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***Chapter 7***

# MERCURY METHYLATION VERSUS DEMETHYLATION: MAIN PROCESSES INVOLVED

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## ABSTRACT

It is well known that mercury presents high toxicity, causing a great damage to the environment and living organisms; however, its properties depend on the mercury species present. Organomercury compounds, where methylmercury is included, cause more concern.

Since 60-70's, several methylation mechanisms are known. Generally, methylmercury can be formed naturally in the aquatic environment by two general pathways: chemical methylation (abiotic) and microbial (biotic) processes. At the same time, methylmercury can be also decomposed abiotically or by the action of several demethylating microbes, or demethylators, ranging from anaerobes to aerobes. Regarding the biotic methylmercury demethylation, two distinct vias - oxidative and reductive - might be used by those microorganisms, differing in the final products obtained. In relation to the reductive processes, two pathways might occur. The first one involves the mercury resistance operon (*mer*) whereas the second one involves sulfide ions; however, the former is considered to be the most common pathway. Regarding the *mer* operon, some bacteria only carry on a narrow-spectrum operon (*mer*A), being only able to reduce inorganic mercury (Hg(II)) to elemental mercury ($Hg^0$). On the other hand, others beyond this operon also carry on a broad-spectrum operon (*mer*B). These microorganisms are able to decompose methylmercury to $Hg^0$.

Taking into account all of these processes, in the present work the most referred methylation and demethylation mechanisms found in aquatic environments are discussed, as well as the environmental factors that influence them. Factors related with the inorganic mercury/methylmercury availability and those that affect directly the activity of methylators and demethylators are also referred. Generally, the relationships encountered are complex and sometimes significant shifts on the microbial communities may be

observed. These changes can alter the processes involving the mercury species, as well as the final products obtained.

In conclusion, the abiotic factors and the type of microorganisms that are present in the environment, including their genetic patrimony, influence significantly the presence and the type of the mercury species. Furthermore, there are environmental factors, such as redox conditions, sulfides and organic matter that also affect the mercury dynamic and the equilibrium existents.

# 1. INTRODUCTION

The amount of mercury in the environment is much higher than the global background level as a result of the anthropogenic activities during the 20$^{th}$ century (Eckley and Hintelmann, 2006). Mercury has been used in several industrial or agricultural applications for ages and mercury species are stable and persistent in the natural systems. In this sense, several harmful situations to the environment and human health have been associated to mercury and its compounds. Methylmercury poisoning in Minamata (Japan), the organic mercury poisoning in Iraq, the methylmercury exposure in the Amazon (Brasil) and the elemental mercury spill in Catamarca (Peru), are examples of real situations that involved mercury species (Gochfeld, 2003).

Mercury properties are well known and have been reported in numerous works (Weast, 1975; Andren and Nriagu, 1979; Nriagu, 1979; Filella *et al.*, 1995; Cutnell and Johnson, 1998; Jackson, 1998; Mukherjee *et al.*, 2004). Mercury can be found in three oxidation states: 0 (elemental), 1+ (mercurous) and 2+ (mercuric) (Andren and Nriagu, 1979; Jackson, 1998), such as in $Hg^0$, $Hg_2^{2+}$ and $Hg^{2+}$; however, the last form is the most common in aquatic environments. This mercury form has strong tendency to form extremely stable coordination complexes and organometallic compounds (Jackson, 1998). Several complexes might be formed between mercury and different ligands. These can be sulfur (Leermakers *et al.*, 1995; Lobinski, 1998; Ravichandran, 2004), namely thiol groups (Carty and Malone, 1979; IPCS, 1991) and sulfides (Jackson, 1998), nitrogen (e.g. R-$NH_2$), phosphorous or carbon (Jackson, 1998). In relation to oxygen ligands, mercury has low affinity to them (Carty and Malone, 1979).

Due to mercury affinity to ligands containing sulfur, low molecular weight thiols, i.e. sulfhydryl containing molecules such as cysteine, are emerging as important factors in the transport and distribution of mercury throughout the body (Rooney, 2007) due to the phenomenon of "Molecular Mimicry" (Bridges and Zalups, 2005), whereby the bonding of metal ions to nucleophilic groups on certain biomolecules results in the formation of organo-metal complexes that can behave or serve as a structural and/or functional homolog of other endogeneous biomolecules or of the molecule to which the metal ion has bonded. When observed with mercury, this phenomenon might cause significant injuries.

## 1.1. Organomercury Compounds

Organomercury compounds are the most toxic mercury species, not belonging to this group of compounds the mercury complexes formed with organic matter originally present in

the aquatic systems. The organomercury compounds can be divided into two groups: one in which mercury atom is linked to an organic radical (RHgX), and another group to that mercury is linked to two organic radicals ($R_2Hg$) (Benes and Havlík, 1979).

The compounds that belong to the first group are soluble in water, dissociating in the $R\text{-}Hg^+$ cation and $X^-$ anion, being the most common the $Cl^-$, $OH^-$, $NO_3^-$ and $SO_4^{2-}$ anions (Benes and Havlík, 1979). Depending on anion nature, the compounds obtained will have different properties. Poorly coordinating anions, such as $ClO_4^-$, $NO_3^-$, $PF_6^-$ and $BF_4^-$ anions confer an ionic character to $RHg^+X^-$ salt (Carty and Malone, 1979), and are correspondingly more hydrophilic (Jackson, 1998), while $Cl^-$, $Br^-$ and $I^-$ anions confer on a linear covalent character (C-Hg-X) (Carty and Malone, 1979), being these methylmercury halides among the more lipophilic methylmercury species (Jackson, 1998).

The second group includes compounds such as dimethylmercury and diphenylmercury (Benes and Havlík, 1979). These compounds are volatile, non polar and have low solubility in water (Benes and Havlík, 1979; Carty and Malone, 1979; Jackson, 1998), not being affected by air, and weak acids and bases (Andren and Nriagu, 1979). These properties might be due to their covalent bonds (Benes and Havlík, 1979; Carty and Malone, 1979; Jackson, 1998).

Both groups of organomercury compounds - RHgX and $R_2Hg$ - are broad-spectrum biocidal agents acting via diverse mechanisms in biological systems. Organomercurials are supposed to induce membrane associated oxidative stress in living organisms through different mechanisms, including the enhancement of the lipid peroxidation and intracellular generation of reactive oxygen species (ROS) (Milaeva, 2006).

Methylmercury is the most common organomercury compound found in aquatic environments. It is also one of the most hazardous mercury species, due to its high stability in combination with its lipid solubility, leading to a high ability to penetrate membranes in living organisms (Beijer and Jernelöv, 1979). Methylmercury is of particular public health concern due to its bioaccumulation and biomagnification within the aquatic food web (Wiener *et al.*, 2002; Orihel *et al.*, 2007; Coelho *et al.*, 2008). In terms of the biomagnification factor that corresponds to the concentration increase for each trophic transfer it is about two- to five-fold for various aquatic ecosystems and to all typical trophic levels, being an order of magnitude higher than the one for inorganic mercury (Meili, 1997).

Although most of mercury emitted to the environment is in inorganic form, nowadays it is well-known that inorganic mercury can be naturally methylated in the environmental ecosystems being it transformed into methylmercury. Since 60-70's, several methylation mechanisms are known. In the aquatic environment, methylmercury can be formed by two general pathways: chemical methylation (abiotic processes) and microbial metabolism (biotic processes) (Celo *et al.*, 2006). On the other hand, methylmercury can be decomposed abiotically, as for example, by light (Jackson, 1998), or biotically, by various free-living demethylating microorganisms (IPCS, 1990; Ebinghaus and Wilken, 1996). As both processes might occur simultaneously, methylmercury presence in the aquatic environments depends on the existing balance of methylation versus demethylation.

## 2. MERCURY METHYLATION/DEMETHYLATION PROCESSES

The knowledge of the efficiency of the different pathways of mercury methylation and demethylation is one of the key steps to predict methylmercury concentrations in the different environmental compartments and to estimate the mercury bioaccessibility to the organisms. However, the factors that influence the competing methylation and demethylation reactions are yet insufficiently understood and little to no attempt has been made to determine end products. The relative importance of each reaction and the resulting net effect will probably depend on the environmental conditions and biological factors with spatial and temporal variations (Hintelmann *et al.*, 2000).

In this sense, it is important to consider that the net amount of biologically available methylmercury is a function of the processes that regulate its formation, degradation and exchanges between compartments. So, methylation and demethylation are two important processes regulating the mercury cycle in natural environments (Rodríguez Martín-Doimeadios *et al.*, 2004; Monperrus *et al.*, 2007a) and they can be driven by both biotic and abiotic mechanisms.

The biogeochemical cycle of mercury has been extensively studied whereas the mechanism of natural mercury methylation in the environment is not still clear. If methylmercury production, for example, is the most significant process that is occurring in the aquatic environment, hazardous effects on living organisms may occur due to methylmercury presence and its related high toxicity. Microbial methylation (biotic processes) is widely accepted as the main conversion mechanism of inorganic mercury into methylmercury in natural environment (Barkay *et al.*, 2003; Eckley and Hintelmann, 2006; Monperrus *et al.*, 2007b; Raposo *et al.*, 2008). Nevertheless, the relative importance of mercury chemical methylation (abiotic processes) is ambiguous. Some authors emphasize that the abiotic pathway is possible in natural environments but it appears to play a minor role (Ullrich *et al.*, 2001; Benoit *et al.*, 2003; Gårdfeldt *et al.*, 2003; Eckley and Hintelmann, 2006; Dominique *et al.*, 2007; Monperrus *et al.*, 2007b), especially photochemical methylation (Dominique *et al.*, 2007). On the other hand, other authors suggest that the biotic processes can not account for all the methylmercury formed naturally (Celo *et al.*, 2006).

