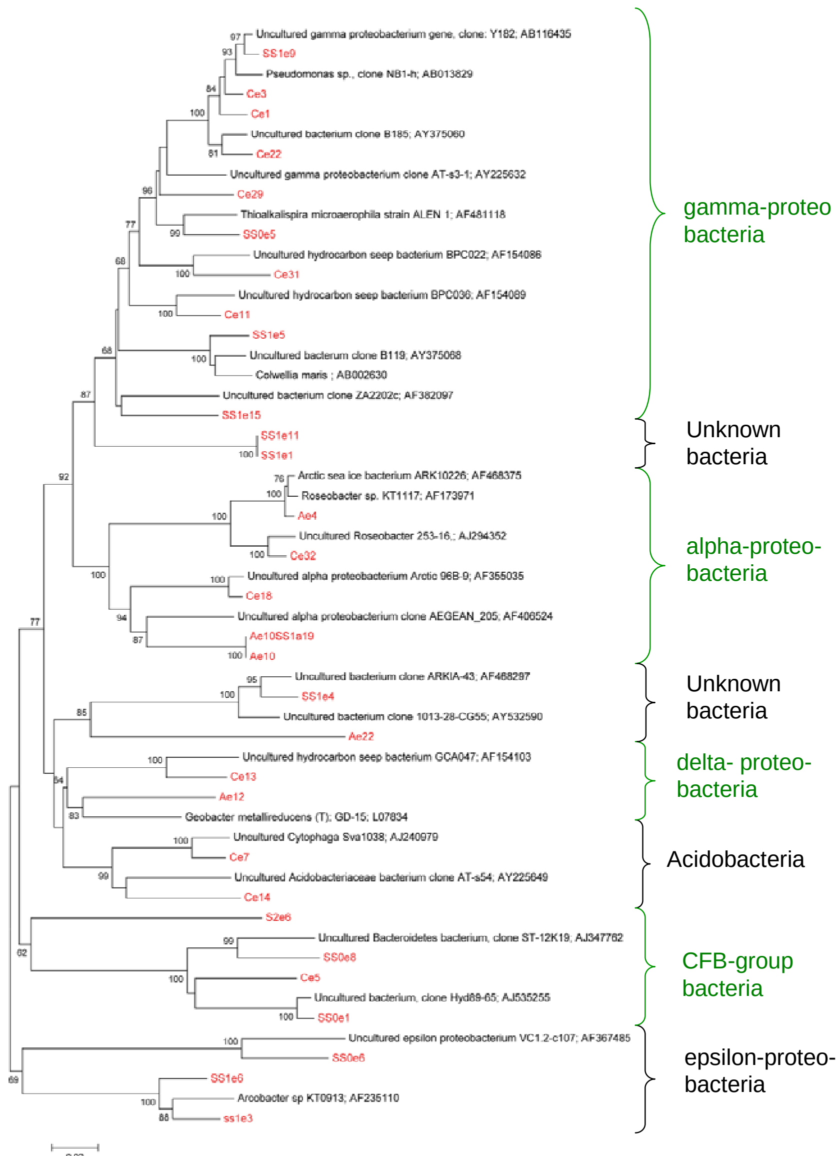


Fig. 13. Dendrogram visualized with the Mega3.1 software showing the phylogenetic affiliation of Venice Lagoon 16 S bacterial clones resulting in 7 deep-branching clusters of Alpha/Gamma/Delta/Epsilon-Proteobacteria, CFB group, Actinobacteria and Unclassified Bacteria. Matrices of evolutionary distance

were constructed by the maximum parsimony method. Numbers beside branching points indicate bootstrap values determined from 1,000 iterations to estimate the confidence of the tree topology.

