SEASONAL CHANGES IN MERCURY SPECIATION AND THE COMPOSITION OF THE MICROBIAL COMMUNITY IN THE SEAWATER IN THE CENTRAL ADRIATIC SEA

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Doctoral Dissertation
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Doctoral Dissertation

SEZONSKE SPREMEMBE SPECIACIJE ŽIVEGA SREBRA IN SESTAVE MIKROBNIH ZDRUŽB V MORSKI VODI V SREDNJEM JADRANSKEM MORJU

Doktorska disertacija

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To my beloved family and friends

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"*L'art de conclure d'après des expériences et des observations consiste à évaluer des probabilités, et à estimer si elles sont assez grandes ou assez multipliées pour constituer des preuves.*" — Lavoisier

Abstract

Mercury (Hg) is one of the contaminants of most concern due to its high toxicity and accumulative behavior in biota. Elevated Hg concentrations can exceed maximal allowed levels in top predator fish, in which Hg is mostly present in its most toxic form monomethylmercury (MMHg). However, the factors controlling its prevalence in fish are largely unknown.

Significant differences in Hg biogeochemistry exist between the coastal and open sea. In coastal waters methylated mercury (MeHg) is primarily produced in coastal sediments, while in open ocean MeHg is formed in a water column. The extent to which MeHg produced in coastal sediments and open waters is accumulated in fish is not well understood.

An overview of the previous research on Hg abundance, distribution and speciation in environmental compartments in the Adriatic Sea revealed that Hg concentrations in seawater are decreased during past decades due to the closure of industries that released Hg to the coastal environments. A decreasing gradient in Hg concentrations from the northern to the southern Adriatic indicates that the presence of legacy mercury still contributes to Hg in seawater. Concentrations of Hg in plankton and mussels follow the spatial and temporal trends observed in water, however, concentrations in fish do not follow these trends indicating the complexity of MeHg formation and accumulation in fish.

The main objective of this work was to better understand the interaction between Hg species and the abundance and composition of microbial community in the central Adriatic Sea, more precisely in the transect from Hg contaminated coastal environment in Kaštela Bay to the Island of Vis. Monthly sampling was performed over a two-year period in collaboration with the Institute of Oceanography and Fisheries in Split and the research vessel *Bios Dva*. Mercury analysis included total mercury (THg), dissolved gaseous mercury (DGM), and MeHg. Microbial structure analysis included high and low nucleic acid bacteria, nanoflagellates, picoeukaryoes, *Prochlorococcus*, and *Synechococcus*. Physico-chemical properties of seawater were also measured.

One of the obstacles to study MeHg dynamics in open waters are its very low concentrations, usually present in the femtomolar range, which represent an analytical challenge. Therefore, the first objective of the work was to develop and validate a method based on MeHg-hydride generation. The selected method was fit for purpose due to low reagent blanks and quantitative recoveries from seawater. Method's limit of detection was 3.94–15.9 fmol L^{-1} and the expanded uncertainty of the method 11.1–21.3%.

The results revealed that Hg methylation in seawater is possibly a metabolism-dependent transformation promoted by the heterotrophic activity of low nucleic acid bacteria under the absence of probable phosphorus limitation ($^{NO-P}$P-limitation). Under these conditions, MeHg was also related with the concentration of nutrients and the abundance of *Prochlorococcus*, whose decay promotes heterotrophic activity. DGM was related with the abundance of heterotrophic bacteria, autotrophic picoplankton and chlorophyll *a* concentration, indicating biotic DGM production that is possibly not metabolically dependent. MeHg biomagnification from microseston to mesozooplankton

was observed through the significant increase of corresponding bioaccumulation factors. Results revealed that Hg uptake by plankton might be enhanced under $^{NO-P}$P-limitation conditions.

Povzetek

Živo srebro (Hg) je zaradi toksičnosti in kopičenja v živih organizmih eden izmed najbolj zaskrbljujočih onesnaževalcev. Koncentracije Hg lahko presežejo najvišje dovoljene vrednosti v plenilskih ribah, v katerih je Hg prisoten večinoma v svoji najbolj toksični obliki kot monometilno živo srebro (MMHg). Faktorji, ki vplivajo na njegovo razširjenost v ribah, so večinoma nepoznani.

V biogeokemiji Hg med obalnim in odprtim morjem obstajajo pomembne razlike. V obalnem morju metilno živo srebro (MeHg) primarno izvira iz obalnih sedimentov, medtem ko se v odprtem morju tvori v vodnem stolpcu. Obseg kopičenja MeHg, nastalega v obalnih sedimentih in v odprtem morju, v ribah ni dobro pojasnjen.

Pregled preteklih raziskav je pokazal, da količina, distribucija in speciacija Hg v Jadranskem morju v preteklih desetletjih pada, predvsem zaradi zaprtja industrij, ki so v obalno okolje sproščale Hg. Od severa proti jugu Jadranskega morja opazimo padajoči gradient koncentracije Hg, kar kaže na to, da Hg iz preteklosti še vedno prispeva k Hg v morski vodi. Koncentracije Hg v planktonu in školjkah sledijo prostorskim in časovnim trendom v vodi, medtem ko v ribah teh trendov ne zaznamo, kar kaže kompleksnost nastanka MeHg in njegovo kopičenje v ribah.

Glavni namen tega dela je bolje razumeti interakcijo med različnimi Hg zvrstmi in sestavo mikrobne združbe v centralnem Jadranskem morju, bolj natančno na transektu med Hg onesnaženim Kaštelskim zalivom in otokom Visom. V sodelovanju z Inštitutom za oceanografijo in ribištvo v Splitu ter raziskovalno ladjo *Bios Dva* smo dve leti izvajali mesečna vzorčenja. Analiza živega srebra je vključevala meritve celokupnega živega srebra (THg), raztopljenega plinastega živega srebra (DGM) in MeHg. Mikrobiološke analize so vključevale bakterije z visoko in nizko vsebnostjo nukleinskih kislin, nanoflagelate, pikoevkarionte, *Prochlorococcus* in *Synechococcus*. Morski vodi smo izmerili tudi fizikalno-kemijske lastnosti.

Med preučevanjem dinamike MeHg so se pojavile ovire, saj se v odprtih vodah koncentracije gibljejo v femtomolarnem območju, kar predstavlja velik analitski izziv. Prav zaradi tega je bil prvi namen razviti in validirati metodo, ki je osnovana na nastanku kompleksa MeHg-hidrid. Izbrana metoda je za morsko vodo ustrezna zaradi nizkih vrednosti slepih reagenčnih vzorcev in kvantitativnih okrevanj. Meja detekcije je bila 3,94–15,9 fmol L^{-1} in negotovost metode 11,1–21,3 %.

Rezultati kažejo, da je metilacija Hg v morski vodi morda odvisna od metabolizma ter poteka preko heterotrofne aktivnosti bakterij z nizko vsebnostjo nukleinskih kislin v odsotnosti verjetne fosfor-limitacije ($^{NO–P}$P-limitacija). Pod temi pogoji je bil MeHg povezan s koncentracijo nutrientov in številčnostjo *Prochlorococcusa*, propad katerega spodbuja heterotrofno aktivnost. DGM je bil povezan s številčnostjo heterotrofnih bakterij, avtotrofnoga pikoplanktona in koncentracijo klorofila *a*, kar kaže na biotsko produkcijo DGM, ki morda ni metabolno odvisna. Preko signifikantnega povišanja faktorjev bioakumulacije smo opazili biomagnifikacijo MeHg iz mikrosestona v mezozooplankton. Rezultati kažejo, da je morda privzem Hg v plankton povečan pod $^{NO–P}$P-limitirajočimi pogoji.