If demethylation of methylmercury is occurring in a significant extent, this is advantageous; however, in some situations the substrate of mercury methylation might be formed, inducing in this way the methylmercury formation. On the other hand, the demethylation process that occurs in the aquatic environments depends also on abiotic and biotic factors. Generally, the existent relationships are quite complex and variable. Nevertheless, in the following sections the most common mercury methylation and demethylation processes found on the environment, as well as the environmental factors that influence these, will be discussed.

### 2.1. Mercury Methylation Processes

#### *2.1.1. Chemical Methylation – Abiotic Processes*

In the case of the abiotic pathway, mercury methylation is possible only in the presence of a suitable methyl donor (Ullrich *et al.*, 2001; Celo *et al.*, 2006). Moreover, this process

may be photochemically induced. The latter reaction mechanism is likely little relevant, since the methyl radicals produced photochemically will be rapidly scavenged by oxygen (Gårdfeldt *et al.*, 2003). Potential methylating agents for abiotic methylmercury formation in natural environments include small organic molecules, such as methyliodide and dimethylsulfide (Celo *et al.*, 2006), and larger organic components of dissolved organic matter, such as fulvic and humic acids (Ullrich *et al.*, 2001; Celo *et al.*, 2006). Transmethylation reactions involving organometallic complexes like methylcobalamin, methyllead or methyltin compounds have also been considered as possible pathways for chemical mercury methylation. Transmethylation reactions can occur as a result of the transference of carbocationic $Me^+$, carbanionic $Me^-$ or radical $Me\cdot$, depending on the chemical properties of the metal component of the methylating agent (Celo *et al.*, 2006). Therefore, a large variety of chemical variables may influence the methylation process (Celo *et al.*, 2006).

Methyliodide (MeI) plays an important role in the biogeochemical cycle of mercury in the marine environment as being it an effective solubilizing agent for mercury sulfides (Minganti *et al.*, 2007). MeI is mainly produced in the marine environment by algae and plankton whereas its dispersion by human activity can be overlooked. So, this compound is present at relatively high concentrations in areas where biomass productivity is high. MeI has not the ability to directly methylate oxidized mercury ($Hg^{2+}$) if methyltin and methyllead species, acting as transferring agents for the methyl group from MeI to mercury, are not present in the medium (Minganti *et al.*, 2007). Hence, the reaction mechanism corresponds to the $Hg^{2+}$-assisted hydrolysis of MeI, resulting in quantitative formation of methanol (Celo, 2003; Celo and Scott, 2005) ($Hg^{2+} + 2\ MeI + 2\ H_2O \rightarrow HgI_2 + 2\ MeOH + 2\ H^+$). The methylation reaction for mercury requires reducing/anaerobic conditions as MeI only methylates reduced forms of metals. Therefore, the oxidative addition ($Hg^0 + MeI \rightarrow MeHgI$) is presumably the methylation mechanism evolved (Celo *et al.*, 2006). In the presence of 200 ng/L MeI, methylmercury formation could therefore be as high as 0.2 pg/L/year (Celo *et al.*, 2006).

Humic matter contains different kinds of functional groups and, besides the linkage of oxidized mercury to thiol groups, it has most likely an additional complexation to neighbouring carboxylic groups. Taking into account that organic acids with methyl groups in the $\alpha$-position show high methylation efficiency for mercury (Falter, 1999a,b), humic matter is the most promising environmental methylating agent as consequence of its high concentration in waters and sediments and of its association with the solubility and thus mobility of mercury in freshwaters and marine waters. Only three humic substances, namely 2,6-di-tert-butyl-4-methylphenol (BHT), *p*-xylene and mesitylene, have the ability to methylate inorganic mercury at pH 3.5 but only at temperatures exceeding 37 °C. At pH 7, only BHT produces methylmercury. In terms of fulvic acids, all of them are able to methylate inorganic mercury but the lower molecular weight compounds (M.W. 200) are the most active ones.

Methylcobalt (III) compounds like methylcobalamin are considered potential mercury methylators because their ability for the transference of a methyl group to free $Hg^{2+}$. Although some authors propose a reaction mechanism based on the enzymatic transference of methyl radicals from methylcobalamin to $Hg^{2+}$ via sulfate-reducing bacteria (Barkay *et al.*, 2003), others reivindicate that the reaction takes place in the absence of biological activity (Celo *et al.*, 2006; Chen *et al.*, 2007). In the latter case, inorganic mercury acts as an

electrophile to attack methylcobalamin with the subsequent transference of a methyl carbanion to the most oxidized mercury specie ($MeCo(dmg)_2H_2O + Hg^{2+} + H_2O \rightarrow Co(dmg)_2(H_2O)_2^+ + MeHg^+$; dmg = dimethylglyoximate). In spite of this, methylcobalamin readily methylates $Hg^{2+}$ in non-environmental matrices but it is unlikely in the aquatic environment because its low abundance.

The reaction products of methylcobalamin and $Hg^{2+}$ are methylmercury and dimethylmercury. The first specie to be formed is methylmercury, the first methylation rate being two times faster than the second one. Chen *et al.* (2007) also studied inorganic mercury methylation by methylcobalamin in aquatic systems and identified methylmercury as the reaction product. On the other hand, kinetic experiments showed that the methylation reaction is fast but the salinity and pH modify the electron density of the methyl donor and the electrophilicity of metal ion in the reaction system, which affects to methylmercury formation (Chen *et al.*, 2007). So, the reaction rate is 0.00612 and 0.000287 $min^{-1}$ for pH 5.0 and 1.5, respectively (Chen *et al.*, 2007). Celo *et al.* (2006) refer that the most favourable environmental conditions to mercury methylation by methylcobalamin are acidic pH, high ionic strength and low chloride concentration that are more usually present in fresh waters. Furthermore, they found that methylcobalamin is unlikely to methlylate in moderate or highly saline environments because it is apparently unreactive towards chloride complexes of $Hg^{2+}$ (Celo *et al.*, 2006). Nevertheless, there are controversies and other authors have also reported that the inorganic mercury methylation by methylcobalamin is possible even in highly saline solutions, which emphasizes its importance in aquatic environments (Chen *et al.*, 2007).

Organotin compounds, particularly methyltin species, are suitable methyl donors and their role in abiotic mercury methylation has been evidenced in the aquatic environment (Rosenkranz *et al.*, 1997). Furthermore, methyltin compounds have been frequently detected in all the environmental compartments of the aquatic system. The favourable conditions for the transmethylation reaction among methyltin compounds and $Hg^{2+}$ ($Me_nSn$ (IV) + Hg (II) $\rightarrow$ $Me_{n-1}Sn$(IV) + MeHg (II)) include alkaline pH and the presence of high amounts of chloride (Celo *et al.*, 2006). Therefore, the greater contribution of this methylation mechanism occurs in seawaters than in freshwaters. Furthermore, organotin compounds are efficient methylators of inorganic mercury only at pH values higher than 6. It can be due to methyltin cations (aqua complexes) are unreactive, while neutral methyltin hydroxide complexes are reactive. Within methyltin compounds, $MeSn(OH)_3$ is the most reactive specie and $Me_3SnOH$ is the least one to transfer methyl groups to $Hg^{2+}$. A transition state involving simultaneous methyl transfer from $Sn^{4+}$ to $Hg^{2+}$ and chloride transfer from $Hg^{2+}$ to $Sn^{4+}$ is suggested. Celo *et al.* (2006) estimate that the mercury methylation rate is 0.5 pg/L/day for typical environmental concentrations of monomethyltin (~ 1200 ng Sn/L) and $Hg^{2+}$ (~ 1 ng/L) under pH and temperature values appropriate for seawaters (8 and 20 ºC, respectively). Evidence that methyllead compounds may also methylate mercury exists (Ebinghaus and Wilken, 1996; Rosenkranz *et al.*, 1997), being methylmercury produced by transmethylation.

The artifactual formation of methylmercury when acetic acid is used as an analytical chemical for mercury speciation has been reported (Falter, 1999a,b). Gårdfeldt *et al.* (2003) investigated the mechanism and kinetics of the methylmercury formation from a solution containing $Hg^{2+}$ and acetic acid. The reaction occurs via mercury acetate complexes [$(Hg(CH_3COO)_n)^{2-n} \rightarrow CH_3Hg^+ + CO_2 + (n-1)((CH_3COO)_n)^-$; n = 1-4]. Since the dominant mercury complexes vary with pH, the reaction rate is dependent on this one. Although there

are controversies in the effect of sunlight or UV radiation on the net rate of methylmercury formation, it is not significantly enhanced by photolysis when methylmercury photodegradation is also considered. Gårdfeldt *et al.* (2003) estimate a maximum methylmercury formation rate of 6.5 pg/dm$^3$/h for $1.5\times10^{-4}$ M acetic acid and $10^{-10}$ M $Hg^{2+}$ at pH 3.6. The main parameter limiting the mercury methylation rate via this reaction mechanism is the presence of other ligands, which may compete with acetate for mercury complexation, e.g. chloride, oxalate and sulfide. Recently, the minimal predicted complexation of inorganic mercury by acetate suggests that the methylation is unlikely to account for the methylmercury found in rainwater, or that the mechanism of this reaction in the atmosphere differs from that previously reported (Conaway *et al.*, 2010).

### *2.1.2. Biotic Processes*

Several microorganisms are able to methylate mercury, such as some sulfate-reducing bacteria (Berman *et al.*, 1990; Rodríguez Martín-Doimeadios *et al.*, 2004; Sunderland *et al.*, 2006; Dias *et al.*, 2008; Duran *et al.*, 2008; Holloway *et al.*, 2009), iron-reducing bacteria (Holloway *et al.*, 2009), sulfide and sulfur-oxidizers (Rodríguez Martín-Doimeadios *et al.*, 2004), among others (Rodríguez Martín-Doimeadios *et al.*, 2004). Nevertheless, the first have been identified as the dominant methylators in the aquatic environments.

