

# Production of methylmercury by sulphate-reducing bacteria in sediments from the Orbetello lagoon in presence of high macroalgal loads

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**Abstract:** Methylmercury is a potent neurotoxin affecting shallow-water ecosystems. Mercury polluted sediment samples were collected at six different sites in the Orbetello Lagoon (central Italy) characterized by high levels of silt, iron, manganese hydroxides, and organic matter originated from the decomposition of macroalgae. Porous water pointed out the presence of sulphates, methylmercury, and sulphides. Slurries arranged in anaerobic conditions from sediment aliquots from the six sites, with the addition of ionic mercury, highlighted the production of methylmercury. Sulphate reducing bacteria (SRB) were quantified in lagoon sediments; furthermore, sediments cultured under anaerobic conditions showed SRBs active in mercury methylation. Anaerobic cultures of SRB, amended with ionic mercury, produced methylmercury during the growth of bacterial cells. The percentage of aerobic mercury resistant bacteria was pointed out at each sampling site, evidencing the presence of bioavailable mercury. Several aerobic mercury resistant bacteria were isolated and their level of resistance to inorganic and organic forms of mercury was evaluated. These isolates may be potentially used for eventual bioremediation processes. Mercury methylation by SRB in the Orbetello Lagoon sediments was described for the first time, focusing the attention on the need for possible bioremediation processes by using autochthonous mercury resistant bacteria. Moreover, the influence of algal biomass on mercury methylation was highlighted for the first time in this lagoon ecosystem. The importance of removing algal biomass, as it represents a source of organic matter favouring the process of mercury methylation, was strongly pointed out in this study.

**Keywords:** Mercury-methylation; sulphate-reducing bacteria; mercury-resistant bacteria; macrophytes; Orbetello Lagoon

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## 1. Introduction

Mercury (Hg) contamination in aquatic systems is a global and serious problem, with consequences affecting both human and environmental health (ICMGP, 2006). Hg compounds in aquatic ecosystems are microbiologically transformed with methylation, leading to methylmercury (MeHg) production, with subsequent biomagnification in food chain, hence increasing health risks for humans and

wildlife (Hosokawa, 1995). Lipoaffinity of MeHg increases its potential to be bio-accumulated, compared to inorganic mercury, improving also bio-magnification in food webs (Gardiner, 1972; Heinz, 1974; Matida et al., 1971). Faust and Ozman (1981) reported that, normally, 90 – 99% of total Hg in the environment is associated with sediments, and less than 1% accumulates in biota. Otherwise, MeHg shows a different distribution and only a fraction of 1 – 10% is associated with sediments, whereas the 90 – 99%

of methylmercury accumulates in the biota. Concerning human exposure to MeHg, the U.S. Environmental Protection Agency (U.S. EPA) reported that the consumption of fish and seafood contaminated by MeHg represents the most important way of exposure to mercury (Environmental Protection Agency, 1997).

The fact that anaerobic bacteria methylate Hg is well known (Jensen and Jernelöv, 1969) and this activity has been attributed to sulphate-reducing bacteria (SRB) present in anoxic sediments (Compeau and Bartha, 1985; Gilmour et al., 1992; King et al., 2000; Petranich et al., 2018). Mechanism of mercury-methylation seems to be related to the acetyl CoA and methylcobalamin ( $B_{12}$ ) production by sulphate-reducing bacteria (Choi et al., 1994), albeit some authors do not agree with this account (Ekstrom et al., 2003; Ekstrom and Morel, 2004). The anaerobic dissimilatory sulfate-reducing bacterium *Desulfovibrio desulfuricans* ND132, isolated from estuarine mid-Chesapeake Bay sediments, was used as a model species for understanding the mechanism of microbial Hg methylation (Gilmour et al., 2011). It is noteworthy to consider that about 50% of *Desulfovibrio* strains have the ability to produce MeHg and that this feature is constitutive and does not confer Hg-resistance (Gilmour et al., 2011). Seaweed addition to sediments affects important bacterial community changes among bacteria involved in nitrogen and carbon cycling, but especially in sulphur one (Aires et al., 2019). The Hg-methylation by iron-reducing bacteria (IRB) was also evidenced (Fleming et al., 2006). Experiments conducted in mangrove sediments suggested that SRB activity could not explain all MeHg formation, this implies the direct or indirect participation of other microorganisms as IRB and methanogens and a complex relationship among these groups (Correia and Guimarães, 2017).  $Hg^{2+}$  methylation by abiotic processes is also reported (Siciliano et al., 2005; Weber, 1993). Further transformation of Hg by microorganisms as demethylation,  $Hg^{2+}$ -reduction,  $Hg^0$ -oxidation, are also well known and allow the construction of a biogeochemical cycle of Hg in different environments (Barkay and Poulain, 2007). Laboratory and *in situ* experiments showed that the main factors affecting the behaviour of Hg are microorganisms, inorganic sulphides, organic matter, iron and manganese hydroxides, redox potential, chlorides and temperature in sediments (Boszke et al., 2003).

In aquatic ecosystems, sediments represent a source of contamination for both water and biota. Hg contamination in marine organisms in the area of North Tyrrhenian Sea has been widely described (Barghigiani et al., 1986; UNEP/FAO/WHO, 1987; Barghigiani and Ristori, 1995). Coastal lagoons could represent critical hotspot of Hg methylation, due to the presence of all of the main factors recognized to affect the behaviour of Hg.

The aims of this study were to highlight in sediment samples from a coastal lagoon the presence of: *i*) Hg-meth-

ylation capability in sediment slurries; *ii*) anaerobic SRB and their methylation activity of  $Hg^{2+}$ ; *iii*) heterotrophic  $Hg^{2+}$ - and  $CH_3Hg^+$ -resistant bacteria, also, achieving their isolation; *iv*) highlight relationships among macrophytes amount and methylmercury production.

## 2. Materials and methods

### 2.1. Study area

The study area was the Orbetello Lagoon (Italy; **Figure 1, Table S1**), located in Tuscany, west coast of Italy ( $42^\circ 26' 34''$  N,  $11^\circ 13' 29''$  E). The lagoon covers an area of ~ 2,300 ha with average water depth of ~ 1 m. The Orbetello lagoon was selected as perfect study area for our purposes due to the large scientific knowledge developed on natural and human pressures by scientific studies developed on this ecosystem (Porrello et al., 2005; Renzi et al., 2009; 2012; Perra et al., 2010; Renzi and Guerranti, 2018) that allow us a better localization of sampling stations. It is a coastal lagoon characterized by eutrophication (Specchiulli et al., 2008) and by a critical coexistence of differing submerged vegetation forms (Renzi et al., 2007; Giovani et al., 2010), with an uneven accumulation of decomposing biomass and phanerogams seed dispersal (Giusti et al., 2010). The use of a mathematical model combining the hydrodynamics and the water quality of the Orbetello Lagoon provides a thorough explanation of the expansion of the rooted vegetation in critical areas (Giusti et al., 2010). The Albegna River allows freshwater inputs (Fibbia canal, W-basin), also, representing a source both of nutrients (ARPAT, 2007a; 2007b) and pollutants (Specchiulli et al., 2008; Guerranti et al., 2017) for the whole lagoon ecosystem. According to literature, sediments are characterized by high levels of trace elements that evidence vertical concentration profiles of As, Cd, Cr, Cu, Hg, Ni, Pb and Zn with local background concentration values (BGVs) well-comparable with mean crustal or shale values for most of the considered elements (Renzi, 2007). On the contrary, Hg (0.87 mg/kg d.w.), and As (16.87 mg/kg d.w.) represent an exception, due to mineralization processes in the catchment basin draining into the lagoon (Romano et al., 2015). Total mercury ( $Hg_{tot}$ ) and MeHg were quantified in several specimens of *Dicentrarchus labrax* and *Sparus aurata* from the east basin of the Orbetello Lagoon, Italy.  $Hg_{tot}$  and MeHg in *S. aurata* ranged between 0.355-1.58 and 0.341-1.53  $\mu g g^{-1}$  wet weight (ww), respectively; in *D. labrax*, their ranges were 0.284-2.54 and 0.214-2.35  $\mu g g^{-1}$  ww. Approximately 90% of the concentrations measured exceeded  $Hg_{tot}$  regulatory maximum level of 0.5  $\mu g g^{-1}$  ww; however, exceedance rate was different in the two species studied (Miniero et al., 2013).

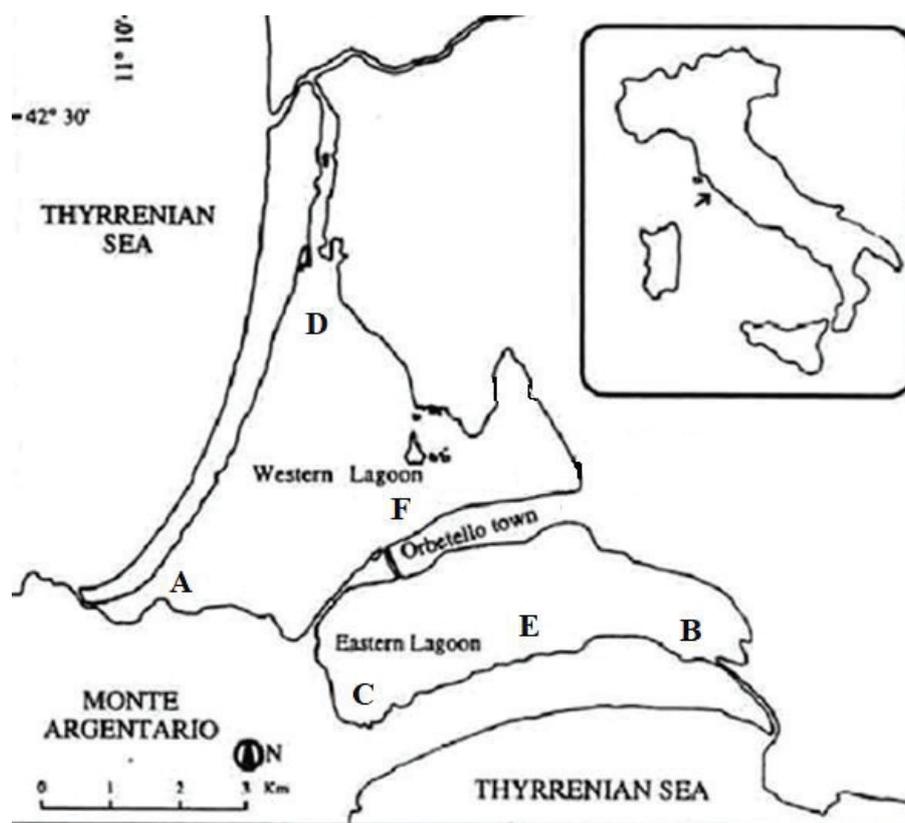


Figure 1. Map of the Orbetello Lagoon, Italy. Letters represent sediment sampling sites; their geographic coordinates are reported in **Supplementary materials (Table S1)**