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Abbreviations

AOU	... apparent oxygen utilization
BAF	... bioaccumulation factor
CV-AAS	... cold vapor atomic absorbance spectrometry
CV-AFS	... cold vapor atomic fluorescence spectrometry
DGM	... dissolved gaseous mercury
DMHg	... dimethylmercury; CH_3HgCH_3
DOC	... dissolved organic carbon
GC	... gas chromatography
GEM	... gaseous elemental mercury
GOM	... gaseous oxidized mercury
Hg	... mercury
Hg^0	... elemental mercury
Hg^{II}	... inorganic mercury(II)
HNF	... heterotrophic nanoflagellate
ISO-GUM	... International Organization for Standardization – Guide to the Expression of Uncertainty in Measurement
k_d	... demethylation rate constant
k_m	... methylation rate constant
k_{pd}	... photodemethylation rate constant
LOD	... limit of detection
MeHg	... total methylated mercury; the sum of monomethylmercury and dimethylmercury
MMHg	... monomethylmercury; CH_3Hg^+
$^{NO-P}$P-limitation	... absence of probable phosphorus limitation
PBM	... particulate bound mercury
POC	... particulate organic carbon
^{P}P-limitation	... probable phosphorus limitation
RM	... reactive mercury
THg	... total mercury; the sum of all mercury species present
$U_{ex,r}$	... expanded relative combined standard uncertainty

Chapter 1

Introduction

Mercury (Hg) pollution poses global human health and environmental risks. Although Hg is naturally present in the environment, human activities have increased the amount of Hg cycling among the land, atmosphere, and ocean by a factor of three to five. Emitted to the atmosphere in its elemental form, Hg travels worldwide before oxidizing to a form that readily deposits to ecosystems (Figure 1.1). In aquatic systems, Hg can convert to monomethylmercury (MMHg), a potent neurotoxin. Humans and wildlife are exposed to mercury as it bioaccumulates up the food chain. Hg continues to circulate in the atmosphere, oceans, and terrestrial system for centuries before it is buried in deep-ocean sediments. Areas of uncertainty in the global biogeochemical cycle of Hg include oxidation processes in the atmosphere, land-atmosphere and ocean-atmosphere cycling, and methylation processes in the ocean. National and international policies have addressed direct Hg emissions, but further efforts to reduce risks face numerous political and technical challenges (UNEP, 2013).

With the signing of the Minamata Convention in 2013, the world's governments have accepted that Hg is toxic and of global relevance; scientific needs will therefore shift towards best implementation practices of the Convention (UN, 2013). Because the majority of Hg emissions emanate from the energy and industrial sectors, governments will have to balance economic and environmental interests. Biogeochemical Hg cycling is complex and superimposed on the strongly perturbed inorganic Hg cycle and natural process of biomethylation that generates methylated mercury (MeHg)[a]. Today, comprehensive multimedia models of the biogeochemical Hg cycle are being developed that capture this complexity to try to evaluate the effectiveness of environmental policy scenarios. For these models to work, they must include realistic descriptions of the Hg transformations and fluxes that occur within and between the Earth's environmental compartments. Despite decades of mercury science, we still lack answers to the most basic questions concerning the fundamental Hg transformations and fluxes (Figure 1.1).

In this complex mosaic of fundamental Hg transformations in the environment, this dissertation focused on processes occurring in the coastal and open seawaters (indicated with red square in Figure 1.1). In the Mediterranean Sea, a substantial amount of research was performed to elucidate the nature of Hg transformations and transport mechanisms between sea and other environmental compartments. These researches have extensively addressed issues of mercury reduction and methylation in the Mediterranean waters (Cossa, Durrieu de Madron, Schäfer, Lanceleur, et al., 2017; Kotnik et al., 2017), and Hg transport between water, sediment and atmosphere (Nerentorp Mastromonaco, Gårdfeldt, & Wängberg, 2017; Ogrinc et al., 2007).

[a] The sum of monomethylmercury and dimethylmercury. Refer to Section 1.1.1 for more details.

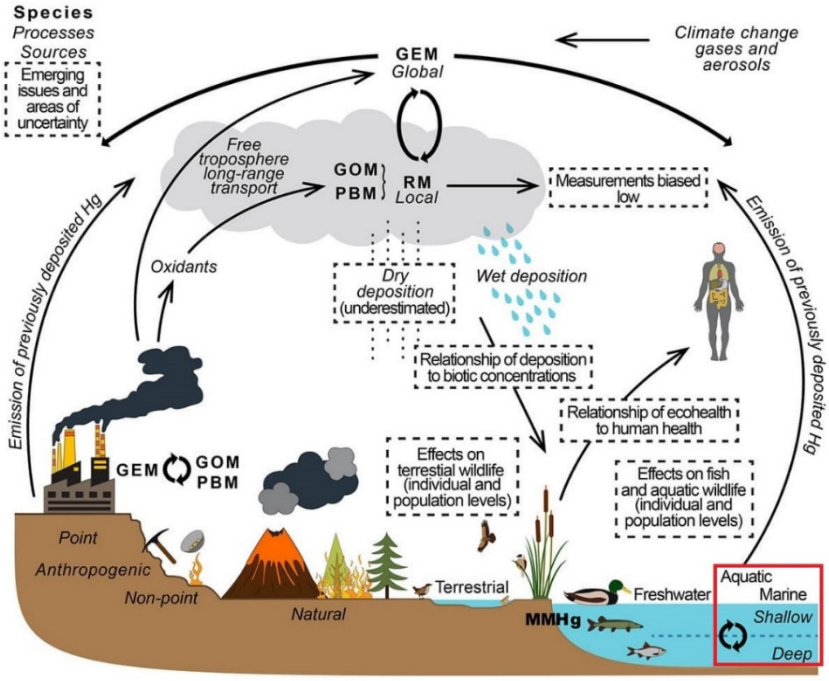


Figure 1.1: Global biogeochemical mercury cycle with the identification of major uncertainties in current understanding (GOM – gaseous oxidized mercury, GEM – gaseous elemental mercury, PBM – particulate bound mercury, RM – reactive mercury, MMHg – monomethylmercury). Modified from Gustin et al. (2016). The focus of the dissertation is displayed with a red square.

The majority of research about mercury distribution and speciation in the Adriatic Sea was performed in the coastal waters of its northern part, where direct mercury methylation has not been observed in the water column, but only in sediments (Hines et al., 2012; Hines, Covelli, Faganeli, & Horvat, 2017). Systematic Hg measurements in the Adriatic are mostly limited to coastal environments, while only few researches were performed in the open Adriatic providing scatter evidences of mercury (bio)transformations. The overviews of these researches in the Adriatic waters, sediments and biota are presented in our review articles (Živković, Kotnik, Šolić, & Horvat, 2017; Živković, Šolić, Kotnik, Žižek, & Horvat, 2017) to assess the extent of mercury contamination, transformation and (bio)accumulation in these compartments, and to compare these results with those from the Mediterranean Sea. In addition, we wanted to investigate whether changes in natural and anthropogenic Hg loadings during the past few decades caused changes in Hg abundance in the Adriatic biota. This research is presented in the dissertation through two review papers (Živković, Kotnik, et al., 2017; Živković, Šolić, et al., 2017) (Sections 3.1 and 3.2).

Our review articles (Živković, Kotnik, et al., 2017; Živković, Šolić, et al., 2017) revealed that the relations between Hg and corresponding (micro)biological transformations have never been clearly demonstrated in the open Adriatic water column, as they were in the Mediterranean Sea (Heimbürger et al., 2010). Researches have shown that microorganisms play an important role in mercury methylation (Barkay & Wagner-Döbler, 2005; Parks et al., 2013). Biological processes at the lowest marine trophic levels are probably connected to Hg methylation in the Adriatic waters, as observed in the western Mediterranean Sea (Heimbürger et al., 2010).

