Methylation of mercury and demethylation of monomethylmercury in saltmarshes – seasonal variation and plant effect

Henrique José Albino Zilhão
Instituto Superior Técnico, Universidade de Lisboa
Lisbon, Portugal

Abstract:

Saltmarshes are known accumulations areas for contaminants, namely mercury (Hg) and has been proven that these environments play a crucial role in its methylation and in monomethylmercury (MMHg) demethylation. In this study, it was used stable isotope tracers of $^{199}Hg^{2+}$ and $CH_3{}^{201}Hg^{+}$ followed by isotope-specific detection with inductively coupled plasma mass spectrometry, to determine methylation and demethylation rates simultaneously in saltmarsh sediments colonized and non-colonized by plants, in two Portuguese aquatic systems (Tagus Estuary and Ria de Aveiro). Also, ambient concentrations of total Hg (THg) and MMHg were obtained. Sediments were sampled with and without vegetation in spring and summer. Vegetated samples contained three specific species of halophyte plants: *Halimione portulacoides* (HP), *Juncus maritimus* (JM) and *Sarcocornia fruticosa* (SF). This allowed to evaluate the plant and seasonal effect in Hg methylation and MMHg demethylation in saltmarsh sediments.Results showed higher concentrations of ambient THg and MMHg in Ria de Aveiro. The highest concentrations of THg was found in Laranjo (LAR) saltmarsh in sediments colonized by JM (58525 ng g^{-1}) and the highest concentration of MMHg was found in Chegado (CHE) saltmarsh in sediments colonized by HP in summer (334,3 ng g^{-1}). The highest methylation rate was also observed in CHE in sediments colonized by HP in summer (0,452 day^{-1}) and the highest demethylation rate was found in Rosário (ROS) saltmarsh in Tagus estuary (25,6 day^{-1}) in spring. In conclusion, results obtained appear to demonstrate that halophyte plants influenced Hg methylation rates and that summer conditions enhanced it possible due to higher microbial activity in the warmer season.

Keywords: Saltmarshes, mercury methylation, monomethylmercury demethylation, sediments, estuaries

1. Introduction

Mercury is considered a global pollutant due to its ability to spread in the environment. It has high mobility and is extremely toxic, being of great importance to understand its biogeochemical cycle, particularly its transport and deposition in the environment (Jitaru & Adams, 2004). Mercuric ion (Hg^{2+}) is the oxidation state in which Hg normally is found in water and soils (Horvat, 1996). Hg^{2+} forms organometallic and/or inorganic complexes that can be methylated by microorganisms or by abiotic factors originating monomethylmercury (MMHg) or dimethylmercury (DMHg) (Barkay, et al., 2012). In aquatic environments, MMHg poses a very serious threat to organisms because it bioaccumulates and biomagnifies in food webs (Kidd et al., 2012). The organometallic forms of Hg are the most toxic ones. Mercury methylation is known to occur in three environmental compartments: water column, sediments and biota (Li & Cai, 2013) and the biomethylation is thought to be the main contributor in the formation of MMHg, happening mainly, due to bacterial activity (Barkay et al., 2012). However, it's important to take in consideration that MMHg content in the environment results from the balance between the methylation and demethylation processes. Biomethylation tends to be higher in suboxic/anoxic conditions and dependent of several factors, such as: microbial activity, abundance of electron receptors, organic matter content, nutrient availability, bioavailability of inorganic Hg and its methylation potential (Barkay et al., 2012). There is also a significant number of abiotic factors that seem to be relevant in the methylation of Hg, such as: pH, temperature, redox shifting,

dissolved oxygen and the presence of complexing agents (Ullrich et al., 2001). All these factors should be taken into consideration when trying to evaluate the environmental factors and seasonal variations in Hg methylation. According to Ullrich et al (2001) sediments are considered to be the main reservoir of Hg in freshwater systems. Commonly being in river basins and estuaries, sediments in saltmarshes tend to be a place for the accumulation of Hg (Jackson, 1998). Saltmarshes are a very important and specific type of environment that occur worldwide in middle to high latitudes. They are present in estuaries, deltas, lakes and bays in intertidal zones being the transition between coastal and marine environments and playing an important part in coastline protection (Silva et al 2013). The main characteristic for plants to thrive in saltmarshes is their ability to live in environments with high values of salinity as is the case of Halophyte plants (Chapman, 1974). They developed an aerenchyma system that allows the plant to live in hypoxic soils, as is the case of wetlands (Caçador & Vale, 2001) and, on saltmarsh sediments, the presence of their roots can increase the organic matter content, which may enhance microbial activity (SRB, FeRB and/or methanogens) and consequently promote conditions for the methylation of Hg (Sun et al., 2011; Canário et al., 2010).

2. Materials and Methods

2.1 Study area

In spring and summer of 2019, Hg methylation and MMHg demethylation activity was studied in different vegetated and non-vegetated cores at two saltmarshes of Tagus estuary (Alcochete (ALC) and Rosário (ROS)), and two saltmarshes of Ria de Aveiro (Laranjo (LAR) and Chegado (CHE)) (Fig. 1). These estuaries, and more precisely these saltmarshes, were selected for this study because they are in places already known for its contamination with anthropogenic Hg. In Tagus estuary, the two saltmarshes chosen to collect the samples are in different areas. ALC, in the northern part of the estuary, is in the border of Tagus Estuary National Reserve, and presents lower to moderate Hg contamination (Canário et al., 2010; Cesário et al., 2016).