Table 1. Bottom coverage (%) by macroalgae and phanerogams and associated Total Biomasses (TB) produced (average data  $\text{g m}^{-2}$  w.w.); SD = Standard deviation. \*data express mean percentage (standard deviation) of the phanerogam fraction of biomass contributing to TB

	Bottom coverage	TB Mean	SD	Phanerogam presence*	Species
A	60	1010.0	775.1	21.5 (37.2)	<i>C. nodosa</i>
B	60	369.0	312.1	7.5 (4.8)	<i>Ruppia</i> spp.
C	100	435.3	322.4	0.0 (0.0)	
D	80	558.0	240.2	47.2 (43.0)	<i>C. nodosa</i>
E	100	570.3	57.5	91.6 (8.0)	<i>Ruppia</i> spp.
F	80	311.7	100.0	0.0 (0.0)	

## 2.2. Sampling activities

The six sampling sites were chosen according to previous studies performed in the Orbetello lagoon (Renzi, 2007). Sediment pH and Eh values were determined in field us-

ing a potentiometric method, following probe calibration with a three-point curve using standard reference solutions (Crison, Eh/pH 25 with combined electrode), while the redox potential (Eh) values were detected after calibration of the system using a +220 mV calibration solution (Crison,

Eh/pH 25 with combined electrode). Samples of water at the bottom-water column interface were collected during samplings by syringe connected with plastic tubes. Sediment samples were collected in August 2007 both from the superficial portion of sediments, 0-5 cm, and at different depths: 5-10 cm, 10-15 cm and 15-20 cm. Superficial samples were collected using a grab sampler with a square section. Sterile portions for microbiological analyses were immediately removed, deposited into sterile sacks (Whirl-Pak, Nasco) and maintained at 4°C in the dark until their arrival in laboratory, where they were processed immediately. Deeper samples were extracted from a core, 50 cm long, sampled by a scuba diver using a soft-sediment plastic-barrel coring device (inside diameter 7 cm). Cores, carefully capped to prevent any loss of moisture, were shipped to the laboratory. Samples for microbiological analyses were aseptically collected from cores, at the cited levels, and maintained at 4°C.

Pore-water was collected by squeezing technique on collected sediments using a core-tube made by Inox steel according to Meson et al. (1998). The core tube was insert until 3 cm of depth into the sediment layer allowing the flux of water from sediment inside the core-tube and collected by a polypropylene syringe. Pore-water such extracted by surface sediments was associated to the sediment layer of 0-5 cm of depth. All the volume amount of pore-water that it was possible to extract from sediments avoiding long air exposure was recovered. Repeated extractions were performed to collect sample quantities need to complete analyses.

During samplings the percentages of coverage of bottom by macroalgae and phanerogams biomasses were determined in triplicates using a 50x50 cm square unit and recovered biomasses were collected to further analyses following methods reported by Renzi (2007) and by Giovani et al. (2010).

### 2.3. Physical-chemical analyses

**Sediment samples.** Core sediments were excised and analysed according to four levels of depth (0-5 cm; 5-10 cm; 10-15 cm; 15-20 cm). Replicates were used to obtain sufficient quantities of samples to perform all analyses. Collected sediments replicate (same depth levels) were homogenized prior to physical and chemical analyses and divided in separate aliquots to allow different treatments of the matrix according to the type of analysis. On collected sediments, water content (ICRAM, 2001-2003), density (ASTM D854), grain-size (ICRAM, 2001-2003; classification according to Udden, 1914; Wentworth, 1922; Krumbein, 1934), total organic carbon (TOC, ICRAM, 2001-2003; Gaudette and Flight, 1974), total carbon, total nitrogen, total sulphur (ICRAM, 2001-2003), total phosphorous (Italian Ministerial decree no. 185/199) levels

were measured. Furthermore, acid volatile sulphides (AVS, Simpson, 2001), Fe, Mn, Hg (US EPA, 2001), and MeHg (Caricchia et al., 1997) were determined. Further details on methods applied to determination of listed parameters in sediments are reported in **Supplementary materials**.

**Water analyses.** The main environmental parameters on water (temperature, salinity and pH) were measured using a multiparametric field probe. Chemical analyses were performed on both bottom water and pore-water samples. Sulphides (APAT-IRSA-CNR, 2003), AVS (Simpson, 2001), Fe, and Mn (US EPA, 2001), bicarbonates and carbonate (APAT-IRSA-CNR, 2003), MeHg (Caricchia et al., 1997) were determined. Further details on methods applied to determination of listed parameters in sediments are reported in **Supplementary materials**.

### 2.4. Determination on biomasses

The whole amount of biomass recovered in 50x50 cm square unit was weighted to determine biomass production by macroalgae and phanerogams in each sampling site as  $\text{g m}^{-2}$  (w.w.).

### 2.5. Microbiological experiments

Analyses were carried out at each sediment sample collected from the six areas chosen at the Orbetello Lagoon. The same four different levels of depth used for physical-chemical analyses were analysed (0-5 cm; 5-10 cm; 10-15 cm; 15-20 cm). Hereby a short description of followed methods is reported; a detailed paragraph explaining experimental conditions for each type of tests was reported in **Supplementary materials**.

Sediment slurries were arranged in the anaerobic culture medium for SRB, with addition of fatty acids as carbon source for SRB growth and of ionic mercury ( $\text{Hg}^{2+}$ ) was added as  $\text{HgCl}_2$  at a concentration of  $100 \text{ ng ml}^{-1}$  to perform experiments on mercury-methylation (King et al., 2000). After 18 days of incubation, 2 ml were collected and MeHg content were determined. Sulphate-reducing bacteria in sediments were revealed after 14 days of incubation by most probable number analyses (MPN). Cultures of SRB were arranged in vials in triplicate for each sample; at the end of the incubation period, 2 ml were collected and analysed for eventual  $\text{Hg}^{2+}$  transformation to MeHg to evaluate mercury-methylation by cultures of sulphate-reducing bacteria. Cells of SRB involved in the methylation process of mercury were counted by microscopic analyses and values expressed as  $\text{cell ml}^{-1}$ . Percentage of mercury-resistant bacteria (% MRB) were determined against total bacteria by incubating (48h at 28°C) surface sediment samples from the different sampling areas both with and without addition of  $10 \mu\text{g ml}^{-1}$  of  $\text{Hg}^{2+}$  added as  $\text{HgCl}_2$ . MRB were isolated and pure colonies were used to prepare liquid

cultures and aliquots were maintained at  $-80^{\circ}\text{C}$  in the presence of sterile glycerol at a concentration of 30%. Bacterial strains isolated on Petri dishes amended with Hg were tested for resistance to the ionic mercury ( $\text{Hg}^{2+}$ ) and monomethylmercury ( $\text{CH}_3\text{Hg}^+$ ), according to the method reported by Amsterdam (1996) to determine minimum inhibitory concentrations MICs were determined after 24 hours of incubation; MICs values allowed to evaluate the minimum concentration both of  $\text{Hg}^{2+}$  and of  $\text{CH}_3\text{Hg}^+$  able to inhibit bacterial growth.

### 3. Results

#### 3.1. Environmental conditions

Results on environmental conditions of water and sediment layers are reported to better contextualize the lagoon environments in the six sampling sites considered in this paper. Detailed data of results described in following paragraphs are reported in **Table S2-S4 of Supplementary materials**.

**Physical analyses of sediments (Table S2).** Sediment samples collected at all sampling sites in Orbetello Lagoon, Italy, evidenced the absence of particles assigned to gravel (diameter  $> 4$  mm). Sites A, C, F showed 22.64 – 27.71% d.w. values of particles within 4-2 mm of diameter. Three sites (B, C, E) were characterized by 59.06 -63.24% d.w. of fine sands (0.250-0.125 mm). Three sites (A, D, F) are characterized by high percentages 55.72-67.56% of mud (0.004-0.063 mm); while clays are low in all sampling sites (0.29-0.72%). The dry weight in all sampled sediment sites resulted included between 5.59-14.27%, and density ( $2.35\text{-}2.64\text{ g/cm}^3$ ) evidenced uniformity.

**Chemical analyses of sediments (Table S3).** Highest Hg levels were recorded in site C ranged within 6.765 (0-5 cm) – 37.630  $\text{mgkg}^{-1}$  d.w. (15-20 cm). Lowest occurred in site A ranging between 0.147 – 0.212  $\text{mgkg}^{-1}$  d.w. Sampling sites B, D, E, F showed higher Hg levels in 0-5 cm than values recorded deeper in the same site. On the contrary, A and C sampling sites showed increasing Hg levels with the sediment depth. Levels measured in the deepest layer of sediments from sampling site C were almost 5 times higher than those recorded in 0-5 cm layer.

Macronutrients (TC, TOC, TN, TS, TP) were determined to evaluate levels and composition of the sedimentary organic matter. The highest value of total carbon (9.00% TC) were recorded in site C were almost half was organic (4.29% TOC). The lowest levels of TC and TOC measured in sediments were 1.95% and 0.20% respectively (site B).