Even though the highest THg concentrations in the Adriatic are found in its northern part, MeHg shows a different spatial distribution. The highest concentrations of methylated mercury species are observed in the southern Adriatic Sea, more precisely, near the south Adriatic Pit (Figure 1.2). These elevated MeHg concentrations in the southern Adriatic during summer 2005 were explained by unknown biological and chemical processes, benthic source, and low MeHg removal from the water column (Kotnik et al., 2015). The lack of exact identification of biological and chemical processes that drive the production of these elevated MeHg concentrations in the southern Adriatic Sea influenced the experimental design of our research.

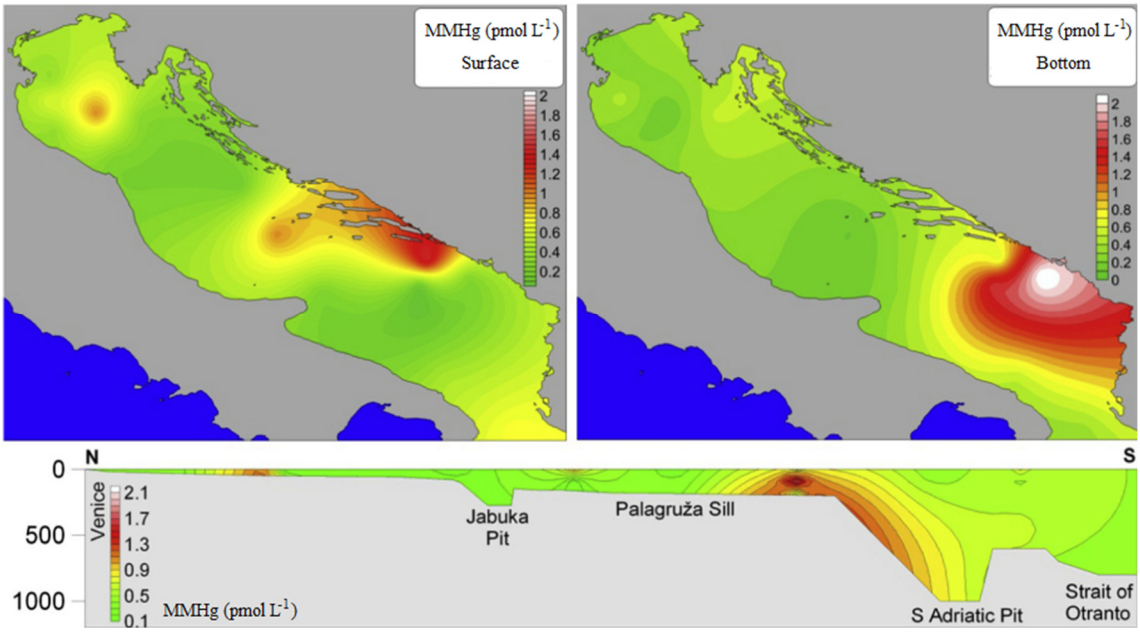


Figure 1.2: Spatial and vertical distribution of monomethylmercury in surface and bottom water layers of the Adriatic Sea during summer 2005. Figure and caption adapted from Kotnik et al. (2015).

In this dissertation, we wanted to determine detailed spatial and seasonal changes in concentrations of Hg fractions, especially MeHg (total methylated mercury), during a two-year period in the transect between Hg contaminated coastal environments (Kaštela Bay) and the open sea (the Island of Vis) (Figure 1.3). As Heimbürger et al. (2010) demonstrated the influence of microorganisms on mercury methylation in the western Mediterranean Sea, we wanted to determine whether this relation exists also in the Adriatic Sea and thus might explains the observed elevated MeHg concentrations in the south Adriatic Pit. Therefore, we created detailed spatial and seasonal profiles of heterotrophic bacteria, autotrophic picoplankton and heterotrophic nanoflagellates in the Central Adriatic Sea. The expertise of the researchers from the Institute of Oceanography and Fisheries in Split was of great importance for this work. Their experience in identification and monitoring of microbial community components is well established (e.g. Ordulj, Krstulović, Šantić, Jozić, & Šolić, 2014; Šantić, Krstulović, Šolić, & Kušpilić, 2011; Šestanović, Šolić, Krstulović, & Ninčević, 2004; Šolić et al., 2010, 2015, 2016, 2017), which helped us create accurate and detailed spatial and seasonal profiles of mercury fractions and microbial community components.

Therefore, the main goal of this dissertation was to determine the main Hg biogeochemical transformations in the coastal and open Adriatic Sea (Figure 1.3), and whether microbial community composition and environmental parameters affect changes in Hg dynamics and speciation. Our intentions were to demonstrate how biological and

environmental parameters could affect Hg methylation and reduction in water column. In addition, we wated to determine whether the composition of microbial food web (abundances of high and low nucleic acid bacteria, nanoflagellates, picoeukaryoes, *Prochlorococcus*, and *Synechococcus*) correlates with the concentrations of mercury species/fractions within the water column. Our results are presented in paper "Relations Between Mercury Fractions and Microbial Community Components in Seawater Under the Presence and Absence of Probable Phosphorus Limitation Conditions" (Živković et al., 2018) (Section 3.4).



Figure 1.3: Sampling area in the Adriatic Sea indicated with a red square (a). Sampling site locations (red pins) mapped using Google Earth Pro 7.1 (b). Yellow pin indicates the location of the former chlor-alkali plant (CAP).

To establish mercury interactions with marine biota we needed accurate analytical methods. Current methods for the determination of low concentrations of total methylated mercury (MeHg; the sum of monomethylmercury and dimethylmercury) in seawater are not accurate, precise or reliable enough. Therefore, in this dissertation we also optimized the method for the determination of low MeHg concentrations and estimated method's uncertainty. These results are presented in paper "Optimization and Measurement Uncertainty Estimation of Hydride Generation–Cryogenic Trapping–Gas Chromatography–Cold Vapor Atomic Fluorescence Spectrometry for the Determination of Methylmercury in Seawater" (Živković, Fajon, Tulasi, Obu Vazner, & Horvat, 2017) (Section 3.3).

This dissertation is therefore organized in the following order. First, the results from our review articles about Hg abundances in the Adriatic seawater, sediments, and biota are presented in Sections 3.1 and 3.2. Optimization and validation of the method for the reliable determination of low MeHg concentrations in seawater (Section 3.3) was used instead of section Materials and Methods. At the end, we presented results of our two-year sampling campaign about seasonal changes of total mercury (THg), dissolved gaseous mercury (DGM) and MeHg, and the abundance and composition of microbial community, which were used for the evaluation of possible existence of microbial mercury transformations (Section 3.4).

1.1 Mercury Cycling in the Environment

The pathways and fate of mercury in the environment are important because in atmosphere, waters, sediments and wetland soils mercury is subject to various chemical and biological processes, changing its speciation, partitioning, mobility and bioavailability. Recent research shows that different mercury species can be found in different environmental compartments (Guédron et al., 2014; Hammerschmidt & Bowman, 2012; Ogrinc et al., 2007; Pirrone & Mason, 2009; Rimondi, Gray, Costagliola, Vaselli, & Lattanzi, 2012; Schartup, Mason, Balcom, Hollweg, & Chen, 2012; Sprovieri, Hedgecock, & Pirrone, 2010; Sprovieri & Pirrone, 2008).

The greatest problem represents the fact that global mercury transport causes elevated Hg concentrations in areas far away from its sources (Baya, Gosselin, Lehnherr, St.Louis, & Hintelmann, 2015; Bratkič et al., 2016; Cossa et al., 2011; Lehnherr, 2014; Nerentorp Mastromonaco, Gårdfeldt, Assmann, et al., 2017; Schartup, Balcom, et al., 2015; Soerensen, Jacob, et al., 2016). Due to transformation and transport processes, mercury can travel between environmental compartments: from atmosphere to water, soil and biota (Figure 1.4).