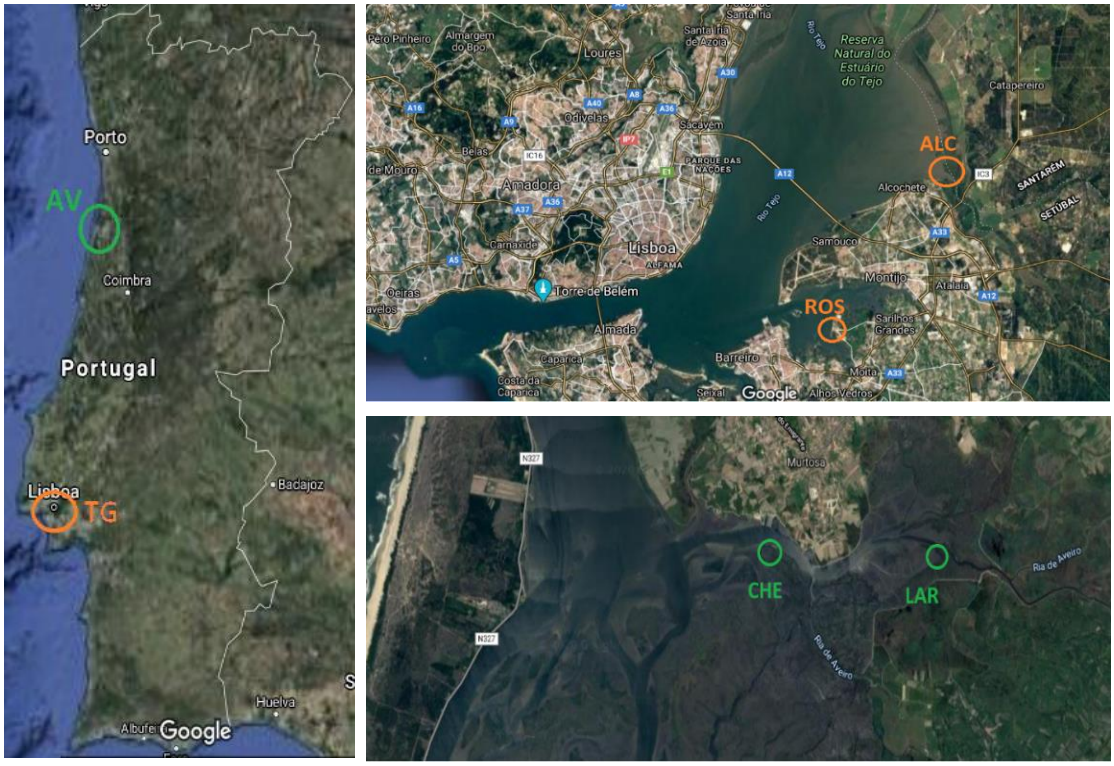

Figure 1 - Geographic location of the two estuaries in Portugal chosen for this study. Tagus estuary (TG) and Ria de Aveiro (AV). The location of each saltmarsh inside each estuary is also presented: In Tagus estuary are Rosário (ROS) and Alcochete (ALC) and in Ria de Aveiro are Laranjo (LAR) and Chegado (CHE).

In contrast, ROS saltmarsh, which is on one of the many coves on the left side of the river with moderate to high Hg contamination (Canário et al., 2007a). It is located between Moita and Barreiro, an area that once was very industrialized. In Ria de Aveiro, two different areas were chosen to collect samples: CHE and LAR. These sites are located between Cacia and Estarreja. Both places are home to several industries that were responsible for the pollution of Ria de Aveiro. LAR site is in greater proximity with the place where effluent discharge of factories took place and it is a more contaminated area (Figueira et al., 2012), compared with CHE, that was chosen to be a reference site inside Ria de Aveiro.

2.2 Sampling procedures

The samples for this study were collected in depth using specific metallic sediment corers. Two types of sediments were sampled: vegetated and non-vegetated. The vegetated cores contained specific species of plants that were chosen for this work. In the saltmarshes of Tagus estuary, the vegetated species in study were *Sarcocornia fruticosa* (SF) and *Halimione portulacoides* (HP), and in the two saltmarshes of Ria de Aveiro the collected vegetated cores were sampled in areas colonized by the plant species HP and *Juncus maritimus* (JM). After the collection of the sediment cores, samples were spiked *in situ* with an isotopic solution of Hg ($^{199}Hg^{2+}$) and MMHg ($CH_3{}^{201}Hg^{+}$) previously prepared in the laboratory. Additionally, three sediment cores were sampled with a different metallic corer with 7 cm diameter and 30 cm depth. The collected samples were to determine the amount of biomass, to measure physicochemical parameters and to analyze the content of metals.

2.3 Sediment Characterization

2.3.1 Water content, Loss of Ignition (LOI) and amount of Biomass

To evaluate the amount of water present in the sediments, approximately 1,5g of sample were weighed in a small aluminum crucible. The samples were put to dry at 105ºC for 24h and weighed again.