Total nitrogen (TN) and total sulphur (TS) showed the same trends recorded for TC and TOC; while total phosphorous (TP) showed a different behaviour and the highest concentration (0.064%) was recorded at the level 10-15 cm of site E while the lowest at level 15-20 cm of site D (0.003%).

Measurements of pH and Eh values revealing a minimum of 7.42 (site E) associated to the lowest Eh value equal to  $-340$  mV, and a maximum of pH of 8.35 (site B) associated to the maximum Eh level of  $-215$  mV. Iron concentrations showed the highest levels, of 36,880  $\text{mgkg}^{-1}$  d.w. in site C (5-10 cm) and of 32,690  $\text{mgkg}^{-1}$  d.w. (site F, level 0-5 cm), whereas the lowest value of 3,339  $\text{mgkg}^{-1}$  d.w. was detected at site B (level 5-10 cm). Manganese concentrations resulted strongly correlated to Fe levels. Mono-methylmercury values were revealed at the superficial level (0-5 cm) at each sampling site, evidencing the highest value of 0.330  $\mu\text{gkg}^{-1}$  d.w. at site D, followed by site E with 0.245  $\mu\text{gkg}^{-1}$  d.w., site A with 0.136  $\mu\text{gkg}^{-1}$  d.w., a value of 0.109  $\mu\text{gkg}^{-1}$  d.w. at site C, and the two sites with lower level of MeHg equals to 0.087  $\mu\text{gkg}^{-1}$  d.w., and 0.077  $\mu\text{gkg}^{-1}$  d.w., sites B and F, respectively. Acid-volatile sulphide evidenced the highest values at site C, with the maximum equal to 41.61  $\mu\text{molg}^{-1}\text{w.w.}$  (15-20 cm) and a minimum of 0.55  $\mu\text{molg}^{-1}\text{w.w.}$  at site A (10-15 cm).

**Pore-water (Table S4).** Pore-water evidencing homogeneity of dissolved sulphates values (3,192 – 3,587  $\text{mg l}^{-1}$ ) with minimum recorded at sites A and maximum at site E. Maximum values of pH, T, and salinity resulted 8.0 udpH,  $28.5^{\circ}\text{C}$ , and 46‰, respectively. Differences were evidenced concerning volatile sulfides, with values ranging within 0.13  $\text{mg l}^{-1}$  (site B)- 12.92  $\text{mg l}^{-1}$  (site C). Manganese concentrations showed the highest level of 0.19  $\text{mg l}^{-1}$  at site F and the lowest corresponding to 0.03  $\text{mg l}^{-1}$  at sites A, B and D. Iron showed homogeneity among sites, the highest concentration of 0.003  $\text{mg l}^{-1}$  was detected at site A and the lowest, equal to 0.001  $\text{mg l}^{-1}$  at sites B, E and F, and site D values were below the detection level. Mono-methylmercury was detected at the highest concentration of 3.641  $\mu\text{g l}^{-1}$  at site E and the minima value of 0.258  $\mu\text{g l}^{-1}$  at site A, no mono-methylmercury was detected in pore water at sites D and F. Hydrogen carbonate ion concentrations evidenced a maximum of 192.8  $\mu\text{g l}^{-1}$  at site C and a minimum of 100.5  $\mu\text{g l}^{-1}$  at site F. The largest differences among levels measured in pore-water and levels measure in water at the bottom interface is related to sulfides and MeHg.

#### 3.2. Biomass production

Sampling sites showed different percentage of coverage of bottoms as reported in Table 1. Sampling site C resulted having 100% of coverage of the bottom. The principal composition of total biomass recovered was represented by the macroalgae *C. linum* species. Sampling site E resulted also 100% but the dominant species was *Ruppia* spp. (phanerogam). The highest mean biomass productivity was recorded in sampling site A also associated to a wide standard deviation suggesting not homogeneous distribution of the biomass coverage. Presence of phanerogams (*Ruppia*

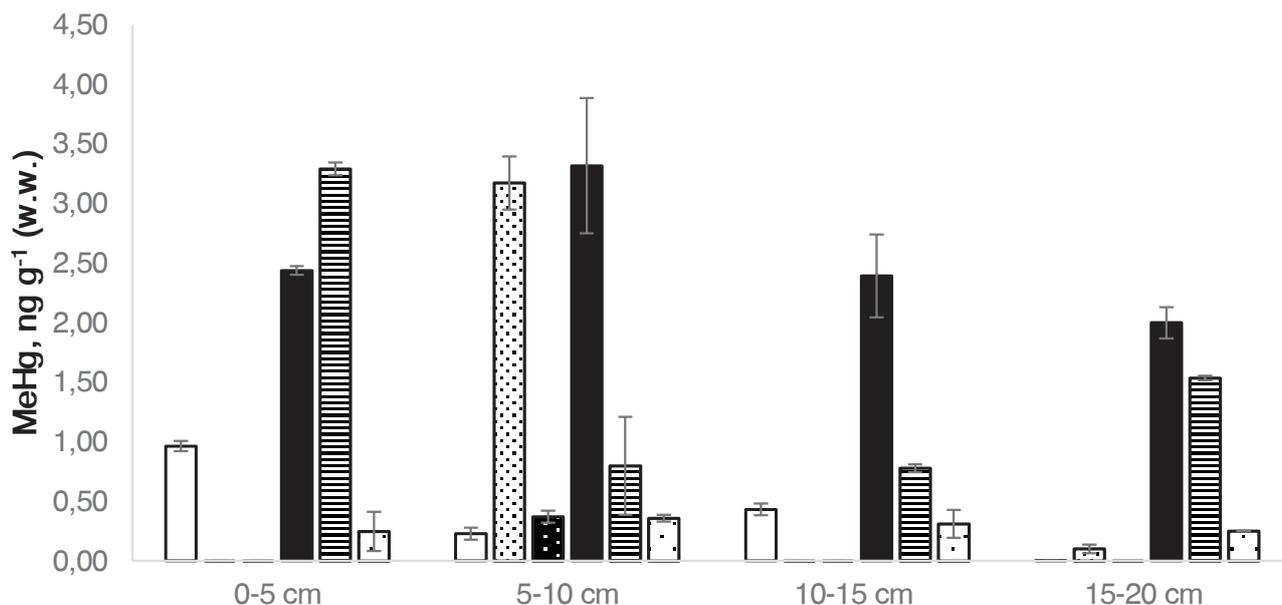


Figure 2. Mercury methylation in slurries arranged with different sediments samples collected from sites A (□); B (⊞); C (▨); D (■); E (⊞) and F (⋯) in the Orbetello Lagoon, Italy.

spp.; sites C, D, E) and *Cymodocea nodosa* (site B) was recorded during samplings.

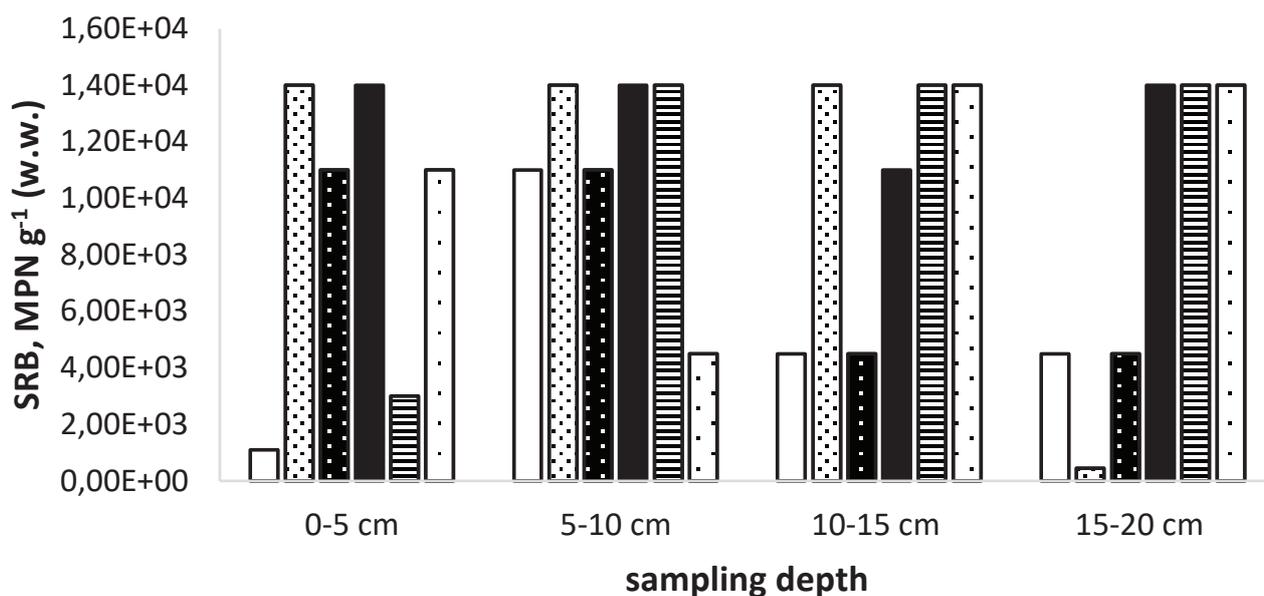
### 3.3. Microbiological aspects

MeHg production of slurries experiments are reported in **Figure 2** comparing performances to sediment level of depth. Slurries arranged with samples collected at level 0-5 cm evidenced the highest MeHg production at site D (2.4 ngg<sup>-1</sup> w.w. of MeHg) followed by site E, A and F, whereas no MeHg was revealed in slurries of sediments of sites B and C. Methylation at level 5-10 cm were the highest, in particular, for sites D (3.7 ngg<sup>-1</sup> w.w.) and B (3.4 ngg<sup>-1</sup> w.w.). Site D evidenced again the highest level of methylation at level of depth 10-15 cm (2.45 ngg<sup>-1</sup> w.w. of MeHg), followed by site E (0.8 ngg<sup>-1</sup> w.w. of MeHg), site A and F with a low methylation. Again, no MeHg was recovered in slurries from sites B and C (levels 10-15 cm). A similar behaviour was pointed out at level of depth of 15-20 cm, where site D still evidenced the highest methylation activity equal to 2.0 ngg<sup>-1</sup> w.w. Low levels of methylation were pointed out in slurries of sites A and C (respectively of 0.25 ngg<sup>-1</sup> w.w. and of no methylation).