Some studies involving mercury methylators have been done in order to get insight on the pathway of carbon and on the nature of methyl donors used. Several pathways have been proposed but one of the most studied and well understood is the referred to the *Desulfovibrio desulfuricans* (Berman *et al.*, 1990; Choi *et al.*, 1994a). The most likely source of the methyl group seems to be the C-3 of serine. This compound is the principal methyl donor to tetrahydrofolate and is formed during the carbon flow from the pyruvate. The proposed pathway is represented briefly in Figure 1 and was adapted from the works performed by Berman *et al.* (1990) and Choi *et al.* (1994a).

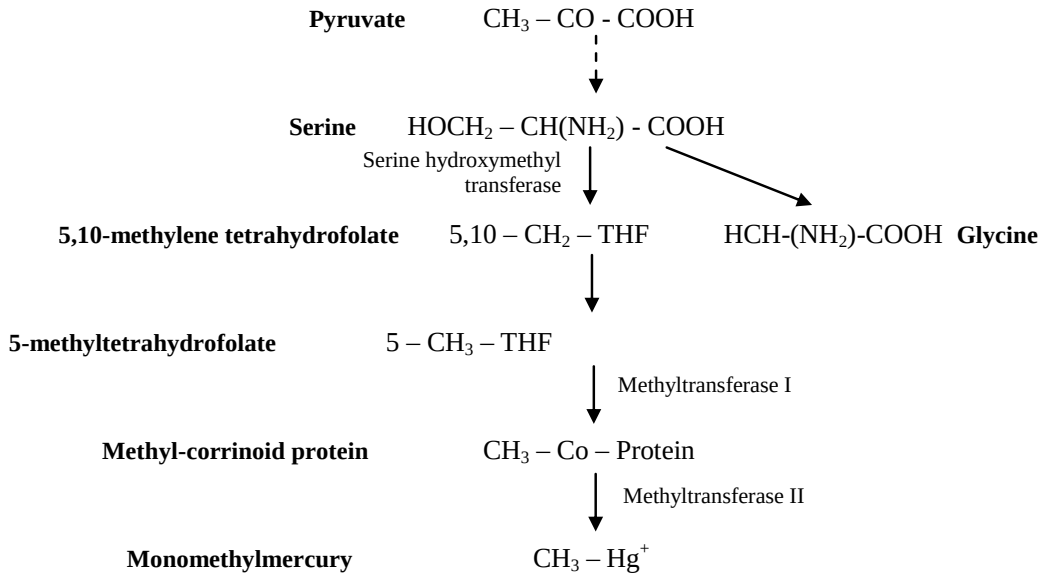


Figure 1. Proposed pathway for methylation of mercury in *Desulfovibrio desulfuricans*.

In more detail, when performing incubations with radiocarbon of C-1-labeled pyruvate, C-3-labeled pyruvate and C-3-labeled serine, the last specie was incorporated into methylmercury with 95% preservation of specific activity, a much higher percentage than those obtained with the other labeled substrates (11 and 21%, respectively) (Berman *et al.*, 1990). These results indicated that the methyl group is probably donated as C-3 of serine to tetrahydrofolate by the action of the enzyme serine hydroxymethyl transferase (Berman *et al.*, 1990; Choi *et al.*, 1994b). After that, tatrahydrofolate transfers the methyl group to cobalamin (vitamin $B_{12}$) or a closely related methyl carrier, such as a corrinoid protein, that finally transfers the methyl group to mercuric ions (Berman *et al.*, 1990; Choi and Bartha, 1993; Choi *et al.*, 1994a). Nevertheless, it has been reported that methylcobalamin is able to methylate spontaneously inorganic mercury (Choi and Bartha, 1993) as referred in the previous section. These observations raised the question of how mercury methylation process occurs *in vivo*. In fact the role of methylcobalamin in the methylation of inorganic mercury in organisms, as well as, its ability of methylating spontaneously mercuric ions, has been readily demonstrated. Therefore, it was necessary to verify if the principal source of methylmercury determined *in vivo* was due to spontaneous transmethylation or to an enzymatically catalyzed process. Choi *et al.* (1994a) proved clearly that inorganic mercury methylation occurring *in vivo* is an enzymatically catalyzed process, rather than a spontaneous transfer of methyl group from methylcobalamin, after observation of saturation kinetics and of the higher rate of inorganic mercury methylation (at pH 7.0) by cell extracts of *Desulfovibrio desulfuricans*, when compared with transmethylation by free methylcobalamin. Therefore, in these processes two methyltransferases seem to be involved (Choi *et al.*, 1994a). Latter on Choi *et al*. (1994b) have further proposed that methyl group may also originate from formate via the acetyl-CoA synthase pathway.

In biological systems, beyond methylcorrinoid derivatives (such as, methylcobalamin), there are two possible other microbial methylating agents: *S*-adenosylmethionine (SAM) and 5-methyltetrahydrofolate (5-MTHF). Nevertheless, Gadd (1993) refers that the major methylating agent involved in mercury methylation is the methylcobalamin.

Due to the important role of the enzymes mentioned before, sometimes it might be difficult to differentiate between biotic and abiotic methylation because it has been suggested that the formation of abiotic methylmercury may result from dead communities of bacteria that can continue to methylate mercury by releasing enzymes (Eckley and Hintelmann, 2006). Thus, these enzymes seem to have potential to promote extracellular methylation (Eckley and Hintelmann, 2006).

Regarding culture conditions, these also play an important role on methylmercury synthesis. It has been reported, for example, that *Desulfovibrio desulfuricans* produced more methylmercury under fermentative than under sulfate-reducing conditions (Choi and Bartha, 1993).

*i. Environmental Factors that Affect Mercury Biotic Methylation*

As biotic methylmercury production is related to microorganism's activity, it will depend on several factors. It must always be remembered that the factors that affect mercury methylation can be separated into those that affect the activity of mercury methylating bacteria and those that affect the bioavailability of mercury to the methylating organisms. The relative importance of these factors is generally difficult to assess.

In relation to the first type of factors, Holloway *et al.* (2009), for example, observed that the soil type influenced more the soil microbial communities than season, when they studied the spatial and seasonal variations in mercury methylation in soils collected in a historic mercury mining area in Yolo County (California). Soil moisture is another factor that might have an important role. When it increases, water-saturated micropores in soils also increase, being induced the reduced environment required by both sulfate- and iron-reducing bacteria (Holloway *et al.*, 2009) that might promote mercury methylation. Owing to this, strong correlations between soil moisture and methylmercury concentrations are sometimes encountered (Holloway *et al.*, 2009). Nutrients are also required by microorganisms and when there is a shift in these compounds concentrations, variations in soil microbial communities are driven (Holloway *et al.*, 2009). Nutrients might be also the reason why higher methylation rates are generally determined in sediments than in water column. This might be due to the highest nutrient and carbon contents of sediments (Eckley and Hintelmann, 2006), leading to more prolific bacteria populations, including mercury methylators. Sediment temperature also affects the activity of the microbial community present and so mercury methylation might vary seasonally (Raposo *et al.*, 2008). Methylation is generally increased with temperature, as stated for sediments collected in Lavaca Bay (Texas) (Bloom *et al.*, 1999), Gulf of Trieste (Hines *et al.*, 2006), Hudson River (Heyes *et al.*, 2006) and New York/New Jersey Harbor (Hammerschmidt *et al.*, 2008); however, methylation might be quite active in Winter (Hines *et al.*, 2006).

Since sulfate-reducing bacteria appear to be the primary mercury methylators in sediments and they are able to reduce sulfate to sulfide (Eckley and Hintelmann, 2006), it is common to find out similar depth distributions for mercury methylation activity and sulfide (Hines *et al.*, 2006). Also, an increase in methylmercury concentrations are generally associated with increasing sulfate concentrations (Muresan *et al.*, 2007; Holloway *et al.*, 2009). Nevertheless, both the availability of sulfate and the presence of high quality carbon (electron donor) in organic matter are the two major variables affecting sulfate-reducing bacteria populations and activities (Heyes *et al.*, 2006) and thus mercury methylation (Mitchell *et al.*, 2008).

As already mentioned, other factors, such as, sulfide and organic matter, affect the bioavailability of mercury to the methylating organisms. The substrate for mercury methylation in sediments, for example, is the inorganic mercury present in pore waters, whose bioavailability for methylating bacteria tends to decrease as sulfide concentrations increase, since dissolved mercury tends to form non-neutral complexes with sulfur (Benoit *et al.*, 1999; Hines *et al.*, 2006; Lambertsson and Nilsson, 2006). At low sulfide concentrations, neutral HgS complexes in pore waters tend to dominate (Lambertsson and Nilsson, 2006). These species can diffuse through bacterial membranes and can be methylated to methylmercury (Hines *et al.*, 2006). On contrary, as dissolved sulfide concentrations increase, these species are replaced by non-neutral complexes, which are not able to pass through the bacterial membranes. Several studies (Heyes *et al.*, 2006; Lambertsson and Nilsson, 2006; Muresan *et al.*, 2007; Hammerschmidt *et al.*, 2008) propose the existence of these processes in natural environments, after considering the methylmercury formation rates and the mercury and sulfide pore waters concentrations; however, some exceptions have been reported. Sunderland *et al.* (2006), for example, verified an increase in methylmercury percentage in high sulfide sediments containing high levels of dissolved organic carbon. This might result of the formation of bioavailable Hg(II) complexes that contains both sulfur and dissolved organic

carbon (Sunderland *et al.*, 2006). Taking into account the important role that sulfate-reducing bacteria, as well as, the sulfide content have on mercury methylation, the relationship between mercury methylation and sulfate/sulfide chemistry is complex (Eckley and Hintelmann, 2006). While sulfate controls microbial activity, sulfide controls mercury speciation.