To determine the amount of organic matter in the sediment samples, it was used the method of Loss of Ignition. This method allows to determine the weight change of the samples after some of its content has been burned at high temperatures, in this case the organic matter. The previously dried samples were put in a muffle furnace at 450ºC for 2h and then weighed again.

The belowground material of each layer was separated from the sediment carefully under a flux of Milli-Q water using a mesh sieve to remove any adhering particulate matter. Sediments and roots were oven dried at 40°C and weighed to determine belowground biomass (Canário et al., 2010).

2.3.2 Total Iron (Fe) and Manganese (Mn) contents

To determine the total Fe and Mn concentrations was necessary to digest the sediment samples and then proceed to metal quantification by flame atomic absorption spectroscopy (AAS-F). The digestion was made accordingly to the method described by Loring & Rantala (1992). It consists in using a total decomposition method with hydrofluoric acid (HF) and aqua regia, that is a mixture of hydrochloric (HCl) and nitric (HNO_3) acids in a proportion of 3:1. The HF is used due to its ability to completely dissolve the silicate lattices and therefore being able to release the associated metals, such as Fe, Mn and Al (Loring & Rantala, 1992). Aqua regia is used to solubilize the metals due to the strong oxidizing power. In the end of the procedure, boric acid (H_3BO_3) is added to neutralize the HF and to prevent the precipitation of fluoride (Loring & Rantala, 1990).

2.3.3 Total mercury determination

In order to use an ICP-MS detector for measuring mercury isotopes, the samples were first subjected to a digestion. Approximately 100 mg of dry sediment was placed in a clear, labelled vial, where were added 7 mL of 7:3 HNO_3 (aq):H_2SO_4(aq) mixed acid solution for complete digestion. The vials were placed on a hot plate which was pre-warmed in increments from 75 °C to 110 °C and covered with marbles that were soaked in HNO_3 acid and rinsed with deionized water. The vials were left overnight for at least 24 hours. Once the solutions become clear, they were diluted with deionized water. Then, the samples were left to cool down and stored at room temperature in the dark until further mercury

analysis. The same process was also done with replicates of the standard reference material (PACS-2) and with blanks for every set of samples. To quantify the mercury isotopes in the digested samples was used a continuous flow cold-vapor generation with ICP-MS (8800 ICP-MS Triple Quad Agilent Technologies) detection. The sample was continually mixed with a solution of stannous chloride 3% (w/v) in 10% (%v/v) HCl by means of a peristaltic pump and the formed mercury vapors were separated from the liquid in a gas-liquid separator (Model L1-2) and the elemental mercury swept into the plasma of the ICP-MS. The concentrations of the mercury isotopes were calculated according to the method described in Hintelmann and Ogrinc (2003) and the following isotopes: $^{202}Hg^{2+}$ (added isotope for the methylation determination), $^{198}Hg^{2+}$ (internal standard) and $^{199}Hg^{2+}$ (to calculate ambient THg) were determined.

2.3.4 Monomethylmercury (MMHg) determination

To evaluate the amount of MMHg in the sediment samples it was used water vapor distillation (Hintelmann et al., 2000). Approximately 200-500 mg of wet sample was weighed into Teflon vials and then added 10ml of distilled water. Prior to distillation the samples were spiked with CH_3HgCl enriched with $^{198}Hg^{2+}$ as an internal standard and then 500 mL of H_2SO_4 (9 M) and 200 mL of KCl (20%) were added to the vials. The teflon vials were put into a heating block at 140ºC and a continuous stream of nitrogen (60 ml min^{-1}) was passing through the sample into to the receiving vials. The distillation was considered finished when at least 85% of the sample was distilled. The same process was also done with blank samples and with a certified reference material (IAEA-158). The quantification of the MMHg in the samples was done by species-specific isotope dilution inductively coupled plasma mass spectrometry using an automated Tekran 2700 system coupled to ICP-MS (8800 ICP-MS Triple Quad Agilent Technologies) and allowed to measured four different isotopes: $^{202}Hg^{2+}$ (methylated Hg), $^{200}Hg^{2+}$ (MMHg demethylation assay), $^{198}Hg^{2+}$ (internal standard) and $^{199}Hg^{2+}$ (to calculate ambient MMHg). The concentration of the individual mercury isotopes was calculated with an Excel spreadsheet that uses matrix algebra, as described in Hintelmann and Ogrinc (2003).

2.3.5 Stable mercury isotope tracer

This work used stable isotopes of mercury at tracer levels to measure the Hg methylation and MMHg demethylation rates. Using a cocktail solution of stock solutions of 511 µg mL^{-1} of $^{202}HgCl_2$ with 91,5% purity (10mL) and 55,7 µg mL^{-1} of $CH_3{}^{200}Hg^+$ in ethanol (0,368mL), several injections were made in the sediment cores. The cores were collected and sustained inside PVC tubes that were already prepared with pre-drilled ports, enabling the injection of the solution. The sediments were injected with 25, 100, 300 or 750 µL of the cocktail at 0-3 cm,6-9 cm and 21-24 depths and incubated *in situ* for approximately 5 hours. This means that the injected amount represented 12322, 49286, 147859 and 369647 ng of $^{202}Hg^{2+}$ and 49, 198, 593 and 1483 ng of $CH_3{}^{200}Hg^+$, respectively. For each saltmarsh, different spikes were chosen accordingly to the already proven Hg contamination of the site. 750 µL spikes in LAR, 300 µL spikes in CHE, 100 µL spikes in ROS and 25 µL spikes in ALC were introduced into the layers mentioned above. To ensure the dilution of the spiked solution, upon injection, the isotopes were dispersed as evenly as possible into each 1 cm layer and then vertical migration within the core diluted the spike further. On average, the total Hg concentration increased less than 10% and MMHg levels increased by a factor of 1,7. In the most contaminated sites, ROS and LAR, increases were lower and in the less contaminated sites, ALC and CHE, increases were relatively higher. Once methylation and demethylation rates were measured during the same period of time and in the same volume of sediment, they are directly comparable.