Concerning sulfate reducing bacteria (SRB), the level 0-5 cm of sediments evidenced the highest concentrations in sites B and D ( $1.4 \times 10^4$  MPN g<sup>-1</sup> w.w.) even if SRB was high also in sites C and F ( $1.1 \times 10^4$  MPN g<sup>-1</sup> w.w.), while sites A and E showed low levels. Level recorded in 5-10 cm was high in site B, D and E ( $1.4 \times 10^4$  MPN g<sup>-1</sup>

w.w.), and in sites, A and C ( $1.1 \times 10^4$  MPN g<sup>-1</sup> w.w.). Site D showed the lowest concentration of SRB ( $4.5 \times 10^3$  MPN g<sup>-1</sup> w.w.). At the sampling depth levels equal to 10-15 cm, the three sites B, E and F evidenced the highest levels of SRB concentrations ( $1.4 \times 10^4$  MPN g<sup>-1</sup> w.w.), followed by site D ( $1.1 \times 10^4$  MPN g<sup>-1</sup> w.w.). Sites A and C showed the lowest SRB concentration ( $10^3$  MPN g<sup>-1</sup> w.w.). Again, three sites out of six, D, E and F, evidenced high levels of SRB ( $1.4 \times 10^4$  MPN g<sup>-1</sup> w.w.) at the deepest sampling level of 15-20 cm. Sites A and C pointed out SRB values of  $4.5 \times 10^3$  MPN g<sup>-1</sup> (w.w.), and a lower concentration ( $4.5 \times 10^2$  MPN g<sup>-1</sup> w.w.) was moreover evidenced at site B (**Figure 3**).

Cultures from site A evidenced Hg-methylation with production of 18 ngml<sup>-1</sup> of monomethyl mercury (MeHg) and  $7.0 \times 10^8$  SRB cellsml<sup>-1</sup> in superficial level of 0-5 cm (**Figure 1Sa**). Site B (levels 0-5 cm and 5-10 cm) showed similar concentrations of MeHg produced in the anaerobic cultures of SRB, with corresponding concentrations of SRB. Surprisingly, at level 10-15 of site B, the high levels of MeHg produced were not supported by equally high concentrations of SRB, thus suggesting a problem in counts or in MeHg detection. Further experiments with sediments at this level of depth are necessary to confirm these results. At the deepest level of 15-20 cm, both MeHg and SRB concentrations were low (**Figure 1Sb**). Concerning site C, production of MeHg in cultures was pointed out in all levels, with the highest value recorded at the deepest one (15-20 cm). A correspondence with SRB levels almost linear, was moreover evidenced (**Figure 1Sc**). A similar be-



**Figure 3.** Sulphate reducing bacteria (SRB) in sediment samples collected at the different sampling sites in the Eastern side: B (⊙); C (⊖) and E (⊕), and in the Western side: A (□); D (■) and F (⋯) of the Orbetello Lagoon, Italy, revealed as Most Probable Number per gram of fresh weight (MPN g<sup>-1</sup> f.w.).

havior was recorded at site D, where MeHg was recovered in all cultures of SRB with the maximum of production at the deepest level (15-20 cm) corresponding to a high concentration of SRB of  $3.8 \times 10^8$  cells ml<sup>-1</sup> (**Figure 1Sd**). Site E evidenced the highest levels of both Hg-methylation and SRB, except in level 5-10 cm, where low concentrations of MeHg were pointed out, along with a low number of SRB. The other levels of site E showed high levels of MeHg, with the highest in cultures from level of 15-20 cm, showing a MeHg concentration equal to 88,5 ng ml<sup>-1</sup> and  $1.2 \times 10^9$  SRB (**Figure 1Se**). In site F, the first two levels, only, evidenced the presence of low concentrations of MeHg and of SRB ( $2,0 \times 10^8$  cells ml<sup>-1</sup>), whereas nor MeHg, neither cells of SRB were detected in the other cultures arranged with deeper levels of sediment samples of the same site (**Figure 1Sf**).

A measure of cultivable total heterotrophic bacteria and of Hg-resistant heterotrophic bacteria was evidenced at each depth level of the different sediment sites. A percentage of mercury-resistant bacteria (MRB) as bioindicators of Hg in bioavailable was obtained at each sediment sampling site. As a results of this investigation, site B evidenced the highest level of percentage of MRB, equal to 8.9%, followed by site E, and with similar level of percentage of MRB at sites C, D and F, with values around 2%, and with the site A evidencing the lowest level of MRB, with a percentage of 0.5 (**Figure 4**).

Fifteen MRB were isolated and maintained at -80°C in sterile glycerol for eventual biotechnological applications. The minimum inhibitory concentrations of the isolates in the presence of both the inorganic and organic forms of Hg, added as HgCl<sub>2</sub> and CH<sub>3</sub>HgCl, respectively, evidenced highest levels of resistance to Hg<sup>2+</sup> of 31.25 µg ml<sup>-1</sup> for isolates B1b, A2a, A2b and A2c, and resistance to CH<sub>3</sub>Hg<sup>+</sup> at 3.12 µg ml<sup>-1</sup> for isolates E2a and F1a (**Table 2**).

#### 4. Discussion

**Effects of environmental conditions.** In Mediterranean coastal lagoons, Hg contamination is widespread, and the knowledge of the Hg biogeochemical cycle is of crucial importance (Coelho-Souza et al. 2006; Han et al. 2007). The Orbetello Lagoon is a heavily human-stressed ecosystem (Renzi et al., 2013) that is affected by Hg contamination due to the presence in the surrounding area of both an inactive volcano (Monte Amiata) and Hg mines. Mines were closed down in the late 1970s but represented a source of contamination for the whole nearby area (Grassi and Netti 2000; Fantozzi et al. 2009). Orbetello Lagoon belongs to the shallow-water lagoons, environmentally sensitive terrestrial-marine interface areas, where Hg is strongly affected by biogeochemical transformations of other elements, especially redox sensitive, microbially important elements

Table 2. Minimum inhibitory concentrations (MIC) of aerobic mercury resistant bacterial strains isolated from sediments samples collected from different sites in the Orbetello Lagoon, Italy.  $\text{Hg}^{2+}$  = ionic mercury ( $\mu\text{g ml}^{-1}$ );  $\text{CH}_3\text{Hg}^+$  mono-methylmercury ( $\mu\text{g ml}^{-1}$ ).

Isolated bacterial strain	$\text{Hg}^{2+}$	$\text{CH}_3\text{Hg}^+$
B1 a	15.62	1.56
B1 b	31.25	0.39
B1 c	7.80	0.39
A2 a	31.25	1.56
A2 b	31.25	1.56
E2 a	31.25	0.78
E2 b	15.62	3.12
F1 a	15.62	3.12
E2 c	3.90	0.19
C1a	7.80	0.19
A2 c	31.25	0.39
E2 d	15.62	0.19
E1 a	15.62	0.39
E1b	15.62	0.39
F1b	15.62	0.09

such as sulphur, iron and manganese and their interaction with organic matter (Faganeli et al., 2012). As evidenced by results reported in this study, a large part of lagoon sediments is represented by clay (diameter ranging within 0.004 – 0.063 mm). According to Förstner and Wittmann (1983) and more recently Dung et al. (2013), this grain size favours the accumulation of contaminants through surface chemical processes. Site D, E and F have high levels of silt and slurries produced methylmercury with constancy at any depth level, this could suggest a higher bioavailability of Hg, as the element is distributed on the surface of these little particles. Instead site C evidenced particles of higher size and a high content in total Hg, but the availability of Hg decreases, as the metal is bound to particle with a larger size (Tessier et al., 1988).

Salinity could affect MeHg production. Boyd et al., (2017) suggested that in low saline habitats an important role is played by both sediment anoxia and microbial sulphate reducers in the production of MeHg; on the contrary, high salinity levels could limit  $\text{Hg}^{2+}$  methylation by influencing the availability of  $\text{Hg}^{2+}$  for methylation. Orbetello Lagoon pore-water evidenced high salinity levels (range 40-46‰); nevertheless, MeHg in pore-water seems not be influenced by this parameter.

Iron and manganese hydroxides are inorganic compounds that can have a significant effect on Hg transpor-

tation (Guentzel et al., 1996). The importance of iron and manganese hydroxides derives from their high sorption capacity, the ability to co-precipitate with Hg compounds, and the ability to release Hg from iron and manganese hydroxides (Gagnon et al., 1996). In this study, site D evidenced high concentration of iron and this suggests a possible involvement of this inorganic compound in the high production of MeHg registered at this site in slurries experiments.

Mono-methylmercury (MeHg) at the superficial level of sediment samples collected at sites of Orbetello Lagoon evidenced a value less than 0.5% at site B, in line with MeHg in shallow water sediments, and close to the limit of 1,5% for bottom sediments at site C (Hamasaki et al., 1995; Kannan and Falandysz, 1998).

The alkalinity in pore water samples of site C showed a quite high value of sulphides, whereas concentrations in sulphates are similar in all the sampling sites. It is known that alkalinity is connected to increase in sulfide and a decrease in sulfate, suggesting that a principal factor affecting alkalinity in pore water is bacterial sulfate reduction (Berner et al., 1970).