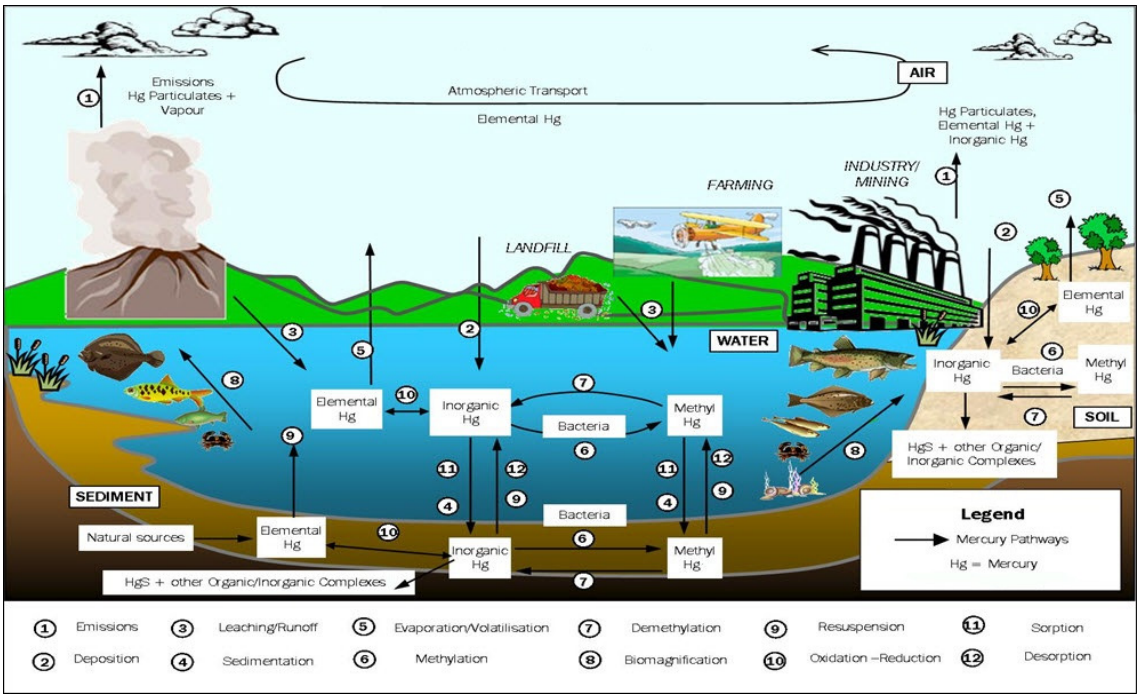


Figure 1.4: Simplified mercury cycle in the environment. Figure adapted from Chrystall & Rumsby (2009).

Mercury cycle can modify natural and anthropogenic Hg inputs in the environment through its transfer across long distances and across different compartments. The atmosphere is the most important medium by which Hg is transported around the globe (Ariya et al., 2015; Mason et al., 2017; Sprovieri et al., 2010). Elemental mercury (Hg^0) is the dominant mercury species in the atmosphere (Gaffney & Marley, 2014), where it has a long residence time (0.8–1.7 years) (Ariya et al., 2015; Driscoll, Mason, Chan, Jacob, & Pirrone, 2013; Pirrone et al., 2010). This represents a major problem because during this prolonged residence time in the atmosphere, Hg^0 can be transported over a large geographical distance, and exchanged with land and surface waters (Mason et al., 2017).

Therefore, mercury contamination must be considered a global environmental problem (UN, 2013).

1.1.1 Mercury cycling in the marine environment

The oceans actively and significantly participate in the transport and transformation of Hg. Therefore, understanding biogeochemical cycling of this toxic element and the impact of anthropogenic inputs in the marine environment are of great importance. Hg cycling in the ocean waters has received scarce attention with almost no long-term, seasonally oriented ocean studies. Even in the near-shore environments, the biogeochemistry of Hg is understudied; estuaries and coastal waters are areas of special interest, due to high biological productivity and fishery activity. Hg cycling in the marine environment is insufficiently studied and undersampled, given the importance of the oceans as a whole in the cycling of elements, and international concerns regarding human exposure to Hg through marine fish consumption (Fitzgerald & Lamborg, 2007).

Mercury that enters marine environments can continue to actively participate in aquatic chemistry, while most of Hg deposited from the atmosphere to terrestrial systems is sequestered. Hg transformations in the marine environments are mutually interlinked and form a web of connected biogeochemical processes (Figure 1.4), resulting in complex marine Hg cycle (UNEP, 2013).

Most marine waters are (super)saturated with elemental mercury with respect to atmospheric Hg^0 concentrations (Gonzalez-Raymat et al., 2017; Kotnik et al., 2017; Mason et al., 2017). Together with dimethylmercury (DMHg), Hg^0 represents the major component of DGM, whose air-sea exchange is a crucial part of the global mercury cycle (Cossa, Durrieu de Madron, Schäfer, Guédron, et al., 2017; Kotnik et al., 2017; Mason et al., 2017; Nerentorp Mastromonaco, Gårdfeldt, & Wängberg, 2017). Through evasion, the ocean prolongs the residence of mercury at the Earth's surface and intensifies the human perturbation of the global mercury cycle (Fitzgerald & Lamborg, 2007).

Even though the mercury emitted from oceanic surfaces is mostly in its elemental form, the majority of Hg deposited to seawater is oxidized mercury (Ariya et al., 2015; Heimbürger, Migon, & Cossa, 2011; Sprovieri et al., 2010). Hg^{II} compounds are easily removed by wet deposition due to higher solubility and have atmospheric lifetimes of days to weeks (Ariya et al., 2015), which limits its long-distance transport. Therefore, the direct deposition from the atmosphere is the dominant pathway by which mercury reaches the oceans. The exceptions are smaller, semi-enclosed basins where river runoff, coastal erosion, and ocean currents account for about half of mercury inputs (UNEP, 2013).

Once deposited to the sea, mercury can bound to particulate matter, which facilitates its downward flux to sea bottom and burying in marine sediments (Lamborg, Hammerschmidt, & Bowman, 2016). A greater affinity of Hg to particles could facilitate faster removal from the oceanic water column and lower concentrations in seawater (Merritt & Amirbahman, 2009). The component of marine particulate matter that mostly influences the adsorption of Hg is organic matter, suggesting that Hg scavenging and associated particle cycling may be region- and time-dependent as the dominant materials in marine particulate matter change due to local biogeochemistry and ecosystem structure (Lamborg et al., 2016). Sinking particles are also locations of enhanced microbial activity and consequently, Hg transformations. Therefore, deep water formation and sinking fluxes represent the largest vectors of different Hg species to deep ocean and the sediments (Cossa et al., 2018; Lamborg et al., 2016).

Settling of mercury represents another major problem, as deposited mercury can be transformed in deep waters and marine sediments to toxic organic mercury species, monomethylmercury (CH_3Hg^+, MMHg) and dimethylmercury (CH_3HgCH_3, DMHg)

(Barkay, Kroer, & Poulain, 2011; Barkay & Wagner-Döbler, 2005; Heimbürger et al., 2010; Lehnherr, St. Louis, Hintelmann, & Kirk, 2011; Mason et al., 2012; Selin, 2009).

Some methods for the determination of total methylated mercury (MeHg; the sum of monomethylmercury and dimethylmercury) presented in this dissertation do not distinguish these two species. In addition, DMHg concentrations in surface waters are mostly below the limit of detection. Therefore, we will use only term MeHg in the dissertation to avoid confusion. In general, MeHg concentrations correspond to MMHg concentrations, except when referring to deep ocean waters where elevated DMHg concentrations can be found.