Organic matter is another important factor that influences methylation because it acts as a terminal electron acceptor and as a carbon source to microorganisms; however, the relationship between dissolved organic matter and mercury methylation is more complex than this. On one hand, dissolved organic carbon has been shown to increase mercury methylation by stimulating microbial activity. On the other, mercury bioavailability might change. It has been suggested in some works that, for example, impoundments cause increases in methylmercury concentrations by creating organic-rich anoxic deposits conducive to mercury methylation (Hines *et al.*, 2000). In fact, the microbial community uses the organic carbon pool to build new cells and to include $CH_3$ that combines with inorganic mercury to form methylmercury. Add to this, high contents of organic matter in the sediment are a prerequisite for maintaining low redox potentials while supplies of "high-quality" organic matter, providing electron donors for the sulfate-reducing bacteria (Lambertsson and Nilsson, 2006). Nevertheless, organic matter also provides complexing agents for $Hg^{2+}$ (Ravichandran, 2004; Eckley and Hintelmann, 2006) and methylmercury (Cai *et al.*, 1999; Ravichandran, 2004), thus influencing both the total sediment and water column concentrations and the partitioning between solid and dissolved phases (Lambertsson and Nilsson, 2006; Sunderland *et al.*, 2006). When organic matter complexes the inorganic mercury, this specie becomes less biologically available for methylation because dissolved organic carbon molecules are generally too large to cross the cell membranes of the bacteria (Ravichandran, 2004). Besides, dissolved organic carbon-mediated reduction of inorganic mercury to the volatile $Hg^0$ species would also reduce the bioavailability of mercury for methylation and subsequent biological uptake (Ravichandran, 2004). Another fact to be considered is that the measurement of total organic matter content might have little relevance in terms of the concentration of organic substrate required really by mercury methylating organisms. These require some specific compounds, such as acetate, and not the total pool (Heyes *et al.*, 2006; Drott *et al.*, 2008b). Moreover, the type of the organic matter present is another important factor that influences mercury methylation because as hypothesized by Ravichandran (2004) when organic matter is largely labile and readily biodegradable, it may promote methylation by stimulating microbial growth and when the organic matter is relatively recalcitrant and consists of high molecular weight humic and fulvic acids, the abiotic methylation may be favored.

Organic matter also affects the redox potential of the sediments (Sunderland *et al.*, 2006). A high content of organic matter in the sediment promotes heterotrophic microbial activity, which consumes oxygen and lowers the redox potential close to the sediment surface (Lambertsson and Nilsson, 2006). On the other hand, the lower the organic matter in the sediment the deeper can oxygen and other competing electron acceptors (mainly manganese and iron) penetrate before being depleted by heterotrophic microbial activity (Lambertsson and Nilsson, 2006).

It must also be referred that not always a positive correlation between total mercury and methylmercury is observed in soils (Holloway *et al.*, 2009) and sediments (Heyes *et al.*, 2006; Lambertsson and Nilsson, 2006). Several hypotheses have been formulated in order to explain these results. One of the possibilities is the occurrence of microbial growth inhibition due to the high mercury concentrations, leading to the inhibition of the biotic mercury methylation

(Holloway *et al.*, 2009). The other is linked to geochemical factors as insufficient supply of new organic matter and inadequate redox conditions that also not favor the mercury methylation (Lambertsson and Nilsson, 2006). As already mentioned, for example, a high organic content in sediment maintains a low redox potential, which is a prerequisite for sulfate reduction (performed by sulfate-reducing bacteria) and concomitant mercury methylation (Lambertsson and Nilsson, 2006). In spite of this, in sediments with high organic content the redox potential required for sulfate reduction can be maintained closer to the sediment surface (Lambertsson and Nilsson, 2006). In sediments with lower organic contents, mercury methylation occurs deeper in the sediment (Lambertsson and Nilsson, 2006).

## 2.2. Mercury Demethylation Processes

### *2.2.1. Chemical Demethylation – Abiotic Processes*

The photolytic decomposition of methylmercury remains the only abiotic demethylation mechanism that is significant in surface waters exposed to sunlight (Sellers *et al.*, 1996; Gårdfeldt *et al.*, 2001; Chen *et al.*, 2003; Hammerschmidt and Fitzgerald, 2006; Monperrus *et al.*, 2007a). However, the overall impact on the aquatic Hg cycle is still unclear and the end products of the methylmercury degradation have not been clearly identified yet. Hammerschmidt and Fitzgerald (2006) demonstrate that the methylmercury decomposition in surface waters is an exclusively abiotic and sunlight-induced process. Monperrus *et al.* (2007b) estimate demethylation rates of methylmercury in coastal and marine waters (6.4–24.5 % $day^{-1}$) and suggest that an important part of the demethylation is mostly driven by sunlight because those rates decrease severely under dark conditions. Monperrus *et al.* (2007b) and Whalin *et al.* (2007) refer that, in coastal and marine surface waters, although methylmercury is mainly photochemically degraded, the demethylation yields observed under dark conditions may be attributed to microbial mediated pathways. Furthermore, higher demethylation potentials are predicted in marine surface waters in comparison with the water masses located deeper in the euphotic zone as the methylmercury degradation is inhibited under dark conditions. In sediments, the abiotic mechanism is also more conductive to the environmental methylmercury decomposition than the biotic one (Rodríguez Martín-Doimeadios *et al.*, 2004).

Hammerschmidt and Fitzgerald (2006) demonstrated that the rate of the methylmercury degradation is positively correlated with the intensity of photosynthetically active radiation (PAR) at a 0.75-6 m depth in the water column. Nevertheless, methylmercury can be degraded more rapidly at lower depths due to the additional influence of the ultraviolet (UV) light. In this sense, other authors suggested that the methylmercury photodecomposition is largely limited to the upper 0.5-1 m layer of surface waters, which is consistent with the penetration of the UV light in the water column (Krabbenhoft *et al.*, 2002). Moreover, Lehnherr and Vincent (2009) attribute the most important driver of the methylmercury photodecomposition to the UV radiation in freshwaters because wavelengths in the visible spectrum degrade methylmercury at a much slower rate than the former. However, they also recognize that the visible light plays an important role in deepening waters as it is attenuated much less rapidly than the UV radiation. Therefore, the modeling of the methylmercury photodecomposition requires the mechanistic knowledge of the role of the UV radiation

versus visible light, since wavelengths in both UV and visible regions of the solar spectrum are attenuated at very different rates in the water column of freshwaters.

It is important to take into account that photodecomposition rates are comparable among several lakes with widely varying water chemistry. It suggests that the kinetics of the methylmercury photodecomposition is not influenced by environmental factors apart from those affecting the light intensity and methylmercury concentration in natural surface waters (Sellers *et al.*, 1996; Hammerschmidt and Fitzgerald, 2006).

Since methylmercury cannot absorb sunlight wavelengths at all and thus the direct photodegradation cannot occur, the only possible mechanism is the indirect photolysis involving the photochemical formation of aqueous free radicals in sunlit natural waters. Chen *et al.* (2003) investigated the kinetics and mechanism of the methylmercury photodegradation mediated by hydroxyl radicals. They used the nitrate photolysis from 285 to 800 nm as the hydroxyl radical source. The products identified were $Hg^{2+}$, $Hg^0$, chloroform and formaldehyde, the main aqueous product being divalent mercury. The effects of chloride concentration and methylmercury speciation have also been investigated. The presence of chloride can lead to a higher methylmercury degradation rate that can be attributed to the chlorine radicals produced during the aqueous oxidation of chloride by hydroxyl radicals. The chlorine radicals formed may also attack the C–Hg bond and lead to an enhanced methylmercury degradation. Although the pH value does not significantly affect the degradation rate constant for reactions induced by hydroxyl radicals, a small decrease in the degradation rate is observed when the pH value increases from 5 to 8.5. It seems to be due to an increase in the relative concentration of methylmercury hydroxide, whose degradation rate is lower than that of methylmercury chloride. The two mechanisms proposed for the methylmercury degradation by hydroxyl radicals are both the dissociation of $CH_3$ group ($CH_3HgCl + {}^{\cdot}OH \rightarrow {}^{\bullet}CH_3 + HgOHCl$) and the dissociation of HgCl ($CH_3HgCl + {}^{\cdot}OH \rightarrow CH_3OH + {}^{\bullet}HgCl$) to form HgOHCl or other divalent mercury products. So, the Hg–C bond is attacked by the electronically excited hydroxyl radicals. Based on the typical concentration of hydroxyl radicals in natural waters, the methylmercury degradation rate was calculated. It ranges from 0.008 to 3.204 ng $L^{-1}$ $d^{-1}$ assuming a methylmercury concentration of 0.9 ng $L^{-1}$ in natural waters, except for seawaters due to their lower OH radical concentration. The methylmercury photodegradation mediated by hydroxyl radicals may be one of the most important pathways in sunlit surface waters.

Other possible mechanisms of indirect photodegradation could involve the singlet oxygen mediated pathway or the organic peroxy radical mediated pathway. However, no laboratory data is available to assess the importance of these reactions for the methylmercury decomposition.

As above mentioned, the methylmercury photodecomposition occurs via indirect photolysis and, therefore, it requires the presence of a photosensitizing species such as nitrate or dissolved organic matter (Chen *et al.*, 2003). Several studies have shown that this reaction is enhanced in the presence of organic compounds (Sellers *et al.*, 1996; Gårdfeldt *et al.*, 2001). Lehnherr and Vincent (2009) showed that the contribution of the UV radiation to the methylmercury degradation is greater in high dissolved organic matter waters (76 %) than in low ones (54 %) where the visible light acquires a similar role (46 %). Within UV radiation, the region A (320-400 nm) is a more important driver of the methylmercury photodecomposition than the region B (280-320 nm). On the other hand, the

photosensitization of dissolved organic matter by wavelengths in the PAR spectrum appears to be an important factor influencing the methylmercury photodecomposition in not very surface photic zones (Hammerschmidt and Fitzgerald, 2006). However, the demethylation mechanism of methylmercury by PAR (400-700 nm) still remains unknown.