2.3.6 Hg methylation and MMHg demethylation rates

The mercury methylation and monomethylmercury demethylation rates were calculated based on the assumption that both processes have first-order kinetics (Cesário et al., 2017). To evaluate the rates, it was calculated the methylation rate constant (K_M) and the demethylation rate constant (K_D), both of them expressed in day^{-1}. To determine the methylation rate

constant K_M (day^{-1}), it was used the following equation:

$$K_M = [MM^{202}Hg^{+}]/([^{202}Hg^{2+}] \times t) \quad (1)$$

Where, [$MM^{202}Hg^{+}$] is the concentration of monomethylmercury (ng g^{-1}) that was formed due to the methylation of the spiked mercury, [$^{202}Hg^{2+}$] is the total concentration (ng g^{-1}) of this mercury isotope and t is the incubation time (day). To determine the MMHg demethylation rate its necessary to take into account that the concentrations of the spiked $MM^{202}Hg^{+}$ decrease exponentially over time, due to the fact that this is a first-order kinetic process. The following equation was used:

$$c\,(t) = c(0) \times e^{(-K_D \times t)} \quad (2)$$

Where c (0) is the starting concentration of $MM^{202}Hg^{+}$ in each sample, c (t) is the initial concentration at time of spiking and t is the incubation time. By solving the prior equation, the following equation is obtained:

$$K_D = \frac{(Ln[c(0)] - L[c(t)])}{t} \quad (3)$$

To solve equation 3 it is first necessary to determine c (0). This value cannot be obtained directly and therefore needs to be estimated by using the ratio (r) of $^{202}Hg^{2+}$ by $MM^{200}Hg^{+}$ in the spike solution. By knowing the measured concentration of $^{202}Hg^{2+}$ in each sample and then diving it by r, the concentration of $MM^{200}Hg^{+}$ at the start of the incubation is obtained, so:

$$c(0) = \frac{[^{202}Hg^{+2}]}{r} \quad (4)$$

3. Results and Discussion

3.1 Sediment Characteristics

Several parameters were analyzed to characterize the sediments, such as: pH, humidity, Loss of ignition (LOI), amount of belowground biomass and content of metals (total Fe and Mn). pH was found to be similar between both seasons in both saltmarshes, but with the saltmarshes of Ria de Aveiro presenting slightly lower values in comparison with Tagus estuary. Water content was found to be higher in sediments from Ria de Aveiro. The difference is probably related to grain size of the soil. In Ria de Aveiro the sediments are mostly constituted by mud and silt and in Tagus estuary sediments are a mixture of mud, silt and sand with the presence of small stones, more pronounced in ALC. Organic matter content, which is a very important parameter to consider when trying to access conditions for the methylation of Hg was also found to be present in higher amounts in Ria de Aveiro, when compared with Tagus estuary. The same trend was observed in the amount of belowground biomass. The analysis of the total metal content showed that between the saltmarshes of the same estuary, total Fe and Mn concentrations were approximately of the same range and no relevant variation was found between seasons or between colonized and non-vegetated sediments.

3.2 Ambient Total Hg

Analyzing the obtained ambient THg concentrations in the saltmarshes of Ria de Aveiro, it's clear that LAR is a much more Hg contaminated saltmarsh than CHE. In LAR, sediments showed some values (e.g.58525 ng g^{-1}) that were more than ten times higher than those recorded in CHE (e.g. 4462 ng g^{-1}). Also, the results indicate that ambient THg concentrations generally increase with depth in both vegetated and non-vegetated sediments, which could be explain by the cease of the contamination source. Natural sedimentation of both saltmarshes can explain why concentrations are higher at bigger depths. Probably, values recorded in past years were closer to the surface and contaminated sediments have become buried. This historical record is consistent with what was found by Pereira et al. (1998). High concentrations of ambient THg were