In the marine sediments, Hg methylation is connected with the activity of sulfate-reducing bacteria (Barkay & Wagner-Döbler, 2005; Compeau & Bartha, 1987). MeHg production by iron-reducing bacteria and methanogens has also been observed (Hsu-Kim, Kucharzyk, Zhang, & Deshusses, 2013). On the contrary, Hg methylation in the open ocean takes place largely at intermediate depths and is linked to organic carbon remineralization (Cossa, Averty, & Pirrone, 2009), chlorophyll maximum (Lehnherr et al., 2011), and oxygen-deficient zones (Cossa, Durrieu de Madron, Schäfer, Lanceleur, et al., 2017; Heimbürger et al., 2010). Photochemical or microbial demethylation is the major removal process for MeHg in the ocean (Monperrus, Tessier, Amouroux, et al., 2007).

MeHg shows strong bioaccumulation (incorporation in organisms by uptake from water) and biomagnification properties (trophic transfer along food chain) (Fitzgerald, Lamborg, & Hammerschmidt, 2007; UNEP, 2013). Among mercury fractions, inorganic Hg^{II} and especially MeHg are readily sequestered by the first trophic level. However, higher trophic levels seem to sequester only organic mercury. In the marine food chain MeHg is bioaccumulated and biomagnified with the highest levels found in predatory fish (Fitzgerald et al., 2007; Lamborg, Bowman, et al., 2014; Mason et al., 2012; Selin, 2009). However, the initial bioconcentration of MeHg by phytoplankton represents the biggest single contribution to bioaccumulation in marine food webs (Mason et al., 2012). It is important to note that dimethylmercury does not bioaccumulate and can be found only in deeper ocean waters due to its (photo)demethylation in the air and surface waters (Lehnherr, 2014).

Sedimentary processes can play an important role that controls Hg release to overlying marine waters (Covelli et al., 2008; Hammerschmidt & Fitzgerald, 2008), especially to those depleted in oxygen (Covelli et al., 2008; Mason et al., 1999). Sediments can therefore represent a source of MeHg to the overlaying waters, which represents a significant problem in shallow estuaries and coastal areas, such as the northern Adriatic Sea (Hines et al., 2012, 2017). In addition, oligotrophic central and southern Adriatic are known for their intensive production of MeHg in the water column (Kotnik et al., 2015) whose transport to open sea can have a tremendous influence on biota.

Based on the overview of literature, we summarized the current knowledge presented above about mercury cycling, transport and transformations in the Adriatic, and implemented it in a review paper "The Abundance, Distribution and Speciation of Mercury in Waters and Sediments of the Adriatic Sea – a Review" (Živković, Kotnik, et al., 2017) (Section 3.1). We wanted to understand the main transport and transformation processes occurring in the Adriatic seawater and sediments, especially in the coastal case studies, and to better comprehend the relations and differences between mercury in the Adriatic and in the Mediterranean. We gathered data from published studies and created corresponding box-and-whiskers plots to obtain a detailed insight on mercury contamination in the Adriatic. This review paper revealed that the highest THg concentrations in the whole Mediterranean Sea can be found in the northern Adriatic Sea; concentration gradients in both waters and sediments can be observed from the northern to the southern Adriatic. In addition, Hg concentrations in seawater decreased during past

decades probably due to the closure of industries that previously released Hg into coastal environments. The spatial distribution of MeHg does not follow that of THg, as the highest MeHg concentrations in the Adriatic seawaters are found in the southern part (Figure 1.2).

From this review paper and from the facts described above, it was evident that a great part of mercury transformations (mainly methylation and reduction) in the Adriatic Sea were still not explained well, especially in the open waters of the central and southern Adriatic. This was a driving motivation for the main research of this dissertation – the determination of major biogeochemical processes in the coastal and open Adriatic Sea.

1.1.2 Understanding mercury bioaccumulation and biomagnification

Understanding factors that control mercury bioaccumulation and biomagnification is of great significance due to its high toxicity. MeHg readily bioaccumulates and biomagnifies along trophic levels (Gosnell, Balcom, Tobias, Gilhooly, & Mason, 2017; Hammerschmidt, Finiguerra, Weller, & Fitzgerald, 2013; Lehnherr, 2014), and therefore presents the most toxic mercury species for humans, through fish consumption.

In a digestive tract, MeHg has almost quantitative absorption and can therefore cause severe adverse effects to human health (Fernandes Azevedo et al., 2012; Park & Zheng, 2012). Consumption of mercury-contaminated fish caused the biggest outbreak in mercury poisoning in Minamata, Japan, during early 1950s (Bank, 2012). Related body disability and impairment became known as Minamata disease/Russell-Hunter syndrome (Fernandes Azevedo et al., 2012; Gochfeld, 2003). Therefore, it is important to understand all factors that lead to elevated MeHg concentrations in aquatic biota.

Microbial mercury methylation is likely to be an intracellular reaction. Thus, transport of inorganic mercury from the microorganism's extracellular surroundings and through the outer and inner membranes is an important step leading to its biomethylation (Hsu-Kim et al., 2013). Current paradigms regarding mercury bioaccumulation are based on observations that MeHg biomagnifies along pelagic food chains. However, exact mechanisms regulating MeHg bioaccumulation in food chain, its initial incorporation at the base of pelagic food chains, and its subsequent trophic transfer remain controversial (Feng et al., 2018). Transport and accumulation of Hg in the food chain start with its adsorption and absorption by phytoplankton, followed by zooplankton and the upper trophic levels through feeding (Feng et al., 2018). Even though phytoplankton represents the entry point of Hg to the higher trophic levels, it has not been extensively investigated (Gosnell & Mason, 2015) due to low environmental MeHg concentrations (Lamborg, Hammerschmidt, et al., 2014).

MeHg bioaccumulation factors (BAF) are about 10^5 for phytoplankton and increase with each following trophic level (Lee & Fisher, 2016). However, the exact mechanisms of MeHg bioaccumulation and biomagnification are still not well understood. Previous researches have reported passive and active diffusion as the most important MeHg bioaccumulation pathways (Chen et al., 2008; Lee & Fisher, 2016; Mason, Reinfelder, & Morel, 1996; Pickhardt & Fisher, 2007), but the exact mechanisms are unknown. Planktonic growth and cell size are reduced in oligotrophic water and inversely related to phytoplankton MeHg concentration (Driscoll et al., 2012). Consequently, a greater BAF for MeHg in phytoplankton can be found in oligotrophic waters (Gosnell et al., 2017; Gosnell & Mason, 2015; Mason et al., 2012).

Even though mercury speciation data in environmental compartments have provided a better understanding of the mercury cycle in the Mediterranean, no definitive answer is provided for the high mercury biomagnification in the Mediterranean fish (Cossa et al., 2012). This is very important, because the Adriatic and Mediterranean fish species usually have higher mercury concentrations compared to the adjacent Atlantic Ocean (Cossa et

al., 2012). Following bioaccumulation in phytoplankton, MeHg is transferred via consumption through the food chain to herbivorous zooplankton, planktivorous fish, and eventually to large predators. As uptake of MeHg from water into invertebrates is small, almost all of the MeHg accumulated by zooplankton originates from diet (Mason et al., 2012). Furthermore, the uptake of MeHg from fish diet accounts for approximately 90% of total uptake, while the remaining part originates from water (Bradley, Barst, & Basu, 2017).