The chemical methylmercury demethylation mediated by selenoamino acids via a bis(methylmercuric)selenide intermediate has been suggested, which is readily degraded to mercury selenide and dimethylmercury (Khan and Wang, 2010). The latter one is then decomposed further to methylmercury. This demethylation reaction can occur in vivo. In the aquatic environment, although there has been no report on the concentrations of selenoamino acids in natural waters, their sulfur counterparts have been reported in surface and sediment pore waters. Similarly, the sulfur-aided demethylation pathway gives mercury sulfide as ultimate reaction product.

### *2.2.2. Biotic Processes*

Microorganisms in contaminated environments have developed resistance to mercury and play a major role in natural decontamination. Mercury resistance occurs widely on Gram negative and Gram positive bacteria, in environmental (Chatziefthimiou *et al.*, 2007; Ramond *et al.*, 2008), clinical (Soge *et al.*, 2008) and industrial isolates. On contrary to mercury methylation that seems to be restricted to a subset of bacteria, mercury demethylation appears to be a process that is more widely spread. Research works on molecular biology shows that methylmercury degradation performed by microorganisms generally proceeds through two distinct vias (Hines *et al.*, 2006), oxidative and reductive, being the last one mainly linked to the mercury resistance (*mer*) operon. Both biotic pathways for methylmercury degradation are encountered in the environment and will be further discussed in the following sections.

*i. Reductive Methylmercury Degradation*

The reductive methylmercury degradation might occur through two pathways, one involving the *mer* operon and other that does not; however, the former process is the most studied and considered to be the most common.

When microorganisms use the reductive pathway via *mer* operon to perform methylmercury degradation, two stages are involved which are catalyzed by two enzymes. The *mer*-mediated methylmercury degradation pathway may be represented easily by:

$$\text{R-Hg} \xrightarrow[\text{Lyase}\ (\textit{mer}\text{B})]{} \text{Hg(II)} \xrightarrow[\text{Reductase}\ (\textit{mer}\text{A})]{} \text{Hg}^0$$

In general terms, the organomercurial lyase breaks the carbon-mercury bond in toxic substrates, such as methylmercury and phenylmercury, being released methane or benzene, respectively, and inorganic mercury (Hg(II)), which is subsequently reduced to $Hg^0$ by the action of the mercuric reductase.

Based on the organization of *mer* operon genes, two modes of mercury resistance are encountered in bacteria (Hines *et al.*, 2006): narrow-spectrum resistance and broad-spectrum

resistance. In the first, Hg(II) is reduced to the less toxic, inert and volatile elemental form ($Hg^0$), by the action of the mercuric reductase (MerA). On contrary, in broad-spectrum resistance, both organic and inorganic mercury will be remediated due to the presence of a *merB* gene that encodes an enzyme organomercurial lyase, beyond the presence of the mercuric reductase.

Genes encoded by the *mer* operon have been reported to be located on plasmids (Summers and Silver, 1978; Brown *et al.*, 1986; Griffin *et al.*, 1987; Radstrom *et al.*, 1994; Osborn *et al.*, 1997; Barkay *et al.*, 2003), chromosomes (Wang *et al.*, 1989; Inoue *et al.*, 1989, 1991), transposons (Kholodi *et al.*, 1993; Hobman *et al.*, 1994; Liebert *et al.*, 1997; Mindlin *et al.*, 2001; Ng *et al.*, 2009), as well as on integrons (Kholodii *et al.*, 1993; Liebert *et al.*, 1997). The mobile elements (plasmids, transposons and integrons) play an important role in the dissemination of mercuric resistance throughout microbial communities (horizontal transfer). Furthermore, these mobile elements may be occasionally combined with other resistance determinants, promoting the spreading of these plasmids with multiple resistance genes. It has been demonstrated that *mer* operon is associated to multidrug-resistance (Soge *et al.*, 2008, Silver and Phung, 2005, Ball *et al.*, 2007). Moreover, it has been verified that mercuric multiple resistant bacteria can effectively transfer the phenotype to potentially pathogenic species (Ball *et al.*, 2007). This proves clearly that it is of extreme importance to study the *mer* operon frequency in the microorganisms present in the environment, in order to obtain valuable data to be used in the prediction of the evolution of drug resistance.

In other hand *mer* operon has been used in mercury bioremediation studies, performed with the aim to decontaminate environments with high levels of mercury. In spite of this, *mer* operon has been used to modify microorganisms which become able to reduce inorganic mercury to elemental mercury (Deng *et al.*, 2008), and plants with the capacity of also degrade methylmercury (Bizily *et al.*, 2003); however, it is still necessary to perform more studies in order to increase the efficiency. Furthermore, the role of *mer* operon over heavy metals and xenobiotics detoxification has also been demonstrated (De and Ramaiah, 2007; De *et al.*, 2008).

*ii. Organization of the Mer Operon*

The *mer* operon(s), as well as the protein gene products found in a Gram negative bacteria are represented in Figure 2. Generally, when a bacteria is in the presence of methylmercury and inorganic mercury that are very toxic species to the cells, the bacteria tries to bring quickly these species to the cytoplasm where they will be converted enzymatically to the volatile and low-toxicity elemental form, $Hg^0$.

Several studies show that once at the cell surface, methylmercury passes through the cell's outer membrane via passive diffusion in its neutral/hydrophobic form(s) (Step 1) (Barkay *et al.*, 2003; Kritee *et al.*, 2009). Simmons-Willis *et al.* (2002), for example, suggests that methylmercury is transported as a complex with molecules containing thiol groups (i.e., cysteine or glutathione); however, more studies are needed in order to better understand the process involved. Nevertheless and considering the detailed work performed recently by Kritee *et al.* (2009), it seems that the uptake rate across the bacterial membrane (*V*) will always be lower than the rate of diffusion across the diffusion boundary layer (*J*) (Step 1'), remaining the cell uptake limited.

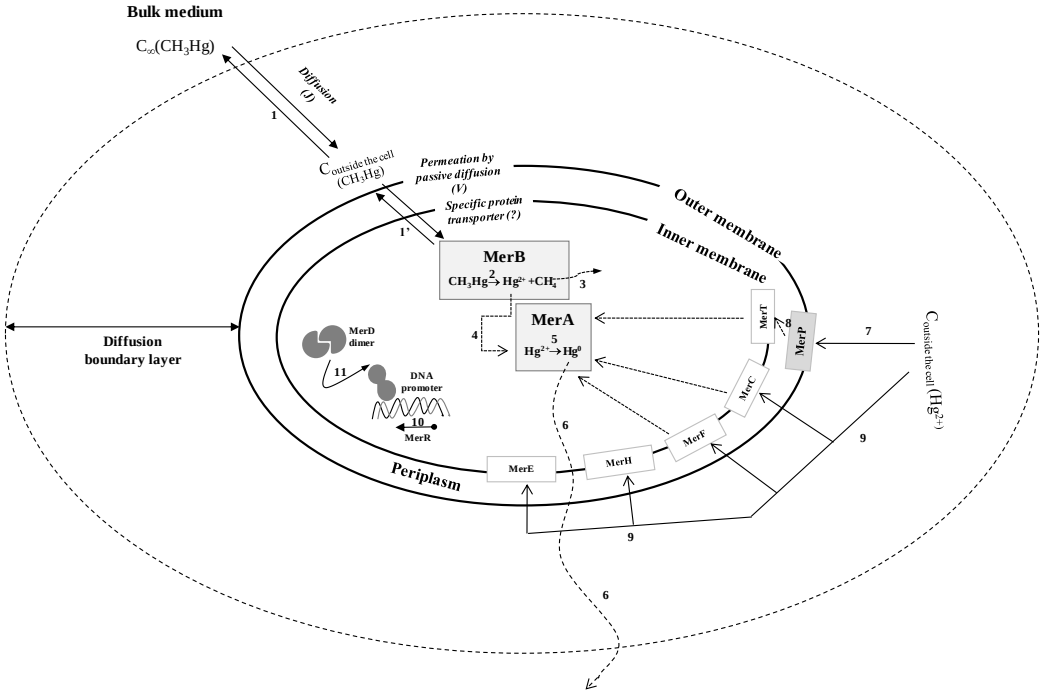


Figure 2. The products of the *mer* operon(s) of a Gram-negative bacteria.

The organomercury lyase (MerB), a small monomeric enzyme that cleaves the Hg-C covalent bond (Step 2) releasing Hg(II) (the substrate of mercuric reductase) (Step 3) and reduced organic compounds, such as methane from methyl mercury (Step 4) (Silver and Phung, 2005), is known as key enzyme in bacterial detoxification and bioremediation of organomercurials species.

The gene *mer*B has been found in the *mer* operon located on the plasmid of some bacteria, such as in *Escherichia coli*, *Pseudomonas* (almost 50%) and *Staphylococcus aureus* with "penicillinase" plasmids (Silver and Phung, 2005). This gene is also found on the chromosomes of methicillin-resistant *S. aureus* (MRSA) and of some *Bacillus* strains isolated from the environment. Frequently, Gram negative bacteria have two copies of *mer*B gene and *Bacillus* has three copies.