The strong affinity of Hg to sulphur regulates its distribution in the environment (Boszke et al., 2003). The solubility of complexes  $\text{HgS}$  in the environment considerably increases with increasing content of sulphide ions  $\text{S}^{2-}$  and

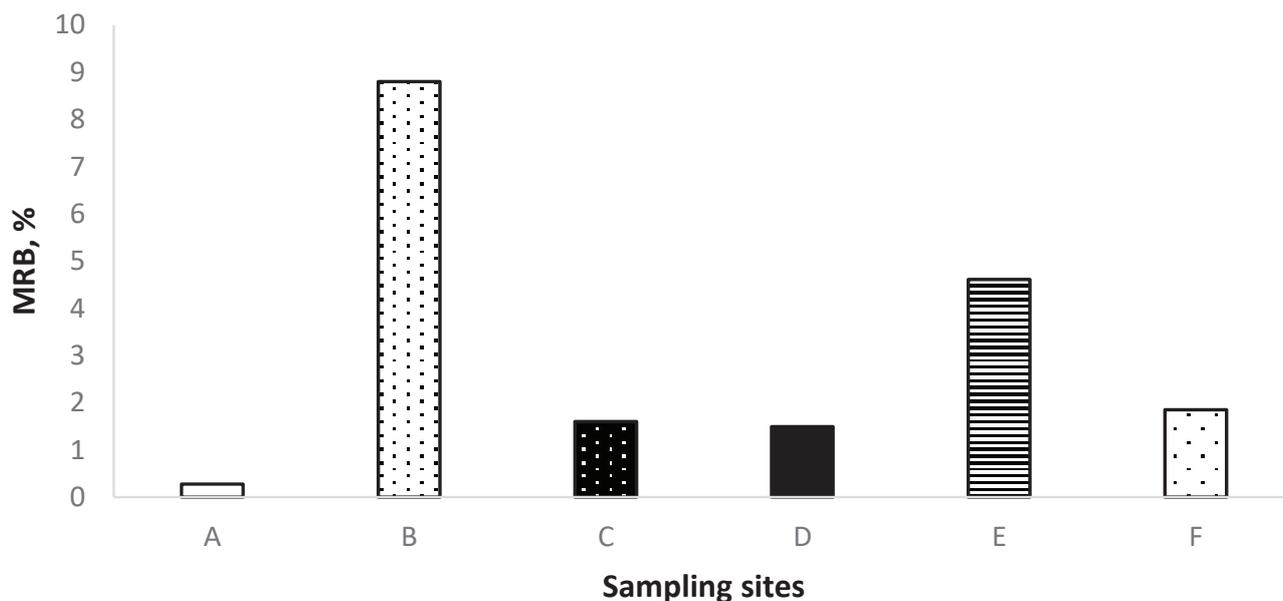


Figure 4. Percentages of mercury resistant bacteria (MRB) isolated from sediments samples collected from sites A (□); B (◐); C (◑); D (■); E (◒) and F (◓) in the Orbetello Lagoon, Italy. Mean actual numbers of MRB were: A =  $1.0 \times 10^4$ ; B =  $3.3 \times 10^3$ ; C =  $2.5 \times 10^2$ ; D =  $3.7 \times 10^2$ ; E =  $3.3 \times 10^2$ ; F =  $2.5 \times 10^1$ . Data are expressed as Colony Forming Units per gram of fresh weight (CFU  $\text{g}^{-1}$  f.w.)

becomes sufficient to form soluble sulphide and disulphide complexes (Jay et al., 2000). This property is responsible for great concentrations of the soluble Hg species in anaerobic zones of the bottom sediment (Fergusson, 1990). Despite high level of Hg which can be conveyed by sulphide ions, organic mercury is frequently absent, due probably by the fact that a very high concentration of Hg inhibits development of microorganisms responsible for Hg methylation. Moreover, absorption of Hg on the AVS can limit the amount of Hg dissolved in porous water in anaerobic conditions (Boske et al., 2003). Highest concentrations of AVS in sediments of the Orbetello Lagoon were found at site C, where methylmercury produced in slurries was present at level 5-10 cm, only. This could represent a case in which Hg in solution is present in very high concentrations, preventing microbial activity. Our results evidenced as site B showed the highest Hg-bioavailability, followed by site E due to both low high size particles % and low iron and manganese concentrations, all features diminishing Hg availability.

Concerning sediment-water column fluxes, a comparative analysis on pore- and bottom- water levels evidenced similar concentrations in almost all sampling sites with the exception of site A and B, where levels in bottom water are almost twice levels recorded in pore-water. This occurrence could be due to different factors: *i*) occurrence of local accumulation of MeHg in A and B bottom water due to the forced current fluxes by the outflow canals of the Orbe-

tello Lagoon (Giusti et al., 2010); *ii*) additional methylation process involving water column. This occurrence could be enhanced by the effluents enriched in soluble organic matter discharged in site A and B by local fish farming plants (Porrello et al., 2005; Specchiulli et al., 2008), *iii*) different turnover routes modulating by the organic matter; *iv*) different MeHg absorption routes/speed by trophic web. Nevertheless, collected data are not sufficient to explain these results and further specific studies are needed to better clarify these aspects.

#### Effects of organic matter loads and bioturbation.

Laboratory experiments with pure cultures, suggested that dissolved organic matter (DOM) may facilitate the uptake of Hg either by acting as a shuttle molecule, transporting the  $\text{Hg}^{2+}$  atom to divalent metal transporters, or by binding  $\text{Hg}^{2+}$  then transported into the cell as a carbon source (Mazrui et al., 2016). Sediment organic matter has been shown to limit MeHg production in estuarine ecosystems, acting as the primary control over MeHg production (Schartup et al., 2013). Nevertheless, when Hg levels in sediments are high, organic matter does not hinder mercury methylation (Schartup et al., 2013). *In vivo* studies performed on the Hg-methylating sulfate-reducing bacterium *Desulfobulbus propionicus* 1pr3 evidenced a time dependence for organic matter-enhanced Hg bioavailability for methylation (Moreau et al., 2015).

Sediment-water interface is considered as the primary methylation site in natural ecosystems (Bravo et al., 2014).

This aspect is sustained from researches evidencing that macrophytes in brackish water stimulate MeHg production as has previously been suggested for other habitats (Aldrige and Gauf, 2003; Canario and Vale, 2012). The origin and molecular composition of organic matter are critical parameters to understand and predict methylmercury formation and accumulation (Bravo et al., 2016). Furthermore, distribution of phanerogams in sampling sites is different as in site C was absent, while, site E showed the 100% of bottom covered by *Ruppia* spp. Phanerogams actively spread the oxygen produced by photosynthesis from the rhizosphere oxidising sediment layers (Sand-Jensen et al., 1982; Viaroli et al., 1996) inducing Eh bioturbation and consequently affecting anaerobic populations. Differences among sampling sites concerning total biomass (TB) and species could explain some differences of MeHg levels reported in this study, nevertheless, further studies are needed to target these relationships.

Organic matter originating from algal blooms strongly increase MeHg production (Bravo et al., 2016), for this reason the high content in algal biomass in the Orbetello Lagoon could represent a serious danger for the whole ecosystem. Mechanic removal of algal biomass from basins of the Orbetello Lagoon could thus be of extremely importance to avoid dystrophic crisis and to reduce MeHg inputs. In Venice Lagoon, Kim *et al.* (2011) evidenced that MeHg/Hg % and specific Hg<sup>2+</sup> methylation rate in sediments increased with the decrease of C/N ratio meaning that the Hg<sup>2+</sup> methylation rate was affected by lability of organic matter. In fact, surface sediments, which contained high levels of fresh algal organic matter (C/N = 5.8-7.8), showed higher Hg<sup>2+</sup> methylation rates than deeper sediment layers (C/N > 10). Kim *et al.* (2011) suggested that C/N ratio can be used as a proxy for the lability of organic matter and of Hg<sup>2+</sup> methylation rate in sulphate-rich sediments (Kim et al., 2011). In Orbetello Lagoon, C/N ratios were always < 10 with the exception of two cases (site A, 0-5 cm; site E, 15-20 cm) (data not shown). This fact confirms possible problems related to the presence of a high algal biomass in this ecosystem that could increase the process of Hg-methylation. MeHg production in macrophyte habitats in presence of Hg contaminated sites request more attention, with regards to the factors controlling the methylation processes (Olsen et al., 2018).

Presence of macroalgae in sediments, represent a source of labile organic matter able to promote microenvironments allowing the activity of sulphate-reducing bacteria and of Hg-methylation. In coastal lagoons, the presence of anoxic conditions and of thriving bacteria associated with the sulphur cycle, could mobilize chemical contaminants present in sediments as in the case of mercury from cinnabar that could be released as methylmercury (Wood and Wang, 1983; Kim et al. 2006). These processes are strictly linked to biomass proliferation. Relationships among or-

ganic matter composition in sediments and methylmercury production in high reductive ecosystems are reported by the literature, supporting the need to monitor a wider range of variables in sediments. Both origin and molecular composition of organic matter are key aspects to understand and predict methylmercury formation and accumulation (Bravo et al., 2016). In brackish water, methylmercury production is stimulated by macroalgae (Aldrige et al., 2003; Canario and Vale, 2012). Thus, next to C/N ratio suggested as a proxy to predict sulphate-reducing bacteria growth and Hg-methylation, analyses of organic matter in sediments of shallow waters are of fundamental importance to predict extremely dangerous phenomena as Hg-methylation processes (Renzi et al., 2019).