To obtain a better insight in bioaccumulation and biomagnification processes described above, we summarized published data of Hg in the most studied Adriatic biota in a review paper "The Abundance and Speciation of Mercury in the Adriatic Plankton, Bivalves and Fish – a Review" (Živković, Šolić, et al., 2017) (Section 3.2). In this paper, we wanted to determine the extent of Hg bioaccumulation and biomagnification in the Adriatic biota compared to the Mediterranean. We gathered data from published research, created corresponding box-and-whiskers plots, and statistically compared Hg concentrations in the most studied Adriatic biota. This review paper revealed that THg is significantly lower in the Adriatic zooplankton than in the Mediterranean zooplankton. However, as MeHg concentrations in zooplankton are similar in both seas, Adriatic zooplankton has the highest percentage of THg present as MeHg. THg concentrations in the Adriatic mussels are not significantly higher than in those from the Mediterranean. Percentage of THg present as MeHg is similar in mussels from the Mediterranean and the pristine areas of the Adriatic Sea. The most studied fish in the Adriatic and the Mediterranean Sea have similar THg concentrations in both seas. Similarly as in seawater, THg in the Adriatic zooplankton and mussels shows a significant decrease with time. On the contrary, statistical analysis showed no significant differences in fish THg concentrations during the past few decades.

It was obvious from this review paper that specific conditions that promote mercury bioaccumulation and biomagnification in the Adriatic biota are still not well understood. Therefore, the results of this review paper and the knowledge described above guided the experimental design of the main research of this dissertation, i.e. the determination of major biogeochemical processes that drive mercury bioaccumulation and biomagnification in the Adriatic Sea.

1.2 Relations Between Mercury and Marine Food Webs

1.2.1 Importance of microbial food web in mercury bioaccumulation

Our understanding of the factors controlling MeHg accumulation in marine food webs is surprisingly limited. Plankton is the most important point where mercury enters marine food webs. Lab and field data show that MeHg is concentrated from ambient water into phytoplankton by at least a factor of 10^5 (Gosnell & Mason, 2015; Hammerschmidt et al., 2013; H. Kim, Van Duong, Kim, Lee, & Han, 2014; Pickhardt & Fisher, 2007), which is the largest bioconcentration step in aquatic food webs. However, the exact mechanisms and conditions of this bioconcentration are still unclear. Previous studies reported passive diffusion, active transport, surrogate transfer, and co-transfer with MeHg ligands into cells (Chen et al., 2008; Lee & Fisher, 2016; Moye, Miles, Phlips, Sargent, & Merritt, 2002; Pickhardt & Fisher, 2007).

In the classical (herbivorous) food web, phytoplankton serves as an enriched MeHg source for zooplankton/herbivores, which can pass this mercury species to animals whose position is higher in the food chain. However, subsequent enrichments in MeHg concentrations are always less than a factor of 10 with increasing trophic level in aquatic food chains (Lee & Fisher, 2016).

Contrary to the herbivorous food web, microbial food web is on average longer, which potentially increases MeHg concentration in zooplankton and higher organisms (Jonsson et al., 2017). The herbivorous food web and the microbial food web represent two different trophic pathways in the marine environment (Figure 1.5) (Froneman, 2004; Legendre & Rassoulzadegan, 1995). The herbivorous food web goes from nitrate to large phytoplankton, zooplankton and fish. This trophic pathway is characterized with a short and simple food web, and high carbon export potential. The uptake of nitrate by large phytoplankton is higher than the corresponding uptake by small phytoplankton in this nitrate-dominated system (Šolić et al., 2016). This shorter food web probably results in lower MeHg concentrations in higher organisms (Jonsson et al., 2017).

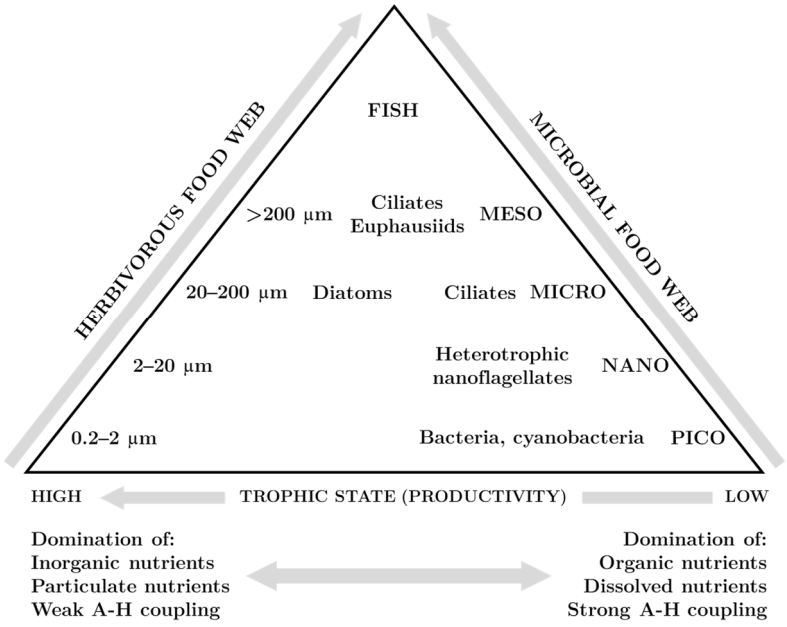


Figure 1.5: The structure of two main trophic pathways in pelagic ecosystems (A-H – autotrophs-heterotrophs). Figure adapted from Šolić et al. (2016).

On the contrary, in the environments dominated by the microbial food web, ammonium uptake sustains small phytoplankton cells (Liu & Dagg, 2003). The conditions that favor the productions of small autotrophic cells lead to the microbial food web. In general, the microbial food web is composed of small eukaryotic algae, cyanobacteria, heterotrophic bacteria and protozoa. These organisms are efficient in organic matter recycling in the euphotic zone sustaining low carbon sequestration rates (Cermeño et al., 2006; Froneman, 2004; Teira et al., 2003). Because MeHg bioconcentration factors increase linearly as the cell surface area-to-volume ratio increases, pico- and nano-dominated communities may lead to larger MeHg accumulation than the one dominated by large phytoplankton (H. Kim et al., 2014). Therefore, in addition to prolonged food web, a small size of the components of the microbial food web might have an influence in enhanced transfer of mercury to animals in higher trophic levels.

The knowledge described in this section and the results from our review paper (Živković, Šolić, et al., 2017) (Section 3.2) influenced the design of our work on Hg bioaccumulation and biomagnification; to determine the extent of Hg contamination in the Adriatic microseston and zooplankton, we sampled these fractions and determined their THg and MeHg content. We compared these results based on sampling month, station, food web composition, and the presence and absence of probable phosphorus limitation conditions

(^{P}P-limitation and $^{NO–P}$P-limitation). These results are presented in the article "Relations Between Mercury Fractions and Microbial Community Components in Seawater Under the Presence and Absence of Probable Phosphorus Limitation Conditions" (Živković et al., 2018) (Section 3.4). The results revealed that THg in microseston and zooplankton generally decreased toward the open sea, similarly like in seawater. On the contrary, the percentage of THg present as MeHg in plankton increased towards the open sea, parallel with the prevalence of microbial food web in seawater. In addition, results showed that mercury uptake by plankton might be enhanced under $^{NO–P}$P-limitation conditions, as ^{P}P-limitation could probably decrease the uptake of Hg related with polyphosphate complexation.

1.2.2 Mercury methylation and microbial food web

Even though marine sediments represent a source of MeHg to the marine water column (Ogrinc et al., 2007), their influence is especially important in the coastal areas, such as in the shallow northern Adriatic Sea (Acquavita et al., 2012; Bratkič et al., 2013; Covelli et al., 2008; Covelli, Langone, Acquavita, Piani, & Emili, 2012; Emili et al., 2012; Emili, Carrasco, Acquavita, & Covelli, 2014; Horvat et al., 1999; Koron & Faganeli, 2012; Petranich et al., 2017). However, these sources cannot account for all the MeHg present in the water column. Therefore, an additional MeHg source has to exist within the water column itself, which can account for these inequalities in MeHg mass inventories. Recent researches have shown that MeHg peaks, which exist within the water column, cannot be explained only by the sedimentary input, but are rather a consequence of *in situ* heterotrophic MeHg production (Cossa, Durrieu de Madron, Schäfer, Lanceleur, et al., 2017; Cossa et al., 2009; Heimbürger et al., 2010). In addition, the amount of MeHg contained in the plankton cannot account for all the MeHg found in the organic matter regeneration zone. Therefore, an internal MeHg source is needed, such as the activity of heterotrophic bacteria (Cossa et al., 2009).