Several *mer*B sequences have been identified in a variety of microorganisms and most of the *mer*B genes are very homologous to each other; however, phylogenetic analysis revealed that MerB is an enzyme without known homologs in prokaryotic or eukaryotic proteins (Silver and Phung, 2005; Pitts and Summers, 2002). Furthermore, the MerB accepts a wide range of substrates. Chien *et al.* (2010) investigated the substrate specificities by resistant strains that have different *mer*B gene(s), and observed that *mer*B1 gene from *Bacillus Megaterium* MB1 conferred the highest volatilization ability to methylmercury chloride, ethylmercury choride and thimerosal, while *mer*B3 conferred the faster volatilization activity to *p*-chloromercuribenzoate; however, further work needs to be done in order to relate the MerB sequences with the wide range of organomercurial substrates known. In the presence of phenylmercury that is other organomercurial extremely toxic to the cells, this enzyme is capable to reduce the phenyl moiety to benzene (Silver and Phung, 2005). The enzymatic

reaction of organomercurial lyase has been studied and explained tentatively by Narita *et al.* (2003), Begley and Ealick (2004) and Silver and Phung (2005), indicating the occurrence of a proton attack on the Hg-C bond. Until now the catalytic mechanism of MerB has been controversial. Recently, Li *et al.* (2010) when studying the degradation mechanism of methylmercury by the tris(2-mercapto-1-*tert*-butylimidazolyl)hydroborate ([$Tm^{t\text{-}Bu}$]) ligand system, which shows a coordination environment that resembles closely the active site of the organomercurial lyase MerB, as well as Lafrance-Vanasse *et al.* (2009) after performing crystal structure studies on MerB in its free and mercury-bond forms, verified that two conserved cysteines, namely Cys-96 and Cys-159 are essential for enzymatic activity, playing a role in substrate binding, carbon-mercury bond cleavage, and controlled product (ionic mercury) release. Moreover, these authors also observed that an aspartic acid (Asp-99) in the active site plays a crucial role in the proton transfer step required for the cleavage of the carbon-mercury bond, acting as a proton mediator. The result of this bond cleavage is the release of methane and the retention of the ionic mercury in the active site. The way that methylmercury is bound to MerB is still under discussion. Nevertheless, the ionic mercury formed seems to be transferred directly to the reductase MerA (Step 3) for conversion to the less toxic elemental mercury (Lafrance-Vanasse *et al.*, 2009). In this manner, MerB and MerA keep the toxic organomercurial and ionic mercury species bound at all times and thus minimize their damaging interactions with other cellular proteins (Lafrance-Vanasse *et al.*, 2009). Indeed, Benison *et al.* (2004) proposed that the carboxyl-terminal cysteines of MerA are involved in removing the mercuric ion directly from MerB. Hg(II) is reduced in MerA to $Hg^0$ by electron transfer from FAD cofactor (Step 5) (Silver and Phung, 2005) and released (Step 6).

Mercuric reductase also reduce Hg(II) from cell outside, which is delivered into cytoplasm by mercuric transporters. A recently developed bacterial two-hybrid protein system showed that N-terminal region of MerA interacts with the cytoplasmatic face of mercuric transporter MerT (Schué *et al.*, 2007); however, in relation to mercuric reductases, diversity among different microorganisms is observed and lies in the N-terminal of the enzyme. MerA protein may have this N-terminal domain once or twice or lack it as for example in *Streptomyces*. The N-terminal domain of mercuric reductase can be proteolytically removed in vivo, and it appears to have little effect on overall rates of mercury reduction, suggesting that mercuric ions can be transferred in a different way (Schué *et al.*, 2008). In more detail, the resistance mechanism against Hg(II) ions (narrow-spectrum resistance) is completed by a mercury transport system. This may be formed by, for example, one (MerC), two (MerT and MerP), or three (MerT, MerP and MerC) proteins, which deliver mercury ions inside the cell cytoplasm where they are reduced to $Hg^0$ (Velasco *et al.*, 1999). Other membrane transport proteins have been reported, such as MerF, MerE and MerH. These transport systems hinder the toxic mercury to be free, not being able to cause damage into the cell. Studies performed in *E.coli* TG2 showed that the highest rate of cellular mercury volatilisation was observed in bacteria that expressed both MerP and MerT (Wilson *et al.*, 2000). MerP seems to make the system more efficient, as it acts not only as a periplasmic mercury binding protein (Step 7), but also as a metallochaperone, delivering Hg(II) to the membrane-anchored protein MerT (Step 8); however, MerP is not essential for Hg(II) transport (Wilson *et al.*, 2000; Nascimento and Chartone-Souza, 2003). Simplified schemes of some of these mercury transporters are represented in Figure 3.

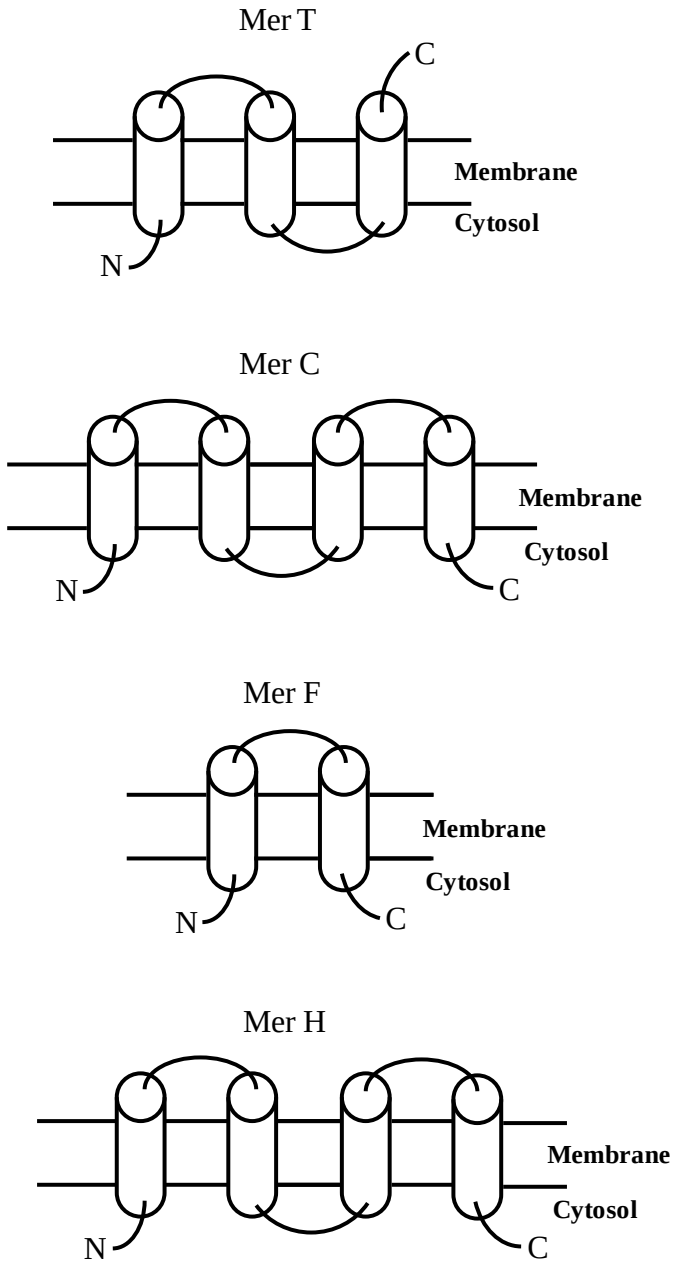


Figure 3. Simplified schemes of the MerT, MerC, MerF and MerH mercury transporters.

MerT has three trans-membrane alfa domains. The first cysteine pair, located in the first *trans*-membrane helix, receives mercury from the periplasmatic MerP. A second cysteine pair, located in a cytoplasmic loop connecting the second and the third *trans*-membrane helices, is important for optimal mercury transport, but not for the interaction with mercuric reductase (Schué *et al.*, 2008).

In relation to other membrane transport proteins, MerC and MerH cross the membrane four times, unlike MerF and MerE that just cross twice. Nevertheless, it is supposed that these

proteins function in a similar way. For acquiring further data over the structure of these proteins we recommend the reading of the works of Wilson *et al.* (2000) and Silver and Phung (2005). MerP does not make any difference to the rate of volatilization in the presence of MerC or MerF (Wilson *et al.*, 2000), indicating that neither of these transport proteins interacts with MerP in vivo (Step 9). It is interesting to observe that among all of the Hg(II) transporters only MerT presents the C-terminal in the periplasm and the N-terminal in cytosol, while all the others present the both terminals in cytoplasm, suggesting no interaction domain with MerP. Moreover, differences have been observed among all these Hg(II) transporters, suggesting that they can have different specificity to different mercury species and/or different $K_m$'s (Wilson *et al.*, 2000; Kyono *et al.*, 2009; Schué *et al.*, 2009); however, further studies must be done in order to clarify the role of these Hg(II) transporters.

The expression of the *mer* operon is regulated by MerR that bind to the upstream operator DNA regions (Step 10). The product of *mer*R is a 144-amino acid MerR protein that represses the transcription of *mer* operon in the absence of Hg(II) and induces it in the presence of Hg(II). MerR also represses its own synthesis whether or not Hg(II) is present (Heltzel *et al.*, 1990). The MerR protein has a distinctive protein fold consisting of a DNA-binding helix-turn-helix motif, followed by another helix-turn-helix motif that communicates between the metal binding and DNA binding domains. Half of the C-terminal of this small protein is a 35-residue leucine-zipper helix that forms the dimer interface as an anti-parallel coiled coil giving the protein an overall shape like a twisted staple (Song *et al.*, 2007). In Gram-negative bacteria the regulatory *mer*R gene is separated from the others genes by the operator-promoter region and is transcribed in the other direction. In Gram-positive bacteria *mer*R is transcribed in the same direction of the other genes of the operon. MerR is an unusual repressor that never leaves its operator that lies in a region of dyad symmetry located between the region consensus -35 and -10, a RNA polymerase recognition site. Moreover, in the absence of Hg(II) it captures an RNA polymerase in an inactive but stable preinitiation complex (Lee *et al.*, 1993). Studies of conformational dynamics of MerR after Hg(II) binding revealed allosteric conformational change from the metal-binding site to the two DNA binding domains leading to distortion of the operator and freeing the pre-bond RNA polymerase to begin transcription of the structural genes (Guo *et al.*, 2010).