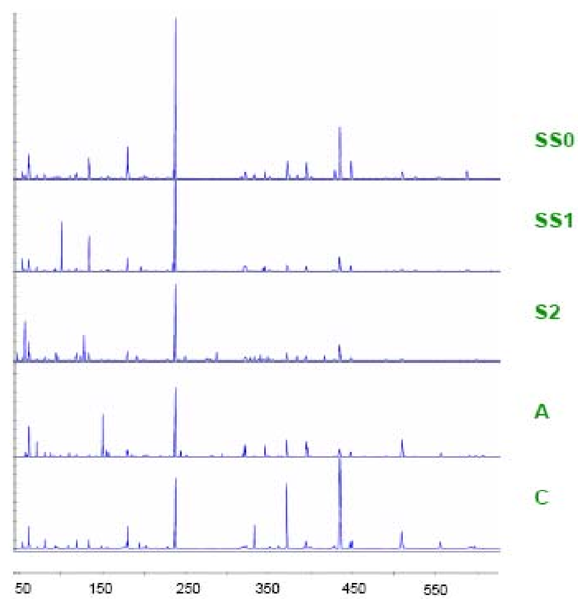


Sulfate-Reducing Prokaryotes distribution and methyl-mercury production

Sulfate-Reducing Prokaryotes reduce sulfate to sulfide as a respiratory process in anaerobic environments. Since prokaryotes capable of this metabolism have a polyphyletic origin (five bacterial and two archaeal phyla, Wagner et al. 2005) and can be closely related to non-sulfate reducers, the 16S gene-based analysis is inadequate for the study of their community (Wagner et al. 2005). The Dissimilatory (bi) Sulfite Reductase (DSR) is a key enzyme, evolutionarily conserved, unique of those prokaryotes capable of dissimilatory sulfate reduction and for this reason can be used as a target gene to study their environmental population (Wagner et al. 1998)

Homogeneous SRP community in different sites is observed specifically targeting the DSR gene (**Fig. 14**) while the sulfate reduction and Hg methylation rates (SRR and MMR) show high variability among the sampling sites (Fig.16 from Han, Obraztsova et al. 2007).

Fig. 14. TRFLP for the sulfate-reducing bacterial community (DSR gene) in June 2005 (month 4, enzyme RSA).



SRR and MMR appear to have similar profiles (**Fig.15**) implying their active metabolism has a major role in MMHg production as widely reported in literature.

A significant presence of potentially active sulfate-reducers in the Venice sediment is evidenced also by MPN data (**Fig.16**) showing a maximum concentration of 10^9 cells/g characteristic of coastal organic rich sediment.

MMHg production, as shown in **Fig.15** occurs in the surface (0-10 cm), right below the water-sediment interface where the anoxic conditions for the SRB growth are created which is below the first millimeter in the Venice lagoon (**Fig.17**). Besides SRP activity, inorganic Hg bioavailability is the other factor affecting methylation. This is influenced by solid organic matter, sulfide and FeS, Fe and Mn oxides and salinity (Hammerschmidt and Fitzgerald, 2004; Mason and Lawrence, 1999; Sunderland et al., 2006; Benoit 1999b; Gilmour et al., 1998; Winfrey and Rudd, 1990, Barkay et al., 1997).

On the upper layer Hg unavailability can be due to the complexation to FeS-organic compounds with Fe(II) possibly produced by Fe- respiring bacteria, among with oxygen inhibiting SRB growth, and the lower methylation boundary can be caused by the precipitation of HgS due to the high concentration of sulfide produced by SRB (Han, Obrastzova et al., 2007; 2008).

Those data are in contrast with the finding of King et al. (2001) who describe MMR being based on the microbial community composition. In their study though, they employed a PCR independent technique based on radio- labeled probes.

Fig.15. (From Han, Obrastzova et al. 2007). Comparison between the background concentration of tot Hg and MMHg (first row) and between the measured Sulfate Reduction Rate and the Mercury Methylation Rate (second row) during the background sampling campaign in June and July 2005.
Both measured background concentrations and activities differ in the different areas even though the bacterial population does not seem to show visible variability.
Sulfate Reduction Rate and Mercury Methylation Rate appear to have similar profile implying sulfate-reducing bacteria have a major role in MMHg production.
SRR is not reported for sample A since it was not possible to obtain the pore water needed for the analysis of sulfate due to the sandy nature of the sediment.