**Microbiological considerations.** Slurries arranged with sediments evidenced methylation of the ionic mercury added. Zhang (2014) reported methylation of Hg in estuarine sediment microcosms amended with dissolved, nanoparticulate, and microparticulate mercuric sulfides. On the contrary, Han et al. (2010) evidenced in slurries from Venice Lagoon active methylation of Hg<sup>2+</sup> occurring under sulfate-limited conditions, evidencing that syntrophic processes can occur between methanogens and sulfidogens bacteria. Anaerobic sulphate-reducing bacteria (SRB) are involved in methylation of Hg with the production of MeHg that is able to bioaccumulate and biomagnificate along trophic chain (Hosokawa, 1995). In this study, SRB was recorded in all sampling sites at similar levels recorded in sediments from the shallow Lagoon of Prévost, French Mediterranean coast (Caumette, 1986). Populations of SRB were recorded in a range from 10<sup>2</sup> to 10<sup>4</sup> MPN from polluted sediments in the Lagos Lagoon (Nigeria) (Esiobu et al., 1991). In the coastal subsurface sediments (5 cm) from the Neuharlingersiel and Gröningen Plate sites (German North Sea), SRB ranged within 10<sup>4</sup> – 10<sup>6</sup> MPN (Köpke et al., 2005). Similar values of SRB values ranging within 10<sup>3</sup>- 10<sup>6</sup> MPN, were evidenced in German Wadden Sea sediments (Llobet-Bross et al., 2002).

Anaerobic cultures arranged with SRB isolated from the Orbetello Lagoon sediments, showed methylation of a known concentration of Hg<sup>2+</sup> added as HgCl<sub>2</sub>. The occurrence of methylation in cultures of anaerobic SRB and microbial methylmercury production was consistent with the literature (Compeau and Bartha, 1985; Gilmour et al., 1992; King et al., 2000; Janssen et al., 2016). A combination of Hg bioavailability and activity of methylating microorganisms is at the base of the processes of methylation of ionic mercury in cultures (Zhang et al., 2014). Cultures of SRB methylating mercury in this study, evidenced concentration of 10<sup>8</sup> – 10<sup>9</sup> cells ml<sup>-1</sup>, by microscopic counts, and similar values of mercury methylating SRB cells were described (King et al., 2000).

In this study, SRB growth and MeHg production were higher in cultures from site E, whereas previous slurry ex-

periments gave site D as the most productive site in terms of methylmercury. This could be due to the fact that, in laboratory condition, when arranging cultures in the medium for SRB, a selection as respect to the natural microbial population is operated, allowing growth of bacteria able to adapt to the growth conditions, but not necessarily the most representative bacteria present in the original sample. It is therefore possible that the comparison of the results obtained in the laboratory and those of the native sediments may not be comparable. Several SRB cultures from sampling sites evidenced mercury methylation at low levels, at similar values detected in sediments slurries, although the high SRB cell concentration could have suggested a major efficiency in methylation. Actually, SRB grew in mixed cultures and at moment of  $\text{Hg}^{2+}$  addition the most effective SRB cells are effective in the methylation process, only.

Hg-resistant bacteria isolated from all sampling sites (40% site E; 20% site B), probably confirm the role played by the Hg bioavailability evidenced by sediment analyses in selecting for Hg-resistant bacteria. Hg-resistant aerobic bacteria were isolated from Tagus Estuary, Portugal, evidencing the capability to reduce  $\text{Hg}^{2+}$  to  $\text{Hg}^0$  followed by  $\text{Hg}^0$  volatilization, along with a low activity of  $\text{Hg}^{2+}$  methylation (Figueiredo et al., 2016). Levels of Hg-resistance were high in some autochthonous isolates from the Orbetello Lagoon, giving insights for future studies of possible biotechnological uses of these adapted bacteria. Studies on mercury-resistant heterotrophic bacteria isolated from sediments of the Orbetello Lagoon were previously carried out, evidencing high levels of resistance and the capability to volatilize both  $\text{Hg}^{2+}$  and  $\text{CH}_3\text{Hg}^+$ , thus suggesting their use in possible bioremediation processes (Pepi et al., 2011, 2013).

## 5. Conclusions

This study evidenced, for the first time, the activity of SRB in sediments of the Orbetello Lagoon and their contribution in methylation processes, with consequent possible serious concerns related to the environmental exposure to MeHg and related biomagnification. The presence of algal biomass resulted a source of labile organic matter able to promote microenvironments favourable for SRB activity and MeHg production. A reduction of Hg-methylation by SRB could be obtained by macrophytes removal. Hg-resistant bacteria isolated from polluted sediments, could represent a possible tool in bioremediation processes of native sites. Further researches could be developed to better understand detailed relationships among MeHg and Hg levels in sediments. Nevertheless, obtained results could be useful to develop well-sized management strategies to improve the whole ecosystem quality.

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Further details on methods and experimental collected data are reported in Supplementary materials.

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## Supplementary material

**Table S1.** Sampling sites coordinates.

Samples	UTM NORD	UTM EST
A	42.436206	11.175701
B	42.428339	11.267111
C	42.419337	11.208132
D	42.469706	11.197925
E	42.427792	11.236087
F	42.444811	11.212199

Notes: A, Western basin of the lagoon, with total Hg in sediments around 0.5 mg kg<sup>-1</sup> (d.w.); B, Eastern basin, showing values of Hg in sediments, in some case higher than 5.0 mg kg<sup>-1</sup> (d.w.);

C, Eastern basin of the lagoon with Hg total values superior to 5.0 mg kg<sup>-1</sup> (d.w.); D, Western basin of the lagoon, with values of total Hg around 0.5 mg kg<sup>-1</sup>; E, Eastern basin with values of Hg around 1 mg kg<sup>-1</sup>; F, Western basin, with values of total Hg of 0.5 mg kg<sup>-1</sup>.

### Methodological details on physical-chemical analyses

**Sediment samples.** Core sediments were excised and analysed according to four levels of depth (0-5 cm; 5-10 cm; 10-15 cm; 15-20 cm). Replicates were used to obtain sufficient quantities of samples to perform all analyses. Collected sediments replicate (same depth levels) were homogenized prior to physical and chemical analyses and divided in separate aliquots to allow different treatments of the matrix according to the type of analysis.

The grain size composition of sediments was determined on separate sediment aliquot according to meth-

ods proposed by ICRAM (ICRAM, 2001-2003). Texture classifications of sediments were expressed as a function of gravel, sand, silt and clay percentages (Krumbein, 1934; Udden, 1914; Wentworth, 1922). Percentage of water was detected according to ICRAM (2001-2003), while, density was obtained according to ASTM D854.

Total organic carbon (TOC) analysis was carried out according to the ICRAM (2001-2003) method, following modifications by Gaudette and Flight (1974). Total phosphorous was determined according to Italian Ministerial decree no. 185 of 199 concerning 'Chemical analysis of soil'. Total carbon, total nitrogen and total sulphur analyses were carried out according to ICRAM (2001-2003) by CHNS/O (2400, Perkin Elmer). Acid volatile sulphides were detected by spectrophotometer at a wavelength of 670 nm (Simpson, 2001), (LOQ = 0.5  $\mu\text{M g}^{-1}$ ). Quantifications of Fe and Mn were carried out by Inductively Coupled Plasma Optical Emission Spectrometry, ICP-OES, (Optima 5000 DV Perkin Elmer). The detection limit for Fe was 1,000  $\text{mg kg}^{-1}$  d.w., and that for Mn 10  $\text{mg kg}^{-1}$  d.w.

Sediment samples were sieved to obtain the <250  $\mu\text{m}$  fraction and mineralised with 65% nitric acid concentrated in a microwave oven (ETHOSD Microwave Labstation, Milestone), according to the US EPA (1994). Hg was determined by cold vapour atomic absorption spectrometry (CV-AAS) (US EPA, 2001). The percentage coefficient of variation (% CV) of three repeated samples was below 5%. Accuracy was tested using Standard Reference Material 1646a, from the National Institute of Standards and Technology (NIST) of the US Department of Commerce, Gaithersburg, MD, USA. The detection limit for Hg was 0.03  $\text{mg kg}^{-1}$  d.w.

Methylmercury content was determined by alkaline digestion from sediments by using a solution of KOH/ $\text{CH}_3\text{OH}$  at 25% in an ultrasonic bath for 45 min, followed by acidification with  $\text{H}_2\text{SO}_4$  (a 4M solution saturated with  $\text{CuSO}_4$ ) and KBr for the extraction of the organic fraction with toluene. The extracted solution was then purified via a clean-up with 1% of cysteine and final extraction by adding benzene,  $\text{CuSO}_4$  and KBr 4M (Caricchia et al., 1997). Aliquots of the final volume extracted of benzene were injected in a gas-chromatograph system with detector for electrons ECD (Autosystem A, Perkin Elmer) for quantification according to the ramp proposed by Baldi et al. (1993). The column (HP-1 Methylsilicone, 30 m with i.d. 0.53 mm and a film of 2.65  $\mu\text{m}$ , Agilent Techn.) was maintained in isotherm conditions at 120°C. The identification was carried out on the retention time of the peaks and the CV% resulted of 6.4%. In particular, the lower limit of detection for the solid matrix was for solid matrix was of 0.039  $\text{ng g}^{-1}$  (LOD) and 0.106  $\text{ng g}^{-1}$  (LOQ).

**Water analyses.** The main environmental parameters on water (temperature, salinity and pH) were measured using a multiparametric field probe. Chemical analyses were

performed on both bottom water and pore-water samples. For the determination of sulphides, a sterile syringe used for the pore-water collection was added by barium chloride according to APAT-IRSA-CNR (2003) to reduce losses due to volatilization processes and to precipitate sulfate ion (LOQ = 1  $\text{mg l}^{-1}$ ). AVS determination was performed on samples fixed with zinc acetate. Zinc sulphide precipitates were measured spectrophotometrically after a colorimetric reaction with *N,N*-dimethyl-*p*-phenylenediamine (LOQ = 0.01  $\text{mg l}^{-1}$ ). Fe (LOQ = 0.001  $\text{mg l}^{-1}$ ) and Mn (LOQ = 0.01  $\text{mg l}^{-1}$ ) levels were determined by Inductively Coupled Plasma Optical Emission Spectrometry, ICP-OES, (Optima 5000 DV Perkin Elmer). Another aliquot of pore water was used to determine levels of bicarbonate ions ( $\text{HCO}_3^-$ ) and carbonate ( $\text{CO}_3^{2-}$ ) according to the APAT-IRSA-CNR (2003), LOQ = 0.1  $\text{mg l}^{-1}$ . MeHg was determined on water as reported for sediments following Caricchia et al. (1997).