In some *mer* operons, a second regulator gene, *mer*D, is present and possibly is an antagonist of *mer*R necessary to turn off expression by binding to the same promoter-operator region to which the Mer R protein bind (Mukhopadhyay *et al.*, 1991) (Step 11).

Regarding the induction of the *mer* operon, Hg(II) and phenylmercury acetate are able to do that (Nucifora *et al.*, 1989); however, this has not been unequivocally demonstrated for methylmercury. Schaefer *et al.* (2004) after performing a very interesting work on the role of the MerB in controlling methylmercury accumulation in mercury-contaminated natural waters, verified that Hg(II) induces quantitatively the expression of both *mer*A and *mer*B. In this work, the bacterium *Pseudomonas stutzeri* OX was used. This bacterium is resistant to inorganic and organic mercury as it carries two discrete *mer* operons, a narrow-spectrum *Tn*501-like operon and a broad spectrum *Tn*5053-like operon. Another interesting point stated by these authors was that at inducing Hg(II) concentrations higher than 2 μM, *mer*A transcript abundance continued to increase while *mer*B transcripts leveled off. One possible explanation for these results is differences in transcription kinetics between *mer*B and *mer*A. Moreover, *mer*A transcripts were consistently 7- to 15-fold more abundant than *mer*B transcripts at a given inducing Hg(II) concentration, which may be expected as this bacteria strain contains

two copies of *mer*A and a single copy of *mer*B. Similar results were achieved by Kritee *et al.* (2009) when studying the strain *Escherichia coli* JM 109 that carries the broad spectrum *mer* operon of the soil denitrifying bacterium previously referred, *Pseudomonas stutzeri* OX. These authors refer that MerB has lower turnover rates (lower $k_{cat}$ – 0.7 to 20 $min^{-1}$ – as compared to MerA – 400 to 800 $min^{-1}$ - i.e. it is less efficient in causing product formation per unit time and therefore the rate of Hg(II) reduction per cell is much higher than the rate of methylmercury degradation). Moreover, there is no evidence until now to suggest active involvement of radical pairs or paramagnetic species in non-photochemical biological reactions involving mercury, either in MerB catalysis and MerA reduction mechanism (Kritee *et al.*, 2009).

Recently, Kritee *et al.* (2009) verified that when performing experiments with a microorganism able to perform the degradation of methylmercury by the action of the *mer* operon, cell density may also have a significant role in the methylmercury degradation rates. These authors observed that at low cell densities, methylmercury seems to be bioavailable for diffusion and uptake into the cells, being the activity of the MerB the rate limiting. As the cell density increases, the bioavailability of methylmercury might be decreased due to sorption of this compound to the bacterial cell surfaces, decreasing methylmercury availability in the cytoplasm, originating lower rates of methylmercury degradation when compared to those obtained with lower cell densities.

Another reductive degradation pathway, a non-*mer*-mediated detoxification, has been proposed. Baldi *et al.* (1993) reported that the sulfate reducing bacteria *Desulfovibrio desulfuricans* might use an alternative anaerobic, non-*mer*-mediated degradation pathway, where methylmercury reacted with microbially produced sulfide to form an unstable dimethylmercury sulfide $(MeHg)_2S$ intermediate, which decomposes to dimethylmercury ($Me_2Hg$) and mercury sulfide (HgS). Dimethylmercury is then degraded to methylmercury and methane. Thus, the production of methane from methylmercury is common to both of the reductive demethylation pathways. Furthermore, as this non-*mer*-mediated degradation pathway implies the reaction of methylmercury with sulfide, it is expected to be most prevalent in sulfide-rich sediments. Such process has been suggested to occur in the environment. In fact, an increase on methylmercury degradation rate has been observed when pore-water sulfide concentrations have also increased (Marvin-Dipasquale *et al.*, 2000).

*iii. Oxidative Methylmercury Demethylation*

Oxidative demethylation is another demethylation pathway of monomethylmercury found in the environment (Oremland *et al.*, 1991; Oremland *et al.*, 1995; Hines *et al.*, 2006) that has been observed under aerobic and anaerobic conditions (Oremland *et al.*, 1991; Marvin-Dipasquale and Oremland, 1998; Hines *et al.*, 2000; Marvin-Dipasquale *et al.*, 2000; Hines *et al.*, 2006). Several kinds of microorganisms have been proposed to be involved in the process; however, the most common are sulfate reducers (Oremland *et al.*, 1991; Marvin-Dipasquale and Oremland, 1998; Marvin-Dipasquale *et al.*, 2000) and methanogens (Oremland *et al.*, 1991; Marvin-Dipasquale and Oremland, 1998; Marvin-Dipasquale *et al.*, 2000).

It seems that methylmercury is demethylated in part by biochemical pathways used for the metabolism of one-carbon compounds, such as methanol (Oremland *et al.*, 1991; Marvin-Dipasquale *et al.*, 2000), methylamines and methyl sulfides, as the addition of this kind of compounds has originated a substantial inhibition in methylmercury demethylation

(Oremland *et al.*, 1991). In oxidative demethylation, methylmercury is converted primarily to $CO_2$ and inorganic mercury, on contrary to the reductive degradation pathway of *mer*-detoxification, characterized by the nearly exclusive production of methane; however, it has been suggested that different microbial groups are capable of oxidative demethylation but with different stoichiometric end-product $CO_2/CH_4$ ratios and/or at different rates (Oremland *et al.*, 1995; Marvin-Dipasquale *et al.*, 2000). For methanogenic bacteria, for example, is expected the production of both $CO_2$ and $CH_4$ during oxidative demethylation, since these are the products of the $C_1$ metabolism by methanogens (Oremland *et al.*, 1995). Moreover, some of the carbon dioxide formed by the demethylators can be fixed into acetate pools by acetogenic bacteria (Oremland *et al.*, 1991).

The following reactions for the oxidative demethylation pathways used by sulfate reducers (Eq. 1) and methanogens (Eq. 2) have been proposed (Marvin-Dipasquale and Oremland, 1998), respectively:

$$SO_4^{2-} + CH_3Hg^+ + 3\ H^+ \rightarrow H_2S + CO_2 + Hg^{2+} + 2\ H_2O \text{ (Eq. 1)}$$

$$4\ CH_3Hg^+ + 2\ H_2O + 4\ H^+ \rightarrow 3\ CH_4 + CO_2 + 4\ Hg^{2+} + 4\ H_2 \text{ (Eq. 2)}$$

Considering Eq. 2, the oxidative metabolism of methylmercury during methanogenis will yield methane and carbon dioxide at a ratio of 3:1 (Oremland *et al.*, 1995), while $CO_2$ will be the only product formed under conditions of sulfate reduction or of respiration of other anaerobic electron acceptors (Oremland *et al.*, 1995). Nevertheless, the knowledge about oxidative demethylation is limited and it is not certain in what form and by what mechanism methylmercury is taken up (Drott *et al.*, 2008a). Further studies on pore water speciation of methylmercury must be performed.

*iv. Environmental Factors that Affect Mercury Biotic Demethylation*

Regarding the microbial communities found in environment, they seem to be quite well adapted to mercury toxicity. Schaefer *et al.* (2004) during the study of microbial adaptation to mercury in two natural waters collected in New Jersey, one water highly mercury contaminated and other much less contaminated, observed that the microbial community found in the most contaminated site was well adapted to mercury toxicity as indicated by the enrichment of Hg(II)-resistant bacteria ($2\text{-}4 \times 10^3$ Hg(II) resistant CFU/ml versus < 80 Hg(II) resistant CFU/ml determined in the less contaminated site), as well as by the presence and expression of *mer*A genes in the microbial biomass.

Some environmental factors seem to play an important role in controlling the magnitude and the pathway of methylmercury degradation (Marvin-Dipasquale and Oremland, 1998; Marvin-Dipasquale *et al.*, 2000) as they influence the microorganism's activity. As expected, the microorganism's activity, for example, increases with temperature. In several studies it has been stated that demethylation of methylmercury in sediments generally increases in summer (Hines *et al.*, 2006).

In relation to nitrate, for example, Marvin-Dipasquale and Oremland (1998) when performing incubations of anaerobic sediments with [$^{14}$C]MeHg observed that nitrate addition did not stimulate methylmercury degradation, indicating that nitrate-respiring bacteria were not directly involved in this degradation process; however, an increase in $^{14}CO_2/^{14}CH_4$ ratio in

some of the sediments was observed, suggesting nitrate inhibition on methanogenesis and possibly on the activity of sulfate reducers (Marvin-Dipasquale and Oremland, 1998). On contrary, addition of sulfate both increased total methylmercury degradation and increased $^{14}CO_2/^{14}CH_4$ ratios to values > 1 at all sites sampled. Similar results were reported by Marvin-Dipasquale *et al.* (2000). Thus, $CO_2$ production from methylmercury degradation seems to be enhanced under sulfate-reducing conditions and suggests that sulfate reducing bacteria oxidize the methyl group of methylmercury entirely to $CO_2$ (Eq. 2), in a similar way that they oxidize acetate (Eq. 3) (Marvin-Dipasquale and Oremland, 1998):

$$SO_4^{2-} + CH_3COO^- + 3\ H^+ \rightarrow H_2S + 2\ CO_2 + 2\ H_2O \quad \text{(Eq. 3)}$$

On contrary, phosphate seems not to have any effect on methylmercury degradation (Marvin-Dipasquale and Oremland, 1998).

Organic matter might play also an important role on the methylmercury degradation, in the same way as mentioned before in mercury methylation. On one hand, dissolved organic carbon might increase methylmercury demethylation by stimulating the demethylators activity. On the other, methylmercury-organic complex formation may be occurring, thereby decreasing methylmercury availability to bacteria (Marvin-Dipasquale *et al.*, 2000).