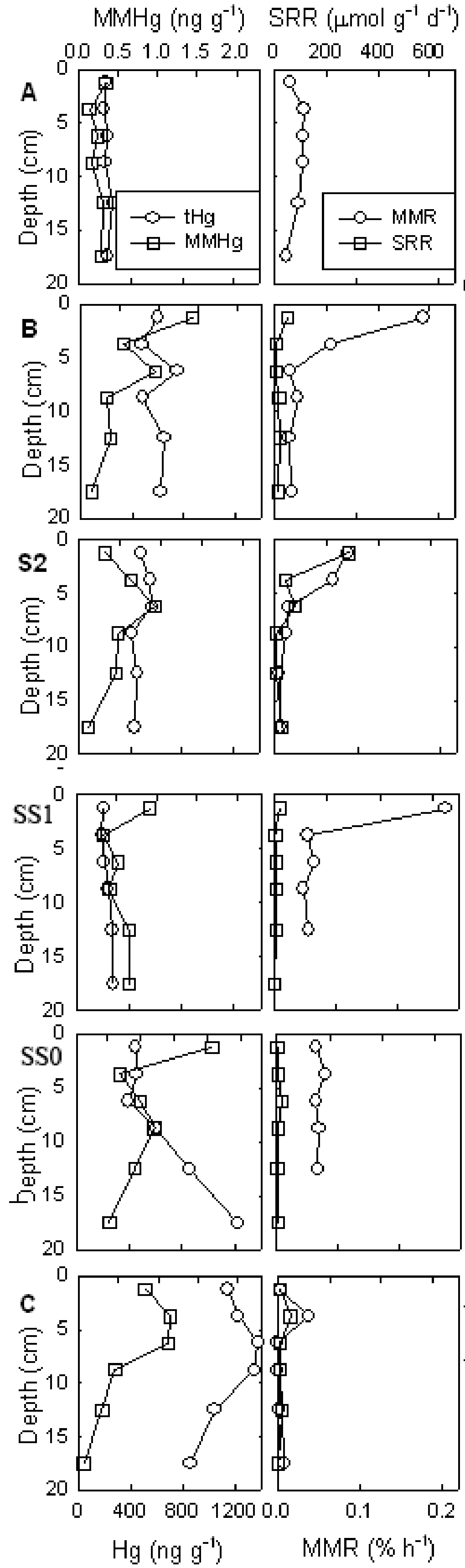
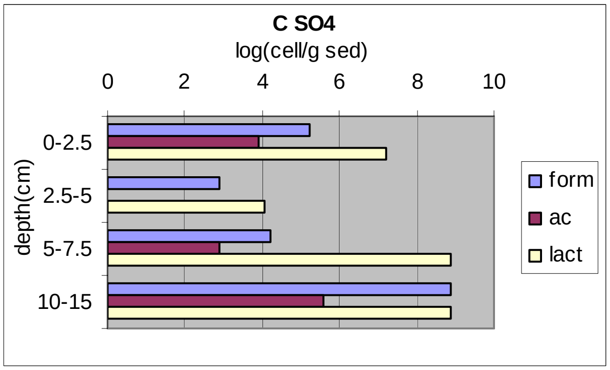
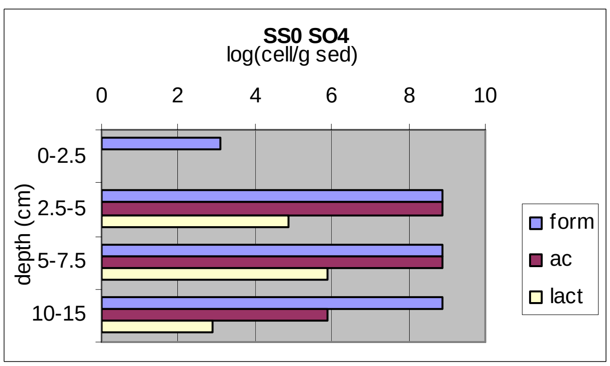


Fig. 16. MPN count for SRB. The plots below show the cell number/g of sediment grown on three carbon sources (formate, acetate, lactate) for the sites C, SS0 and SS1. The depth profile comprehend the first 15 cm top layer.