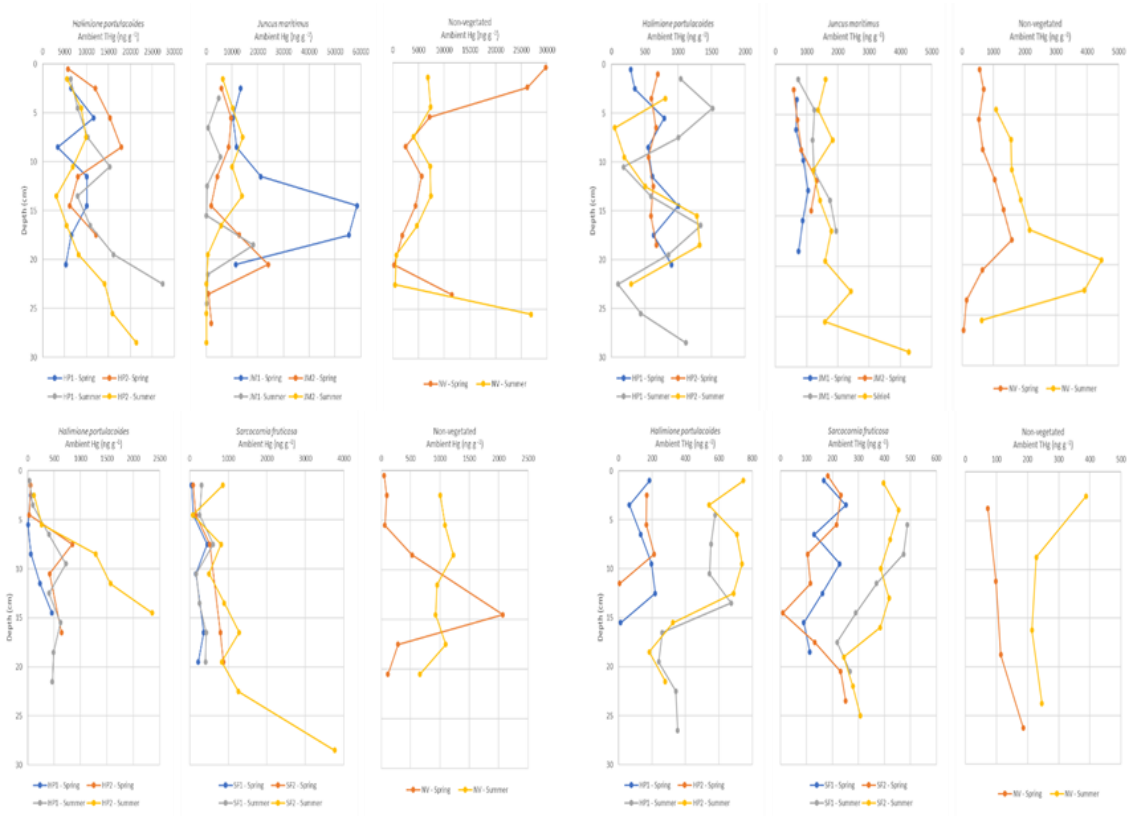

Figure 2 - Vertical profiles of ambient THg (ng g^{-1}) from sediments collected in Laranjo, (LAR) (top left), Chegado, (CHE) (top right), Rosário (bottom left) and Alcochete (ALC) (bottom right) saltmarshes, from Ria de Aveiro and Tagus estuary.

found at depths with higher content of organic matter and, in the case of LAR, some values were associated with the presence of belowground biomass. More belowground biomass raises the organic matter content which in turns increases the accumulation of Hg. This indicates that retention occurs mainly in the rooting sediment layers, which shows the influence of plants in the sequestration of Hg. In a similar study, conducted by Micaelo et al (2003) also in Laranjo bay, higher results of Hg concentration were also found to be related with the presence of roots in the sediments. Between seasons, it wasn't seen any significant variation.

Analyzing the obtained ambient THg concentrations in the saltmarshes of Tagus estuary it was seen, as expected, that ROS is more Hg contaminated than ALC – located on the border of the Tagus National Reserve. The degree of contamination is in line with previous studies conducted at these locations (Canário et al., 2007a; Cesário et al., 2017). Variation with depth showed different patterns. In ROS ambient THg concentrations tended to increase with depth, but in ALC the trend is reversed and the highest values appear close to the surface (Figure 2) because contamination is probably more recent and happened due internal estuarine water circulation resultant from the dynamics of Tagus estuary (Cesário et al., 2016). High ambient THg concentrations were associated with the presence of Fe. The presence of Fe around roots may enhance the accumulation of Hg, which then can precipitate with Fe oxyhydroxides. In terms of seasonal variation, in ROS the values were similar in both seasons, but in ALC summer concentrations of ambient THg were generally higher.

3.3Ambient MMHg Concentrations

As expected, MMHg concentrations were higher in LAR in comparison with CHE. Concentrations presented a broad range of values, with the highest ones being found in the summer in all sediment cores. In both saltmarshes, appears to exist seasonal changes, with higher temperatures having an impact in MMHg production, especially in colonized sediments. These results are in

Ambient THg and Ambient MMHg (ng g-1)								
	Laranjo (LAR) - Ambient THg		Laranjo (LAR) - Ambient MMHg		Chegado (CHE) - Ambient THg		Chegado (CHE) - Ambient MMHg	
Sediment Cores	Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer
HP1	3428 – 11629	6393 – 27360	6,3 – 17,2	65,5 – 115,5	289 – 1003	99 – 1514	4,6 – 17,7	9,9 – 18,9
HP2	5800 – 17892	3117 – 21354	7,7 – 39,1	34,5 – 101,1	555 – 695	52 – 1321	6,9 – 37,5	15,7 – 334,3
JM1	10362 – 58525	88 – 18275	22 – 77,6	143,9 – 260,5	659 – 1046	726 – 1942	18 – 27,2	25,3 – 42,3
JM2	853 – 24030	69 – 14148	27,5 – 80,8	75,2 – 256,5	584 – 1319	1208 – 4263	13,8 – 23,4	18,2 – 67,7
NV	264 – 29698	466 – 26881	8,3 – 27,9	31,4 - 165	47 – 1588	629 – 4462	3 - 7	2,6 – 12,8