### Microbiological experiments

Analyses were carried out at each sediment sample collected from the six areas chosen at the Orbetello Lagoon. The same four different levels of depth used for physicochemical analyses were analysed (0-5 cm; 5-10 cm; 10-15 cm; 15-20 cm).

**Mercury-methylation in sediment slurries.** Slurries arranged in the anaerobic culture medium for SRB, with addition of fatty acids as carbon source for SRB growth and of ionic mercury ( $\text{Hg}^{2+}$ ) added as  $\text{HgCl}_2$  at a concentration of 100  $\text{ng ml}^{-1}$ , was carried out. One gram of sediment collected was added to 9 ml of filtered lagoon water in vials for anaerobic cultures under a nitrogen flux, obtaining slurries from the different sediment samples according to King et al. (2000). At each slurry, sodium lactate 20 mM, sodium acetate and sodium pyruvate at a concentration of 10 mM, each were added as electron donors for anaerobic bacteria growth. These operations were carried out according to the Hungate techniques for anaerobic culturing, and vials containing slurries were incubated at 28°C in the dark. After 8 days of incubation, 100  $\text{ng ml}^{-1}$  of  $\text{Hg}^{2+}$  were added to each vial from a solution of  $\text{HgCl}_2$  at a concentration of 100  $\mu\text{g ml}^{-1}$ . Samples were incubated in the same conditions for other 10 days. Then aliquots of 2 ml were collected from each vial and treated for the extraction of the MeHg content, according to the extraction method previously reported for sediments. Again, in accordance with previous reported methods (Baldi et al., 1993), MeHg concentrations in the aliquots were also detected.

**Sulphate-reducing bacteria in sediments revealed by most probable number analyses (MPN).** In this case, sampling procedures were carried out on a dedicated sediment core under a constant nitrogen flux to maintain anoxic conditions, and respecting sterility rules. On these ali-

quots microbial analyses were carried out in order to detect the sulphate-reducing bacteria (SRB) content, according to the method of the Most Probable Number (MPN). Experiments were carried out in vials containing Postgate medium for growth of anaerobic SRB. Medium preparation and procedures of inoculum were carried out according to the Hungate techniques for anaerobic cultures, in the presence of a constant nitrogen flux. Inocula were carried out both from fresh non-diluted sediment samples and from the relative serial dilutions. Cultures were then incubated at 28°C and growth was detected after 14 days of incubation. Evaluation of microbial growth was carried out according to the numeric code of the McCrady tables based on the positive and negative growth in the different vials and growth expressed as MPN per gram of fresh weight. Composition of the Postgate medium, per litre of double distilled water, for enrichment cultures of SRB was the following: NaSO<sub>4</sub> 2 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 2 g, NH<sub>4</sub>Cl 1 g, KH<sub>2</sub>PO<sub>4</sub> 0.5 g, yeast extract 1 g, ascorbic acid 0.1 g, thyo-glicolic acid 0.1 g, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g, sodium lactate 10 ml from a solution 1 M, sodium pyruvate 10 ml of a solution 1 M, sodium acetate 10 ml from a solution 1 M, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.06 g, NaCl 2.5%, Pfenning's trace element solution 10 ml, and the final pH was adjusted to 7.3 ± 0.2. Composition of the Pfenning's solution, per litre of double distilled water, was the following: ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g, MnCl<sub>2</sub>·4H<sub>2</sub>O 0.03 g, H<sub>3</sub>BO<sub>3</sub> 0.3 g, CoCl<sub>2</sub>·6H<sub>2</sub>O 0.2 g, CuCl<sub>2</sub>·2H<sub>2</sub>O 0.01 g, NiCl<sub>2</sub>·6H<sub>2</sub>O 0.02 g, Na<sub>2</sub>Mo<sub>4</sub>·2H<sub>2</sub>O 0.03 g, and FeCl<sub>3</sub> 0.05 g.

**Mercury-methylation by cultures of sulphate-reducing bacteria.** Cultures of SRB were arranged in vials containing Postgate medium with an inoculum (1:20) from original cultures of anaerobic SRB previously used for counts, in triplicate for each sample. Cultures were incubated at 28°C and 100 ng ml<sup>-1</sup> of Hg<sup>2+</sup> as HgCl<sub>2</sub> were added after three days. The incubation was continued for another three days, then aliquots of 2 ml were collected and analysed for eventual Hg<sup>2+</sup> transformation to MeHg, according to the previously reported method.

**Sulphate-reducing bacterial strains counts.** Cells of SRB involved in the methylation process of mercury were counted by microscopic analyses and values expressed as cell ml<sup>-1</sup>. Aliquots of 0.5 ml of sulphate – reducing bacterial cultures were harvested and 0.5 ml of a 4% formaldehyde were added, and incubated at room temperature for 15 min to allow fixation of bacterial cells. Each suspension was then centrifuged at 4,000 r.p.m. for 25 min, and pellets washed twice in a physiological solution with 0.85% of NaCl, and re-suspended in 0.5 ml of the same washing solution. Aliquots of 30 µl from each suspension were introduced in a counting chamber (Neubauer Improved, Germany), containing a reticulum on the surface of the chamber. Considering the area in each square, and noted the distance from the reticulum and the cover-object slide, the volume

included in each square was known. The number of cells present in each square of the reticulum was counted, then converted in number of cells in each millilitre of suspension multiplying for a conversion factor based on the volume of the counting chamber. Bacteria for each sample present in four different squares were counted, then medium values and relative standard deviations were calculated.

**Percentage of mercury-resistant bacteria.** Surface sediment samples from the different sampling areas were used to prepare serial dilutions by using filtered water lagoon. Aliquots of 100 µl from each dilution were spread plated on the surface of Petri dishes containing Marine Agar medium (Difco), both with and without addition of 10 µg ml<sup>-1</sup> of Hg<sup>2+</sup> added as HgCl<sub>2</sub>. Petri dishes, three with and three without Hg<sup>2+</sup> per each sample, were then incubated at 28°C and colonies counted after 48 hours. The number of mercury-resistant bacteria was then revealed calculating the percentage of Hg-resistant bacteria relative to the total considered as the 100%, according to the following formula: % Mercury Resistant Bacteria (% MRB) = MRB × 100 / Total Bacteria.

**Mercury-resistant bacteria isolation.** Colonies developed on the Petri dishes surfaces in Marine Agar in the presence of 10 µg ml<sup>-1</sup> of Hg<sup>2+</sup> were collected according to rules of sterility and streaked for at least three times on Petri dishes containing the same culture medium in the presence of Hg<sup>2+</sup>. Pure colonies were used to prepare liquid cultures and aliquots were maintained at -80°C in the presence of sterile glycerol at a concentration of 30%.

**Minimum inhibitory concentrations of isolated bacterial strains.** Bacterial strains isolated on Petri dishes amended with Hg were tested for resistance to the ionic mercury (Hg<sup>2+</sup>) and mono-methylmercury (CH<sub>3</sub>Hg<sup>+</sup>), according to the method reported by Amsterdam (1996). Bacterial strains were grown in Nelson Medium (Nelson et al., 1973) containing the following components, per litre of bi-distilled water: casamino acids 5 g, D-glucose 2 g, yeast extract 1 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g, and NaCl 0.9 g. Cultures at inocula values of 2 × 10<sup>4</sup> to 10<sup>5</sup> CFU ml<sup>-1</sup> for each mercury-resistant bacterial strain were added to 96 wells plates. Hg<sup>2+</sup> was added from a solution of HgCl<sub>2</sub> at different concentrations: 0, 0.98, 1.9, 3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, and 500 µg ml<sup>-1</sup>. Mono-methylmercury was added from a solution of CH<sub>3</sub>HgCl at concentrations of 0, 0.09, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 µg ml<sup>-1</sup>. Cultures were then incubated at 28°C in the dark. Each tested strain was plated on the surface of a Petri dish in order to control sterility of bacterial cultures. After 24 hours of incubation plates were read and MICs values were evidenced as the minimum concentration both of Hg<sup>2+</sup> and of CH<sub>3</sub>Hg<sup>+</sup> able to inhibit bacterial growth.

Table S2. Physical characterization of sampling sites.

Samples	Fr >4000	Fr >2000	Fr >1000	Fr >500	Fr >250	Fr >125	Fr >63	Fr 4-63	Fr <4	H	$\gamma_s$
A	0	22.64	4.97	2.52	3.93	7.61	2.21	<b>55.72</b>	0.41	12.51	2.35
B	0	7.50	1.91	1.28	14.97	<b>63.24</b>	2.25	8.48	0.38	8.88	2.55
C	0	24.55	4.72	1.79	1.36	<b>59.06</b>	0.86	7.37	0.29	8.27	2.33
D	0	8.77	3.36	2.05	3.20	9.26	5.08	<b>67.56</b>	0.72	14.27	2.64
E	0	3.40	0.91	0.58	6.42	<b>62.34</b>	1.37	24.56	0.42	5.59	2.52
F	0	27.71	5.63	2.25	2.04	4.22	1.34	<b>56.50</b>	0.32	12.44	2.60

Notes: Fr > 4000 = particles with a diameter greater than 4 mm (gravel,% d.w.); Fr > 2000 = particles with diameter greater than 2 mm (% d.w.); Fr > 1000 = particles with diameter greater than 1 mm (% d.w.); Fr > 500 = particles with diameter greater than 0.5 mm (% d.w.); Fr > 250 = particles with diameter greater than 0.25 mm (% d.w.); Fr > 125 = particles with a diameter greater than 0.125 mm (% d.w.); Fr > 63 = particles with a diameter greater than 0.063 mm (d.w.); Fr 4- 63 = particles with diameter ranging from 0.063-0.004 mm (sludge,% d.w.); Fr <4 = particles with a diameter less than 0.004 mm (clay,% d.w.); H = dry weight = 100-water content (%);  $\gamma_s$  = density = mass/volume (g cm<sup>-3</sup>).