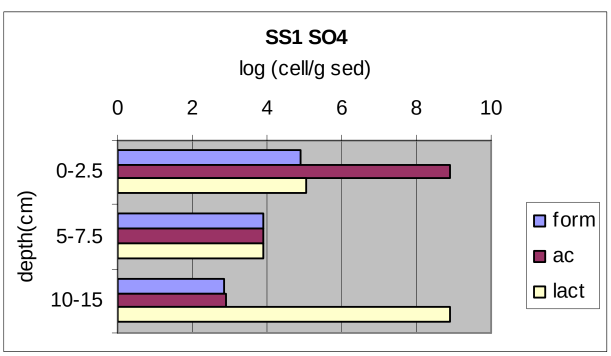
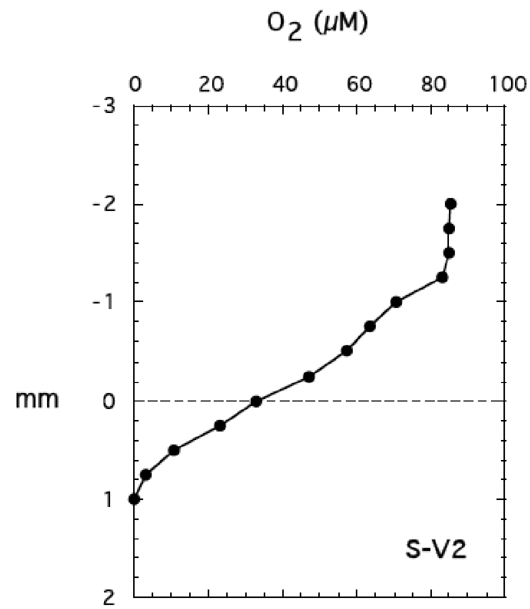


Fig.17. Oxygen concentration profile in sediment collected from the bank V2 in May 2007



Archaea population

Archaea were never proven to be active methylators but MMHg production have been noticed by methanogen’s cell extract (Wood et al., 1968) and by a consortium of SRB and methanogens when sulfate was lacking (Pak and Bartha, 1998). In addition, SRB are well characterized methylator but sulfate respiring prokaryotes are also found among the Archaea group (Wagner et al. 2005) and their methylating activity have never been explored. TRFLP analysis on the Archaea community was performed only on sediment sampled fromV2 bank in November 2005. A lesser species abundance is revealed by the fingerprinting showing a smaller number of peaks (**Fig.18**) Preliminary data obtained from 16S gene cloning and sequencing suggest abundant presence of *Nitrosopumilus* *maritimus*, an ammonia oxidizing organism belonging to the group Crenarchaeota (data not shown), which physiology has been studied by Könneke et al. (2005). This could play an important role in the consumption and detoxification of ammonia after the building of the banks since this contaminant has been detected in highly toxic level (Joris Gieskes personal communication). All the other Archaea found by 16S gene sequencing belong to unknown species (**Fig.19**). Experiments involving Archaea resistance with Hg were not performed in the present study.

Fig. 18. Same Archaeal community represented with 2 TRFLP profiles obtained with HhaI (A) and HaeIII (B). Sediment sample is from site S2 (2.5 – 5 cm) collected in December 2004.