Ambient THg and Ambient MMHg (ng g-1)								
	Rosário (Ros) – Ambient Total Hg		*Rosário (Ros) – Ambient MMHg*		*Alcochete (ALC) – Ambient THg*		*Alcochete (ALC) – Ambient MMHg*	
Sediment Cores	Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer
HP1	5 – 455	33 – 721	1,3 - 6,0	3,7 - 24,0	12 – 219	240 – 674	0,79 - 0,46	1,65 - 6
HP2	26 – 843	115 – 2363	3,4 - 4,7	6,4 - 14,0	7 – 213	184 – 747	0,97	5,95 - 8,29
SF1	48 – 457	171 – 612	1,7 - 6,6	20,2 - 131,5	90 – 252	218 – 489	1,88 - 5,36	2,54 - 6,60
SF2	92 – 890	84 – 3762	1,4 - 3,0	10,3 - 12,5	9 – 251	244 – 455	1,7 - 3,4	1,71 – 5,63
NV	49 – 2071	657 – 1227	0,9 - 3,5	14,3 - 42,4	72 - 186	214 – 388		1,79 – 17,86

Figure 3 - Range of ambient THg and MMHg concentrations (ng g-1) in sediments from Laranjo (LAR), Chegado (CHE), Rosário (ROS) and Alcochete (ALC) saltmarshes, colonized by Halimione Portulacoides (HP1 and HP2), Juncus Maritimus (JM1 and JM2), Sarcocornia fruticosa (SF1 and SF2) and non-vegetated ones (NV).

line with other studies where MMHg concentrations were also found to be higher in summer months (Hiltelmann & Wilken, 1995; Canário et al., 2007a; Monteiro et al., 2016; Cesário et al., 2016; 2017). Warmer temperatures may enhance microbial activity and, as a result, increase the methylation of available Hg to MMHg. In the non-vegetated sediments, the increase of ambient MMHg in LAR during the summer season was significant (from 27,9 ng g^{-1} to 165 ng g^{-1}) and comparable to the colonized sediments, however in CHE the ambient MMHg concentration only varied slightly between seasons (from 7 ng g^{-1} to 12,8 ng g^{-1}). One possibility, that can explain the differences in ambient MMHg concentration between CHE and LAR non-vegetated sediments is that the non-vegetated core from LAR collected in summer, was not completely devoid of roots. Comparing the vegetated cores by plant species, the ones colonized by *J. maritimus* normally have higher values of ambient MMHg concentrations. These happened in both saltmarshes, with different degrees of contamination, which appears to indicate that this specific plant species enables better conditions for the methylation of Hg. In terms of ambient MMHg variation with depth, the higher values recorded in LAR were generally between 5 to 10 cm depth. In CHE, ambient MMHg concentrations were normally higher closer to the surface, in the first 5 cm of the sediment, but it was detected a high concentration of MMHg at ~15 cm depth (HP2 – Summer Core). In this layer, the percentage of ambient MMHg was 26,1% of ambient THg. This may corroborate the hypothesis that, in colonized sediments, exists preferential layers of retention of Hg or MMHg with optimal zones for methylation (Canário et al. 2007b).

In Tagus estuary, when comparing the ambient MMHg concentrations between both sites, the more contaminated site – ROS – shows higher ambient MMHg concentrations. The relation between seasons observed in Ria de Aveiro is also present here, with ambient MMHg concentrations being higher in summer. In ROS and ALC, doesn't seem to exist any significant difference between vegetated sediments and non-vegetated sediments. In the case of ALC, the highest concentration of ambient MMHg was found in a non-

Methylation rates K_M (day^{-1})

	Laranjo (LAR)		Chegado (CHE)	
Sediment Cores	Spring	Summer	Spring	Summer
HP1	0,0042 – 0,0052	0,0381 – 0,1717	0,0031 – 0,0494	0,0086 – 0,1241
HP2	0,0031 – 0,0067	0,0653 – 0,1019	0,0089 – 0,0217	0,0284 – 0,4521
JM1	0,0099 – 0,0168	0,0305 – 0,3120	0,0110 – 0,0347	0,0240 – 0,1640
JM2	0,0045 – 0,0298	0,0770 – 0,2834	0,0482 – 0,0918	0,0157 – 0,0570
NV	0,0054 – 0,0104	0,0517 – 0,1220	0,0087 – 0,0181	0,0014 – 0,1260

Methylation rates K_M (day^{-1})

	Rosário (ROS)		Alcochete (ALC)	
Sediment cores	Spring	Summer	Spring	Summer
HP1	0,0224 – 0,0711	0,0372 – 0,1192	0,0231	0,0093 – 0,0204
HP2	0,0065 – 0,0525	0,0185 – 0,0837	0,0301	0,0041 – 0,0153
SF1	0,0178 – 0,0698	0,0306 – 0,0829	0,0071 – 0,0474	0,0141 – 0,0234
SF2	0,0138 – 0,0281	0,0102 – 0,0328	0,0107 – 0,0169	0,0072 – 0,0370
NV	0,0018 – 0,1518	0,0319 – 0,0611	x	0,0264 – 0,1354