Mercury methylation has been connected to the oxic marine environments and the abundance of heterotrophic bacterial communities in the open seawaters (Cossa et al., 2009; Heimbürger et al., 2010; Lehnherr et al., 2011) and estuarine waters (Schartup, Balcom, et al., 2015). This pathway seems to be related with the heterotrophic remineralization of sinking organic matter (Heimbürger et al., 2010). In general, previous oceanic research showed correlations between MeHg production and biological productivity, and the remineralization of sinking particulate organic carbon (POC) and associated microbial activity (Cossa, Michel, Noel, & Auger, 1992; Mason & Fitzgerald, 1993; Mason, Lawson, & Sheu, 2001; Mason, Rolfhus, & Fitzgerald, 1998; Mason & Sullivan, 1999; Monperrus, Tessier, Amouroux, et al., 2007).

It seems that heterotrophic MeHg production in the water column is not limited only to seawaters. MeHg production within the water column has been observed also in the freshwater systems (Gascón Díez et al., 2016). These authors showed for the first time that MeHg could be produced in particles sinking through oxygenated water column of lakes. In freshwater ecosystems, mercury methylation can also occur in oxic water column likely due to the presence of fresh labile organic matter and micro anoxic environments in settling particles, as it was previously reported in pelagic seawaters. Besides the type of organic matter, which determines the quality of the electron donors for mercury methylating bacteria, the redox potential within these micro anoxic environments likely affects mercury methylation processes in settling particles (Gascón Díez et al., 2016).

Heimbürger et al. (2010) clearly showed that the vertical MeHg profiles in the northwestern Mediterranean pelagic ecosystems show a double-peak pattern (Figure 1.6), which coincides with the occurrence of microbial food web in the euphotic zone (< 100 m)

and microbial loop in the aphotic zone (> 100 m) (Tanaka & Rassoulzadegan, 2002). In the microbial food web bacteria use phytoplankton-derived dissolved organic carbon (DOC) as the primary carbon source, while in the microbial loop dissolution of the sinking POC delivers DOC as the bacterial carbon source (Heimbürger et al., 2010).

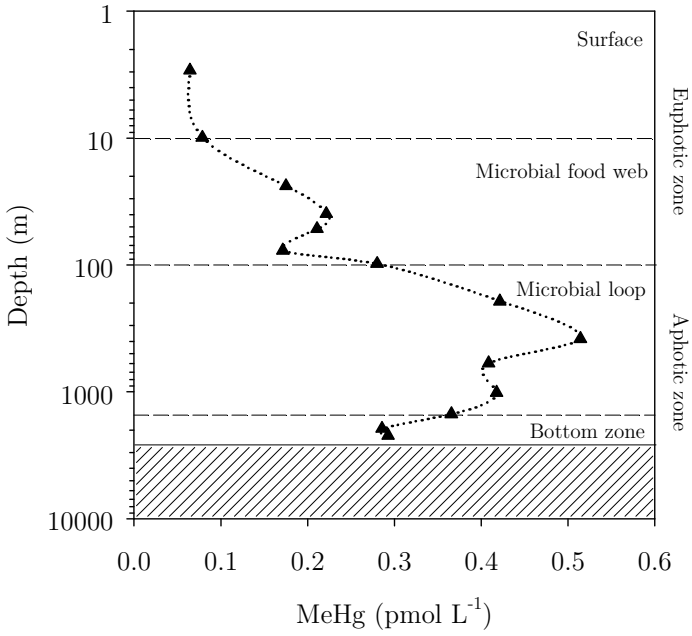


Figure 1.6: Derived mean vertical MeHg profile in the northwestern Mediterranean Sea reflects the existence of two distinct methylation zones associated with two different heterotrophic active layers. Figure adapted from Heimbürger et al. (2010).

The vertical distributions of MeHg concentration and the abundance of phytoplankton indicate that oligotrophic conditions (dominated by nano- and picoplankton) promote microbial loop activity and favor Hg methylation. The microbial community associated with picoplankton creates an appropriate environment for microbial remineralization of settling particles and bacterial Hg methylation (Heimbürger et al., 2010). Therefore, microbial loop and microbial food web can promote conditions that favor mercury methylation in open seawater. The term microbial loop describes the closed system of heterotrophic bacteria and their grazers, which release dissolved organic matter used by bacteria (Legendre & Rassoulzadegan, 1995). In addition to these organisms, the microbial food web also includes autotrophic pico- and nanoplankton. The microbial loop is common in oligotrophic environments characterized with limited nutrients influx in which bacterial biomass surpasses phytoplankton biomass (Legendre & Rassoulzadegan, 1995; Teira et al., 2003). Under these conditions large phytoplankton cells account for a small part of overall phytoplankton biomass. In addition, large zooplankton shows low grazing rates of small plankton, which is too small to be effectively ingested (Finlay & Roff, 2004; Vargas & González, 2004). Therefore, small phytoplankton is grazed by small microzooplankton, whose fecal pellets sink more slowly compared to those produced by larger zooplankton. Their longer residence time in the water column favors the microbial degradation of sinking organic matter, which promotes microbial mercury methylation (Heimbürger et al., 2010).

Under oligotrophic conditions, low nutrient concentrations and high content of dissolved carbon favors the increasing abundance of prokaryotic heterotrophs over phagotrophic heterotrophs (Cotner & Biddanda, 2002). Therefore, small prokaryotic organisms represent the dominant part of plankton biomass under oligotrophic conditions

(Ducklow & Carlson, 1992). This was a basis for our hypothesis that mercury methylation in the photic zone of the oligotrophic central Adriatic Sea depends on characteristic structure of the marine food web in which prokaryotic heterotrophs prevail.

Based on the knowledge that MeHg production is related with the activity of heterotrophic bacteria in the western Mediterranean Sea, we wanted to test if these relations occur also in the open Adriatic Sea. This was a guiding hypothesis for the experimental design of our research. Even though elevated MeHg concentrations were previously observed in the southern Adriatic Sea, as summarized in our review paper (Živković, Kotnik, et al., 2017) (Section 3.1), the exact sources are unknown. Therefore, in this work we measured MeHg concentrations and the abundance of heterotrophic bacteria in the transect from the coastal to open sea during a two-year period. Using statistical analysis, we tried to connect MeHg concentrations and the abundance of different groups of heterotrophic bacteria under different conditions. This analysis is presented in paper "Relations Between Mercury Fractions and Microbial Community Components in Seawater Under the Presence and Absence of Probable Phosphorus Limitation Conditions" (Živković et al., 2018) (Section 3.4). The results revealed that MeHg concentration was related only with the abundance of low nucleic acid bacteria, which are the most abundant bacterial group in oligotrophic environments, and only under $^{NO-P}$P-limitation conditions. This indicates that mercury methylation in the Adriatic water column might be a metabolism-dependent transformation.

1.2.3 Remineralization of organic carbon and mercury methylation

Microbial remineralization of organic carbon has been related with MeHg production within the water column of the open ocean (Figure 1.7) (Lehnherr et al., 2011). Remineralization of settling organic carbon stimulates the water column Hg methylation by providing a source of Hg^{II} to microbially active subsurface waters and by delivering organic substrate to heterotrophic bacteria. Remineralization of POC also creates a geochemical gradient in the water column between reducing conditions and elevated concentrations of dissolved oxygen.