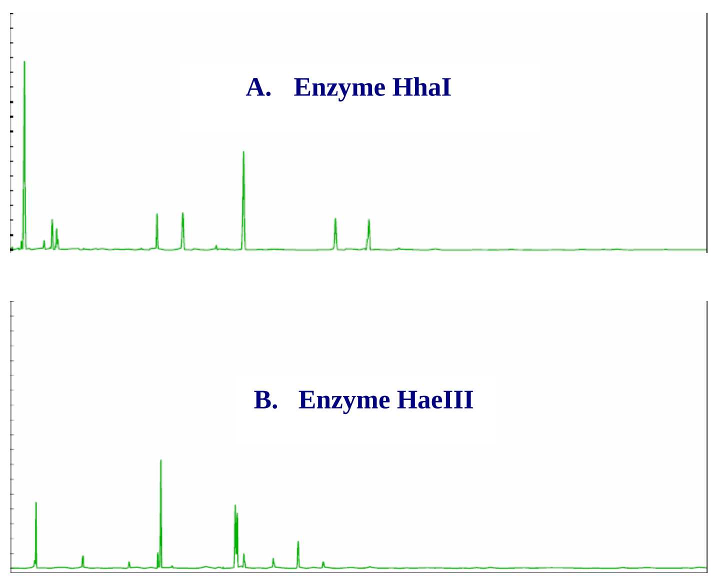
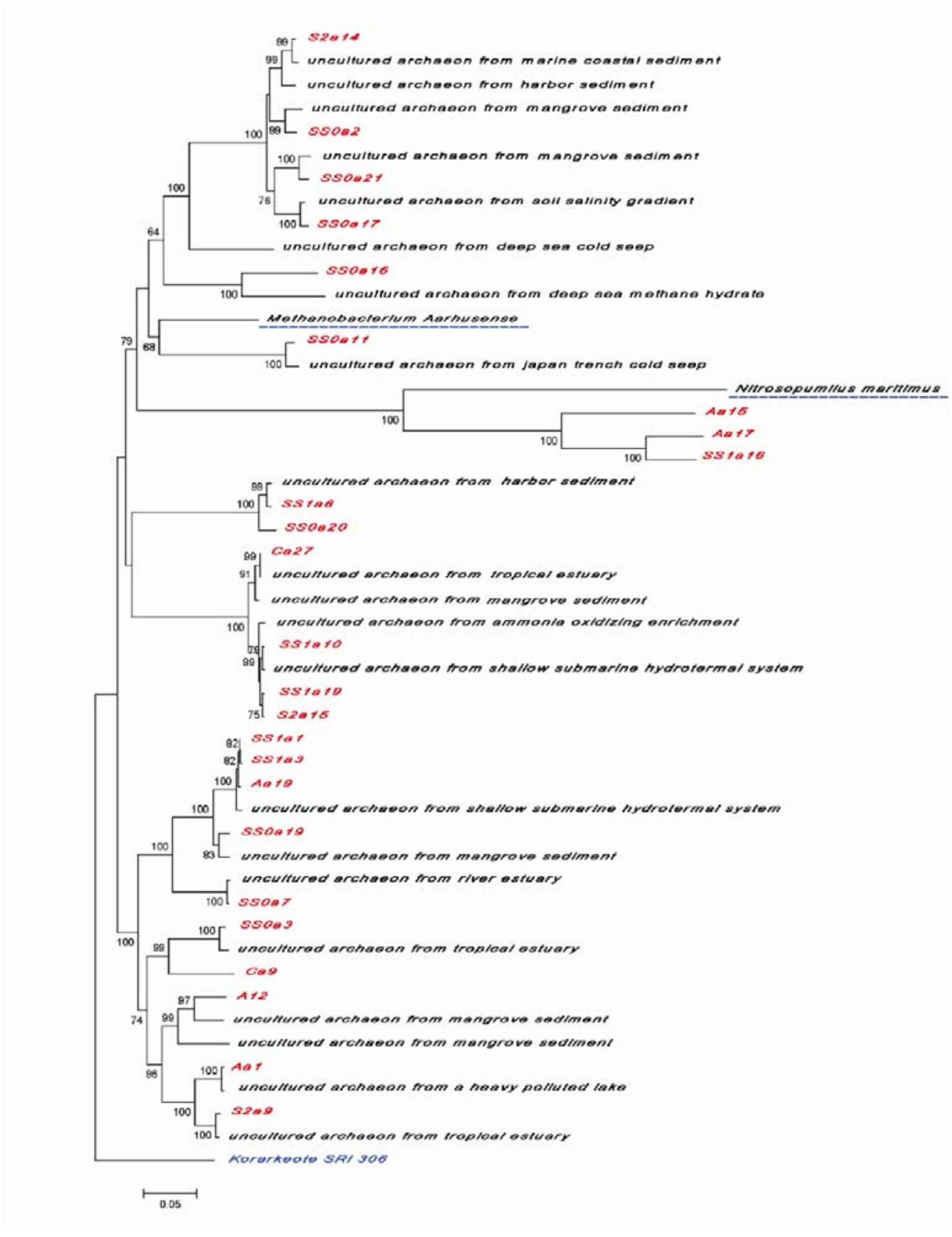