Figure 4 - Range of methylation rates KM (day^{-1}) for sediments collected in Laranjo(LAR), Chegado (CHE), Rosário (ROS) and Alcochete (ALC) saltmarshes, colonized by Halimione portulacoides (HP1 and HP2),Juncus maritimus (JM1 and JM2), Sarcocornia fruticosa (SF1 and SF2) and non-vegetated ones (NV).

vegetated core, representing 4,6% of the ambient THg. In the case of ROS, ambient MMHg concentrations are similar in colonized and non-vegetated sediments in both seasons, but the highest MMHg concentration was recorded in a vegetated sediment colonized by *S. Fruticosa* representing 30,4% of the ambient THg. Comparing plant species, both seem to present ambient MMHg concentrations in similar ranges in both seasons within each saltmarsh. However, a very high ambient MMHg concentration was found in the sediments of a *S. fruticosa* colonized core collected in ROS, but the unique high value may also indicate that along seasonal variation, there is also spatial variation. (Monteiro et al., 2016). Because data is shorter for MMHg concentrations, relation with depth is more difficult to determine. In the non-vegetated sediments, values generally appear do decrease with depth. In vegetated sediments, it doesn't seem to exist any specific relation, with higher values being recorded closest to the surface and also deeper, in some cases between 15 to 20 cm in depth.

3.4 Methylation Rates

The employed method in this experiment allows both methylation and demethylation rates to be directly comparable because they were measured during the same amount of time (5 hours) and in the same amount of sediment. To better understand MMHg concentrations in the environment, a method that allows both rates to be comparable is essential, because the pool sizes of MMHg are controlled by both Hg methylation and MMHg demethylation (Cesário et al. 2017).

In LAR, methylation rates had a big increase from spring to summer, in both vegetated and non-vegetated sediments, with a particularly big difference in the sediments colonized by *H. portulacoides.* The highest value recorded in summer (0,1717 day^{-1}) was 25 times higher than the highest value recorded in spring (0,0067 day^{-1}). In sediments colonized by *J. maritimus*, as well as non-vegetated sediments, the highest K_M in summer (JM: 0,3120 day^{-1}; NV: 0,1220 day^{-1}) was approximately 11 times higher than the highest K_M recorded in the spring (JM: 0,0298 day^{-1}; NV: 0,0104 day^{-1}). In CHE, the highest K_M were also observed in summer. However, the discrepancy between seasons wasn't so big. In sediments

colonized by *H. portulacoides*, the highest K_M in summer (0,4521 day^{-1}) was 9 times higher than the highest K_M in spring (0,0494 day^{-1}), but in *J. maritimus* was only approximately 2 times higher in summer (0,1640 day^{-1} in summer and 0,0918 day^{-1} in spring) and in non-vegetated sediments was 7 times higher in the warmer season (0,1260 day^{-1} in summer and 0,0181 day^{-1} in spring). These findings corroborate what was previously mentioned – in several works, that - summer conditions enhance methylation of Hg (Canário et al., 2010; Monteiro et al., 2016). It seems clear that the higher values of ambient MMHg found in summer can be explain by the higher methylation rates. The increase in microbial activity is thought to be related with the increase of temperatures, in fact optimal methylation conditions within a cell of a specific type of SRB were reported to be 35ºC and pH 6.5 (Ullrich et al., 2001). Once pH doesn't seem to vary much between seasons in both saltmarshes of Ria de Aveiro, the temperature may be a critical factor. Also, in summer, primary production is maximal which enhances nutrient availability for microbes and greater abundance of organic matter rich in Hg (Canário et al., 2007a). Comparing methylation rates between the two different types of plants, it's possible to see that the sediments colonized by *J. maritimus* seem to have higher methylation capacity. In LAR saltmarsh, the average values for methylation rates were higher in sediments colonized by *J. maritimus* in both seasons (HP – Spring: 0,0048 day^{-1}, JM - Spring: 0,016 day^{-1}, HP – Summer: 0,0910 day^{-1} and JM – Summer: 0,175 day^{-1}. However, in CHE saltmarsh, the same trend is noticeable in spring (HP: K_M=0,0181 day^{-1} and JM: K_M= 0,0438 day^{-1}), but in summer, sediments colonized by *H. portulacoides* presented a higher average value (HP: K_M=0,1251 day^{-1} and JM: K_M=0,0592 day^{-1}). It's also seen in both saltmarshes that the difference between seasons is more relevant in colonized sediments by *H. portulacoides*. Another important factor to consider is that K_M from LAR are comparable with those obtained in CHE, although existing a big difference in the degree of contamination between both saltmarshes. This is an indication that ambient THg concentration may not be a decisive factor because not all the Hg found in sediments may be available for methylation.