Table S3. Chemical characterization of sediments at different levels used for microbiological analyses

Sample	Depth cm	Hg	TC	TOC	TN	TS	TP	pH	Eh	Fe	Mn	MeHg	AVS
A	0-5	0.178	6.26	2.48	0.13	0.05	0.029	7.65	-326	7,668	794	0.136	16.87
	5-10	0.168	4.34	0.52	0.09	0.09	0.008			7,771	893		2.48
	10-15	0.147	3.81	0.87	0.22	0.03	0.023			11,900	1,011		0.55
	15-20	0.212	4.75	0.56	0.10	0.11	0.013			16,290	1,003		1.11
B	0-5	0.440	3.16	0.70	0.22	0.01	0.016	8.35	-215	4,127	346	0.087	6.78
	5-10	0.224	3.27	0.41	0.19	0.01	0.009			3,339	297		0.61
	10-15	0.278	1.95	0.27	0.14	0.01	0.010			4,549	257		0.69
	15-20	0.261	2.05	0.20	0.10	0.01	0.014			10,490	497		0.61
C	0-5	6.765	8.49	3.00	0.98	0.37	0.052	7.58	-331	34,350	1,07	0.109	32.31
	5-10	10.180	8.27	4.29	0.94	1.08	0.051			36,880	1,647		31.63
	10-15	15.920	8.66	3.09	1.01	1.11	0.047			32,700	2,743		25.21
	15-20	37.630	9.00	3.74	0.90	2.12	0.041			27,890	3,977		41.61
D	0-5	0.815	5.48	1.82	0.71	0.51	0.042	7.64	-250	22,200	656	0.330	25.04
	5-10	0.539	6.48	1.37	0.41	0.67	0.036			18,080	650		12.68
	10-15	0.346	4.78	1.47	0.38	0.96	0.023			18,310	590		2.90
	15-20	0.105	4.79	0.91	0.37	0.09	0.003			4,629	113		0.75

Sample	Depth cm	Hg	TC	TOC	TN	TS	TP	pH	Eh	Fe	Mn	MeHg	AVS
E	0-5	1.748	5.85	1.84	0.54	0.09	0.042	7.42	-340	11,770	814	0.245	26.36
	5-10	0.149	3.26	0.63	0.11	0.03	0.009			5,087	320		1.07
	10-15	0.122	5.61	0.39	0.06	0.11	0.008			7,765	257		0.79
	15-20	0.164	2.74	0.52	0.02	0.14	0.008			8,504	250		1.45
F	0-5	1.209	5.77	1.66	0.63	0.31	0.047	7.59	-315	32,690	1,015	0.077	6.07
	5-10	0.581	6.64	1.89	0.39	0.27	0.047			26,960	1,065		9.01
	10-15	0.884	5.11	1.33	0.45	0.57	0.064			31,830	933		2.93
	15-20	0.654	6.69	1.56	0.73	0.75	0.026			29,850	550		1.26

Notes: Hg = mercury (mg kg<sup>-1</sup> d.w.); TC = total carbon (% d.w.); TOC = total organic carbon (% d.w.); TN = total nitrogen (% d.w.); TS = total sulfur (% d.w.); TP = total phosphorus (% d.w.); pH (udpH); Eh = oxidation reduction potential (mV); Fe = iron (mg kg<sup>-1</sup> d.w.); Mn = manganese (mg kg<sup>-1</sup> d.w.); MeHg = monomethyl mercury (µg kg<sup>-1</sup> d.w.); AVS = acid-volatile sulfide (µmol g<sup>-1</sup> f.w.); d.w. = dry weight; f.w. = fresh weight.

Table S4. Characterization of water.

Pore-water	Dissolved sulfates	pH	T	Salinity	[S <sup>2-</sup> ]	Mn	Fe	MeHg	HCO <sub>3</sub> <sup>-</sup>
A	3192	7.6	27.7	43	12.92	0.03	0.003	0.258	149.3
B	3355	7.9	27.7	44	0.13	0.03	0.001	0.346	153.7
C	3420	7.6	28.5	42	6.42	0.10	0.002	0.730	192.8
D	3215	7.6	28.5	40	0.57	0.03	<0.001	<0.231	149.6
E	3587	7.9	27.7	46	0.19	0.08	0.001	3.641	140.3
F	3308	8.0	28.5	44	2.45	0.19	0.001	<0.231	100.5
Bottom water	Dissolved sulfates	pH	T	Salinity	[S <sup>2-</sup> ]	Mn	Fe	MeHg	HCO <sub>3</sub> <sup>-</sup>
A	2727	7.9	27.7	43	0.52	0.01	0.008	0.991	104.9
B	3387	8.3	28.5	46	0.78	0.01	0.002	0.747	149.3
C	3424	7.9	29.2	43	1.76	0.14	0.003	0.745	165.9
D	3285	8.3	28.5	42	1.21	<0.01	0.001	<0.231	151.3
E	3494	8.2	28.5	46	0.79	0.01	0.002	3.380	135.4
F	3448	8.3	28.5	44	8.74	0.04	0.002	<0.231	141.5

Notes: Data are referred to 0-5 cm of depth. Dissolved sulfates (mg l<sup>-1</sup>); pH (udpH); T = temperature (°C); salinity (‰); [S<sup>2-</sup>] = volatile sulphides (mg l<sup>-1</sup>); Mn = manganese (mg l<sup>-1</sup>); Fe = iron (mg l<sup>-1</sup>); MeHg = mono-methylmercury (µg l<sup>-1</sup>); HCO<sub>3</sub><sup>-</sup> = alkalinity (mg l<sup>-1</sup>). Eh values in bottom water were: A = within -200 and -300 mV; B and C = <-300 mV; site D, E, F = -200 mV.

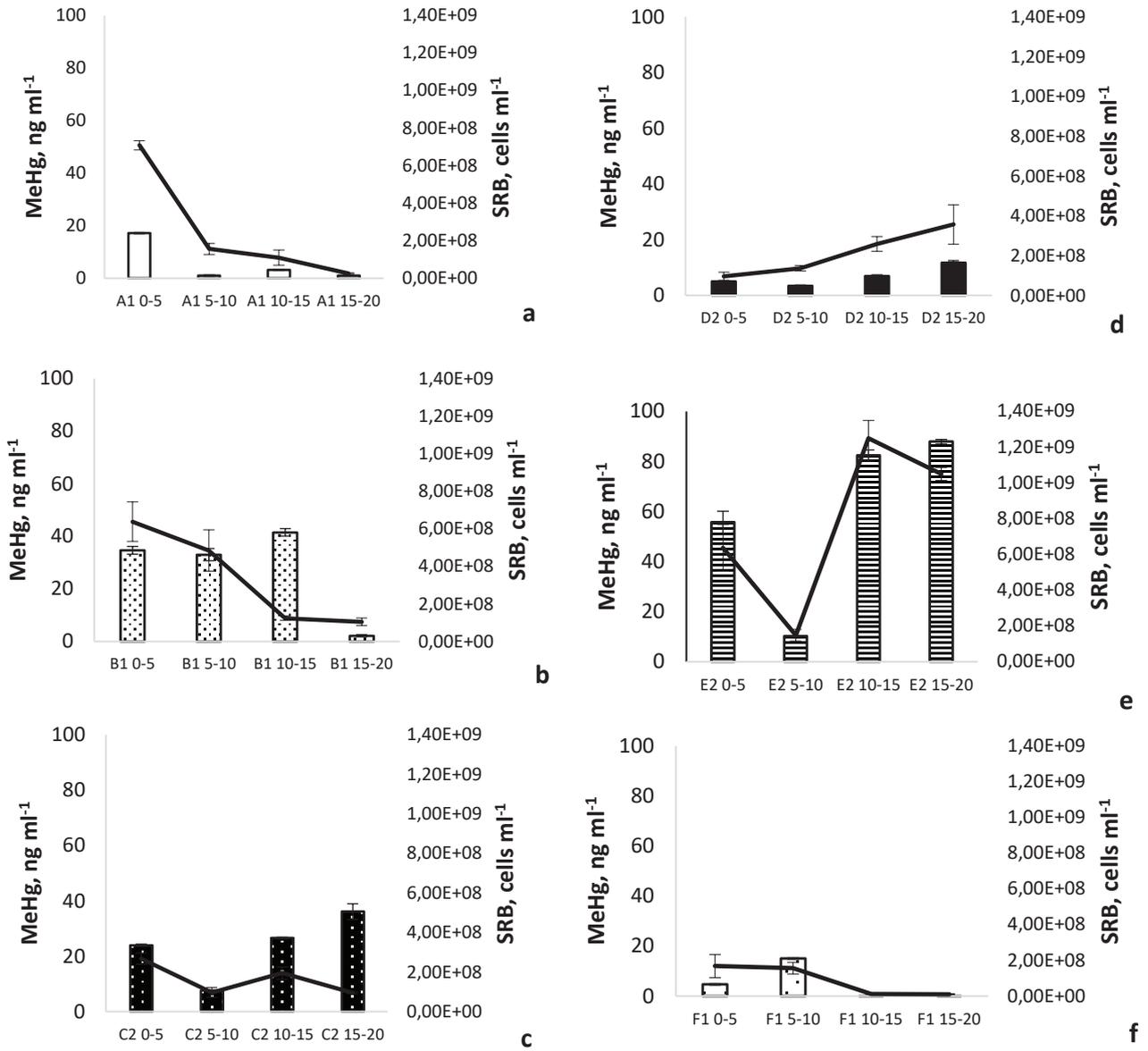


Figure 1S. Mercury methylation revealed as MeHg, ng ml<sup>-1</sup> in cultures of sulphate reducing bacteria (SRB) from sites A (□); B (◐); C (◑); D (■); E (◒) and F (::) in the Orbetello Lagoon, Italy, and related number of cells per ml of culture (SRB, cells ml<sup>-1</sup>) (—)