Fig. 19. Dendrogram visualized with the Silva software showing the phylogenetic affiliation of Venice Lagoon 16S archaeal clones. Matrices of evolutionary distance were constructed by the maximum parsimony method. Numbers beside branching points indicate bootstrap values determined from 1,000 iterations to estimate the confidence of the tree topology.



SRB enrichments and total sediment community

The comparison of TRFLP profiles for the DSR gene (**Fig. 20**) obtained from sediment and enrichment total DNA shows, as extensively reported in the literature, the cultivable bacteria are not the most represented in the environment. The most representative peak at 110 bp in the enrichments with lactate and acetate is weakly present in the fingerprinting obtained from the sediment DNA while the one at 529 bp in the culture grown with lactate is absent. DSR gene sequences display the presence of SRP belonging to well known methylating species of *Desulfovibrio sp* and *Desulfatibacillus sp* (**Fig. 21**). Since the high presence of uncultivable SRB in the environment, mercury methylation could be due to unknown bacteria which metabolism still has to be explored.

Fig. 20. Comparison between the SRB population (DSR gene) in the bulk sediment and in the enrichments with the three carbon sources formate, acetate, lactate (Enzyme RSAI).

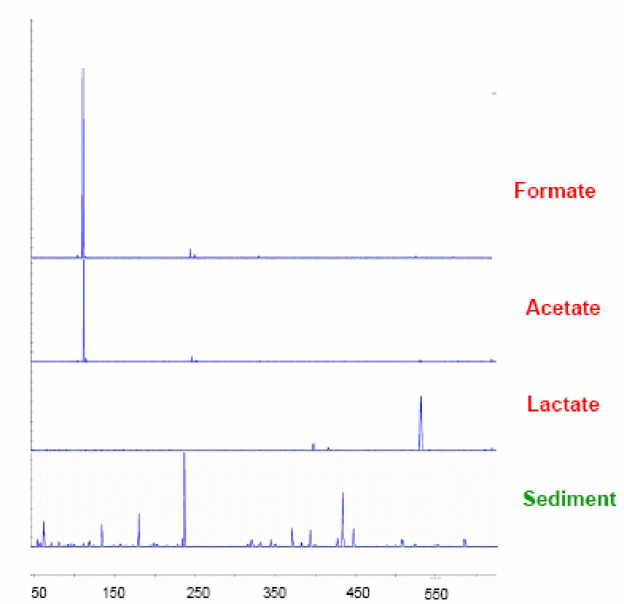
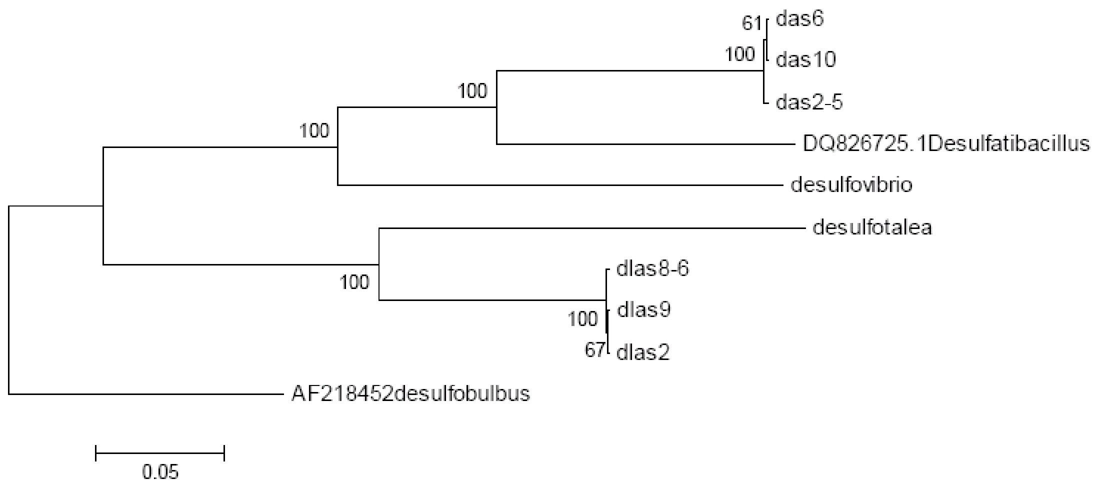