Evaluating the methylation rates of Tagus estuary, it's noticeable that the range of K_M values is very similar in both saltmarshes (0,0018 – 0,1518 day^{-1} in ROS and 0,0041 – 0,1354 day^{-1} in ALC) which, once again, may indicate that methylation isn't dependent on the degree of Hg contamination, but rather on its bioavailability and on the microbial community present in the sediments. Comparing the obtained values in terms of seasonal differences, it's noticeable that the difference between spring and summer is not so evident in Tagus estuary as it was in Ria de Aveiro. Looking at the results from ROS, in the colonized sediments it was summer samples that presented the higher rates of methylation (HP – 0,1192 day^{-1}, SF – 0,0829 day^{-1}), however the difference between seasons greatly decreases, with K_M values from spring being comparable with those from summer. In ALC the trend is reversed and the higher K_M values in the colonized sediments are obtained in the spring (HP – 0,301 day^{-1}, SF – 0,0474 day^{-1}). In non-vegetated sediments, samples from ROS showed the highest K_M in spring, but in ALC isn't possible to make a comparison, although a very high K_M was found in summer. What was observed in Ria de Aveiro for non-vegetated sediments, doesn't appear to happen in Tagus estuary. Higher methylation rates were not found consistently closer to the surface. It was also observed that in Tagus estuary the presence of plants doesn't appear to be enhancing methylation, because the highest methylation rates obtained in ROS (0,1518 day^{-1}) and ALC (0,1354 day^{-1}) were for non-vegetated sediments. It was expected that colonized sediments were enhancing methylation, so these values present themselves as unexpected. However, explanations can be proposed. The first is that the non-vegetated core in fact contained roots from a nearby plant. Another possible explanation is that the plant rhizosphere had contributed to the formation of cinnabar (HgS), that can be formed when sulfate-reducing bacteria reduce sulfate to sulfide (Patty et al., 2009). HgS precipitates and can immobilize Hg in the sediment. Comparing plant species, *H. portulacoides* seems to better enhance the methylation of Hg. In

ROS, it was in a sediment colonized by *H. portulacoides* that was found the highest value in vegetated sediments (0,0837 day^{-1}). Also, the average K_M in sediments colonized by *H. portulacoides* (Spring – 0,041 day^{-1} and Summer – 0,055 day^{-1}) were higher than the average K_M in sediments colonized by *S. fruticosa* (Spring – 0,032 day^{-1} and Summer – 0,036 day^{-1}). In ALC, the same trend is observed in spring (HP – 0,027 day^{-1} and SF – 0,019 day^{-1}) but in summer the trend is reversed (HP – 0,012 day^{-1} and SF – 0,020 day^{-1}) and the highest value recorded in a vegetated sediments was in one colonized by *S. fruticosa* (0,0474 day^{-1}).In ALC, the same is observed in summer.

3.5 MMHg demethylation rates K_D

To evaluate the pool size of MMHg is necessary to understand the demethylation process, because it's also depending on it that the concentration of MMHg and of bioavailable Hg is increased or diminished. Due to technical problems, in this study was only possible to obtain the K_D for some sediment cores from spring season. Analyzing the obtained values, the most significant conclusion is that demethylation rates are significantly higher than methylation rates, sometimes 1, 2 or 3 orders of magnitude higher. These results show the importance of the demethylation process. If MMHg can be demethylated so fast, it means that this process is essential to assure that concentrations of this toxic compound do not raise to higher levels. It also shows the high capacity of the microbial community present in these estuarine environments in the demethylation process. Comparing methylation with demethylation rates, it is also possibly to say that high K_M values don't necessarily mean high K_D values. If so, values from ROS and ALC saltmarshes would be significantly lower than those recorded in LAR and CHE, because observed K_M values in Ria de Aveiro were higher than those in Tagus estuary. However, K_D values form both estuaries are comparable. Also, high MMHg concentrations don't appear to influence the K_D values, exactly for the same reason. Ria de Aveiro showed significant higher concentrations of ambient MMHg when compared to Tagus estuary, but didn't show significantly higher K_D values.

Demethylation rates K_D (day^{-1})

	Laranjo (LAR)	Chegado (CHE)
Sediment Cores	Spring	
HP1	5 – 9,6	3,5 – 4,3
HP2	9,1 – 17	1,0 – 4,1
JM1	9,7	2 – 8,7
JM2	5,6 – 13,4	-
NV	5,4 – 7,2	8,6 – 15,2

Demethylation rates K_D (day^{-1})

	Rosário (ROS)	*Alcochete (ALC)*
Sediment cores	Spring	
HP1	6,2 – 11,7	5,2 – 7,8
HP2	12,1 – 25,6	9
SF1	-	4,3-12,9
SF2	7,3 – 13,7	7,8 – 13,6
NV	8 – 20,8	-

Figure 5 - Range in demethylation rates KD (day^{-1}) in sediments from Laranjo (LAR), Chegado (CHE), Rosário (ROS) and Alcochete (ALC) saltmarshes, during the spring season.

4. Conclusions

First, this study allowed to verify the degree of contamination that these four saltmarshes are subjected. In these ecosystems of great importance in the estuarine/coastal environments was once again proved the ability to accumulate Hg and to provide conditions for its methylation. Second, the results obtained in this study showed that Hg methylation and possible MMHg demethylation rates were affected by seasonal changes and by plant presence. The results were not consistent in all four saltmarshes but appear to be similar within the same estuary, which indicates that the difference may reside in biogeochemical factors that differ from one to another.

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