Methylmercury concentration can also influence the type of bacterial community present. In some sediments an increase of $^{14}CO_2/^{14}CH_4$ ratios with methylmercury concentration has been observed (Marvin-Dipasquale and Oremland, 1998). This fact may reflect a shift from methanogen-dominated demethylation at the low concentrations to sulfate reducers-dominated demethylation at higher concentrations (Marvin-Dipasquale and Oremland, 1998); however, Marvin-Dipasquale and Oremland (1998) stated that above ~800 ng of methylmercury /(g of dry sediment), the $^{14}CO_2/^{14}CH_4$ ratio remained constant, suggesting that the individual contributions of both groups to total methylmercury degradation did not varied.

In relation to sediment depth, some shifts on demethylation processes might also occur due probably again to changes in the bacterial community present. Hines *et al.* (2000), for example, when studying methylmercury demethylation in sediments in the Gulf of Trieste observed that methylmercury was mainly demethylated oxidatively with carbon dioxide as the primary carbon end product, indicative of the action of sulfate reducers, following Eq. 1; however, the percentage of carbon recovered as methane increased with depth, probably due to the enhancement in methanogens activity (Eq. 2).

Regarding the two distinct vias, oxidative and reductive, involved in the methylmercury degradation, the recent use of isotopic labelled methylmercury in determinations of demethylation rates allowed to find out the total mercury concentration is an important parameter that promotes shifts on these processes. In severely mercury contaminated environments, with total mercury concentrations in sediments around 22 to 106 nmol/g, the reductive methylmercury degradation via the *mer* operon seems to dominate, while in less contaminated environments (total mercury concentrations determined in sediments of 0.01-63 nmol/g) the oxidative demethylation is the main process (Marvin-Dipasquale *et al.*, 2000). Similar results were also reported by Schaefer *et al.* (2004) after determining the $^{14}C$-MeHg demethylation in two natural waters collected in New Jersey. The MerB-mediated reductive demethylation was the dominant process in the most contaminated sample whereas oxidative demethylation process was predominant in the less contaminated water (Schaefer *et al.*, 2004). All of these results indicate that reductive demethylation pathway seems to be

triggered when Hg and/or methylmercury contents surpass a determined value; however, it still remains uncertain which threshold of Hg and/or methylmercury concentrations is required for reductive *mer*-mediated demethylation to dominate.

Sediment redox potential and sometimes its conjunction with mercury concentration are important parameters that also control the mercury demethylation processes (Marvin-Dipasquale *et al.*, 2000; Schaefer *et al.*, 2002; Rodríguez Martín-Doimeadios *et al.*, 2004). The reductive pathway seems to dominate in aerobic incubations or under anaerobic incubations of highly contaminated sediments (Marvin-Dipasquale *et al.*, 2000; Schaefer *et al.*, 2002). Similar results were reported by Hines *et al.* (2006) for Gulf of Trieste sediments. These authors verified that demethylation was restricted to the oxidative pathway as evidenced by the production of $CO_2$, being only observed one exception in winter. In this occasion, the sediment tended to harbour a deeper oxidizing region at the surface, since oxygen consumption was slow, becoming more oxidizing and inducing the occurrence of the reductive demethylation pathway. So that, in the surficial sediments of Gulf of Trieste an increased contribution of reductive demethylation in winter was observed when oxidizing conditions penetrated further into the sediment (Hines *et al.*, 2006).

Another important aspect that must be referred is the inverse relationship that sometimes is observed between the proportion of total mercury present as methylmercury and the concentration of total mercury, known as "mercury accumulation paradox" (Schaefer *et al.*, 2004). This phenomenon might be due to the higher number of mercury resistant bacteria found in these mercury contaminated environments, to the existence and expression of *mer* genes in those organisms and to the occurrence of MerB-mediated reductive demethylation. All these factors will lead to a decrease in methylmercury concentration, even high total mercury concentrations are found. Nevertheless, alternative explanations for these results exist, such as enhanced methylation in less contaminated sites due, for example, to the water acidification that can stimulates Hg(II) transport into bacterial cells. This will lead to an increase on methylation rates and so higher methylmercury concentrations may be found in less mercury contaminated environments.

Moreover, the reductive and oxidative methylmercury degradation processes should be regard with great care. The effect of Mer-B mediated methylmercury degradation and the immediate reduction of Hg(II) to $Hg^0$ by MerA, corresponds to a net loss of mercury from the system via the volatilization of $Hg^0$ (Schaefer *et al.*, 2004). On the other hand, the end product of the oxidative demethylation is thought to be Hg(II) that is the substrate for methylation. In spite of this, methylmercury might be produced and its concentration increases, resulting in higher toxicity.

In order to evaluate the relative importance of biotic processes versus photochemical degradation of methylmercury in a given ecosystem, mercury isotope studies seem to be very promising. The detailed study performed recently by Kritee *et al.* (2009) provided evidence that the evaluation of mass dependent and independent fractionations, MDF and MIF, respectively, will allow to differentiate between microbial and abiotic mercury transformation pathways (Kritee *et al.*, 2009). For example, the extent of MDF evaluated by the ratio of $\alpha_{202}/\alpha_{198}$, will be different if microbial or abiotic mercury transformation pathways are occurring, being equal to 1.0004 and 1.0016, respectively (Kritee *et al.*, 2009). In relation to MIF, if microbial mercury transformations are occurring, MIF will not be observed (Kritee *et al.*, 2009). On contrary, if photochemical transformations exist, MIF will occur.

## 3. CONCLUSIONS

The methylation/demethylation processes that occur in the aquatic environments establish a methylmercury pool continually available for bioaccumulation. This is of great concern as methylmercury is one of the most toxic mercury specie. Both processes - mercury methylation and methylmercury demethylation - can involve abiotic or biological processes, the last one involving the action of microorganisms. Generally, the biological processes are more significant; however, sometimes the abiotic processes might have also an important role. In fact, the relative importance of mercury abiotic methylation is controversial. Some authors emphasize that the abiotic pathway appears to play a minor role in natural environments whereas others suggest that the biotic processes can not account for all the methylmercury formed naturally.

In relation to the biological processes, methylmercury demethylation appears to be a process that is more widely spread across the microbial genera in comparison to mercury methylation. Sulfate-reducing bacteria are considered to be the most important methylators present in the aquatic ecosystems. Moreover, these bacteria are able to donor a methyl group by the C-3 of serine (which is a compound formed during the carbon flow from pyruvate) or by formate via the acetyl-CoA synthase pathway.

The photolytic decomposition of methylmercury remains the only abiotic demethylation mechanism that is significant in surface waters exposed to sunlight. However, the overall impact on the aquatic mercury cycle is still unclear and the end products of the methylmercury degradation have not been clearly identified yet. In sediments, the abiotic mechanism is also more conductive to the environmental methylmercury decomposition than the biotic one.

Regarding the biotic methylmercury demethylation, two distinct vias - oxidative and reductive - might be used by microorganisms. The former is mainly conducted by sulfate reducers and methanogens. In this process, methylmercury is primarily converted to $CO_2$ and inorganic mercury; however, sometimes methane is also formed. The reductive process might occur through two pathways, one involving the *mer* operon and other does not. Nevertheless, the process involving the *mer* operon is the most studied and it is considered the most common pathway. In this process, two enzymes participate - the MerB (organomercurial lyase) and the MerA (mercuric reductase) - and so methylmercury will be converted to elemental mercury. In spite of this, the mentioned process represents a net loss of mercury ($Hg^0$) from the system, while the oxidative process might be only a source of substrate to mercury methylation due to the formation of Hg(II). Thus, if oxidative demethylation is not associated with a subsequent Hg(II) reduction, this has major implications in natural systems where oxidative demethylation dominates. In fact, this mercury can be remethylated to methylmercury if conditions are appropriate. Oxidative demethylation is presumed to result in Hg(II) as an end product, so no net elimination of Hg(II) takes place in this process, in contrast to the reductive demethylation.

The *mer* operon shows that the genetic patrimony of the microbial community is very important and affects significantly the methylmercury presence in the aquatic environments. In fact, there are bacteria that only transcribe MerA (narrow-spectrum resistance) and so they are only able to reduce Hg(II) to $Hg^0$, but there are others that have MerB and MerA (broad-spectrum resistance) and the latter are able to decompose methylmercury to $Hg^0$. Both

microbial mercury resistances might assume nowadays an important role, for example in the remediation of mercury contaminated environments. Therefore, the identification of mercury resistant strains is essential to the development of technological mercury bioremediation strategies.

Moreover, *mer* operon is present in various taxonomic groups and microbiocenoses, and *mer* genes are localized essentially in DNA mobile elements, promoting the horizontal transfer. It has been demonstrated that the *mer* operon is associated with multidrug-resistance and so the frequency study of this operon in the microorganisms present in the environment will give valuable data to be used in the prediction of the drug resistance evolution.

In terms of the action way of the *mer* operon, more research work is needed in order to better understand how the mercuric ion transporters work and the interaction between all *mer* products, as well as the role of different mercury species in induction of *mer* operon and its own regulation.

In this work, it was also stated that the mercury methylation and methylmercury degradation via abiotic and biotic pathways are affected by several environmental factors that influence inorganic mercury/methylmercury availability, as well as the activity of methylators/demethylators. These relationships are often very complex. Furthermore, these environmental factors might also change the microbial communities present, leading to shifts, for example in the biotic processes involved in methylmercury demethylation. As discussed in the present work, total mercury and the redox potential are factors likely to induce these changes. So, for example, the reductive demethylation pathway seems to dominate in both anoxic severely mercury contaminated and aerobic environments. Nevertheless, further works are needed to be performed in order to get more knowledge on these aspects.

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