Fig.21. Dendrogram visualized with the Mega3.1 software showing the phylogenetic affiliation of Venice Lagoon DSR bacterial clones resulting in 2 deep-branching clusters. Matrices of evolutionary distance were constructed by the maximum parsimony method. Numbers beside branching points indicate bootstrap values determined from 1,000 iterations to estimate the confidence of the tree topology.



Enrichment's MMHg production

Culturing experiments were performed to better understand the physiology of the organisms present in the lagoon in regard to Hg biogeochemical cycle. Enrichment for sulfate-, iron-, nitrate-, and manganese-reducing bacteria, all physiological groups inhabiting the upper sediment layer where Hg methylation occur, were obtained from SS0 sediment using formate, acetate, and lactate as carbon source. All the enrichments have been tested for MMHg production and positive results were obtained only for sulfate-reducing bacteria (**Fig.22**). The concentration of produced MMHg decreased with the carbon sources formate> acetate> lactate. Methylation has been noticed also for Fe-reducing bacteria growing on formate but this result has not been confirmed (data not shown). A further time series experiment was performed to better investigate

MMHg production by sulfate-reducing bacteria based on the utilized carbon source which suggests demethylation, probably due to the *mer* operon, rather than methylation is influenced by the electron donor's nature (**Fig. 23**). The demethylation rate increment over time followed the order formate < acetate < lactate, results in agreement with the former experiment. Further experiments need to be performed to investigate this finding.

Fig. 22. Monomethyl Hg production by enrichments of aerobic, and sulfate-, iron-, manganese- and nitrate-reducing bacteria.

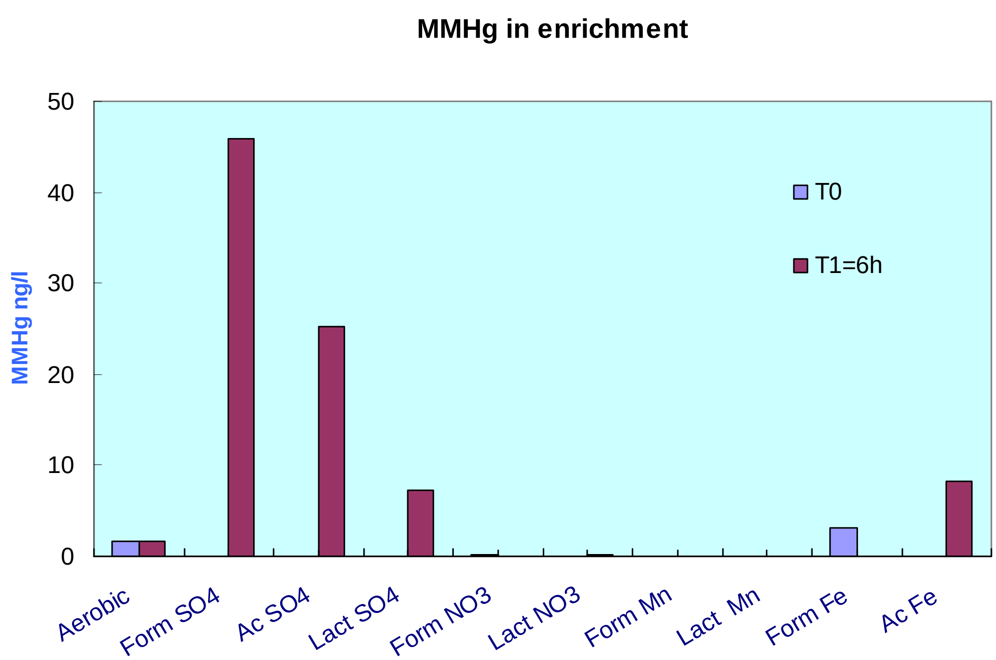
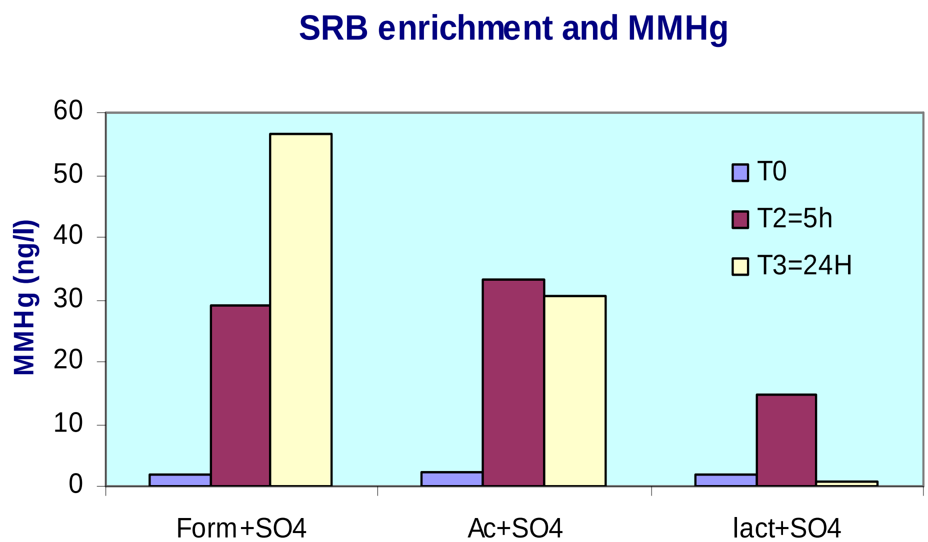


Fig. 23. Time series experiment for MMHg production by sulfate-reducing bacteria (SRB) utilizing different carbon sources.



CONCLUSIONS

The microbial community inhabiting the Venice lagoon sediment shows a similar species composition in the sites of interest for the SIOSED project which are dislocated in a large area in the lagoon. Homogeneous composition was observed in different areas (before and after banks construction), over time and in depth profile (up to 10 cm) through TRFLP targeting the 16S bacterial gene for the study of the total community. Each plot was characterized by a large number of reproducible peaks and observed variations concerned only the presence or absence of peaks of minor intensity. Similar bacterial population among distant areas in the lagoon can be due to the presence of an internal water circulation transporting suspended particle from the inlets to the entire basin surface. Furthermore, since the long history pollution caused mostly by the chemical plant in Marghera, it is likely that microbes in the sediment have been selected for metals and other contaminants resistance which can be preserved when the toxic substance contamination cease. Microbial species dwelling in the Lido area can be the same inhabiting the sediment close to Porto Marghera since both populations probably have been exposed to pollutants and transported by the current.

Similar results were obtained in the surface depth profile (up to 10 cm) where the sediment is re-suspended mainly by tidal flow and boat activity and between the banks and surrounding areas meaning if a change occurred due to prokaryotes living in the dredged SS0 deeper sediment, the close areas population is restored in short time.

SRP community was investigated performing TRFLP targeting the DSR gene evidencing the common similarity in species composition between the interested sites before the construction of the banks.

The results above described though do not give any information on the microbial activity. Prokaryotic cells can be present as not active or dead and their 16S will be retrieved and amplified by PCR. Measured metabolic activity (SRR and MMR) show indeed evident differences in different areas, over time and in depth profile due probably to different availability of nutrient and mercury and different temperature. Furthermore MMR and SRR show correlated profiles meaning that MMHg is produced by SRB as shown also by the methylating capability of the enrichments.

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