Water column MeHg production in pelagic marine waters is enhanced by the presence of microzonal redox gradients inside fecal pellets and marine snow aggregates, since they have very low internal oxygen levels (Sunderland, Krabbenhoft, Moreau, Strode, & Landing, 2009). Low-oxygen, thermocline waters characterized by high activity of heterotrophic bacteria and a positive relationship between MeHg concentrations and organic carbon remineralization rates point to a link between organic carbon utilization and Hg methylation in the open ocean.

Significant correlations of MeHg concentrations with the concentrations of nutrients, especially phosphate, and apparent oxygen utilization (AOU) in world oceans further corroborate relations between mercury methylation and organic matter remineralization (Cossa et al., 2011, 2009; Cossa, Durrieu de Madron, Schäfer, Lanceleur, et al., 2017; Heimbürger et al., 2010; Lehnherr et al., 2011; Sunderland et al., 2009). AOU is a proxy for the heterotrophic activity and its values are the highest in areas where the highest MeHg concentrations are observed in the western Mediterranean Sea (Cossa et al., 2009). The MeHg–AOU relation is therefore interpreted as a proxy for the *in situ* methylation capacity of the water column and reflects the bioavailability of Hg for methylating bacteria (Cossa et al., 2011). In addition, the effects of organic carbon remineralization on mercury methylation can be observed through the similar regression coefficients between the concentrations of MeHg and phosphate in the particulate and the dissolved phases (Cossa, Durrieu de Madron, Schäfer, Lanceleur, et al., 2017).

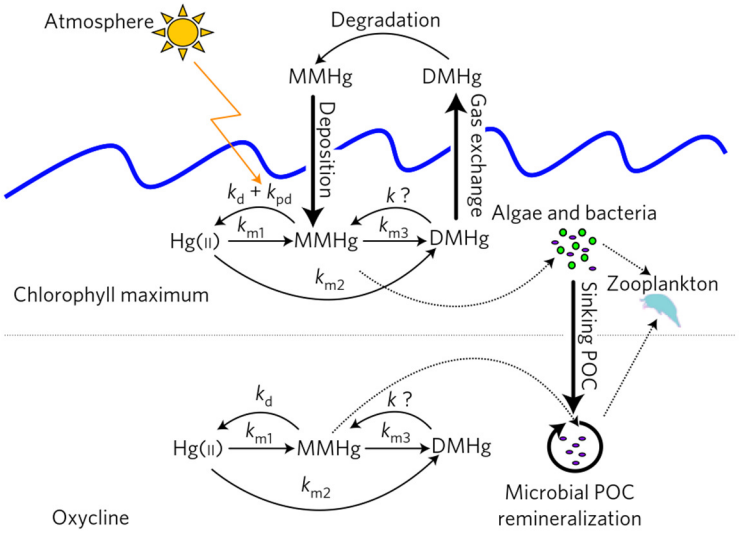


Figure 1.7: Conceptual model of Hg methylation/demethylation in marine waters. Mercury methylation and (photo)demethylation pathways (thin arrows) governed by respective rate constants (k_m – methylation, k_d – demethylation, k_{pd} – photodemethylation), with associated biogeochemical processes (thick arrows) and bioaccumulation/biomagnification (dashed arrows). Figure and caption adapted from Lehnherr et al. (2011).

In addition to the correlation between MeHg concentrations and physico-chemical parameters, the quality of organic matter itself reveals a significant role in remineralization processes. The importance of organic matter composition on Hg methylation is demonstrated in laboratory and field studies (Bravo et al., 2017; Chiasson-Gould, Blais, & Poulain, 2014; M. Kim, Han, Gieskes, & Deheyn, 2011; Schartup, Ndu, Balcom, Mason, & Sunderland, 2015). Remineralization of fresh and energy-rich labile organic matter causes higher mercury methylation rates than remineralization of degraded refractory organic matter both in sinking particles and sediment (Bravo et al., 2017; Gascón Díez et al., 2016). Recently, molecular composition of organic matter from the boreal lake sediment revealed that compounds produced by the remineralization of fresh chlorophylls, proteins and phytoplankton cell wall lipids enhance mercury methylation (Bravo et al., 2017).

Labile DOC causes the increase of MeHg concentration in water, while humic DOC causes its decrease. The positive correlations between the concentrations of MeHg and labile DOC show that labile DOC is a proxy for organic matter remineralization rate in the water column, which is in accordance with *in situ* MeHg production during organic matter remineralization. On the contrary, humic matter seems to decrease Hg^{II} bioavailability to methylating microorganisms and limit MeHg production (Soerensen, Schartup, Skrobonja, & Björn, 2017). Mercury methylation rates (Table 1.1) can exhibit high temporal variability due to changes in organic carbon availability related with remineralization of plankton biomass (Lehnherr et al., 2011) and due to different sources of organic matter (Soerensen et al., 2017).

Based on the facts described in this section, we wanted to test whether MeHg production could be related to organic carbon remineralization in the Adriatic Sea. Therefore, our two-year research included determinations of dissolved oxygen concentrations (from which AOU was calculated) and concentrations of nutrients (nitrate, nitrite, ammonium, phosphate, silicate). Using statistical analysis, we tested the presence of relations between the concentrations of MeHg and nutrients and AOU at different stations and under different conditions of phosphorus limitation. This research is presented

in our final paper (Živković et al., 2018) (Section 3.4). Results revealed that MeHg concentrations were correlated with AOU only at the coastal station.

Table 1.1: Mercury methylation and demethylation rates (k_m and k_d) in sediments and seawaters.

	Location	k_m (% d^{-1})	k_d (% d^{-1})
sediment	Venice Lagoon [a]	0.00–5.60	n/a
	Gulf of Trieste [b]	0.00–0.66	n/a
	Gulf of Trieste [c]	0.25–2.10	0.92–10.4
	Gulf of Trieste [d]	0.23–4.86	3.95–17.0
	Marano and Grado Lagoon [e]	0.00–5.40	1.00–55.0
	Strait of Otranto [f]	0.10–0.54	n/a
	Mediterranean Sea [f]	0.10–1.08	n/a
	Thau Lagoon [g]	0.25–1.32	n/a
	Lake Geneva [h]	0.10–0.68	6.40–33.0
	Long Island Sound [i]	1.40–8.20	n/a
	Bothnian Bay [j]	1.00–11.5	5.00–20.0
sea-water	Thau Lagoon [k]	0.30–6.30	6.40–24.5
	Canadian Arctic [l]	0.06–1.30	23.0–59.0
	Baltic Sea [m]	0.00–0.04	3.00–17.0

[a] M. Kim et al. (2011); [b] Bratkič, Koron, Ribeiro Guevara, Faganeli, & Horvat (2017); [c] Hines et al. (2000); [d] Hines et al. (2017); [e] Hines et al. (2012); [f] Ogrinc et al. (2007); [g] Monperrus, Tessier, Point, et al. (2007); [h] Gascón Díez et al. (2016); [i] Hammerschmidt & Fitzgerald (2004); [j] Lambertsson & Nilsson (2006); [k] Monperrus, Tessier, Amouroux, et al. (2007); [l] Lehnherr et al. (2011); [m] Soerensen, Schartup, et al. (2016); n/a – not available.

Generally, there were no significant differences between MeHg–AOU correlations under ^{P}P-limitation and $^{NO–P}$P-limitation, probably because primary production prevails over organic matter degradation in the photic zone. However, MeHg concentration was significantly correlated with the concentrations of all major nutrients under $^{NO–P}$P-limitation and not under ^{P}P-limitation, indicating that possible Hg methylation in the water column might be a nutrient co-dependent transformation during organic carbon remineralization, as described above.

1.2.4 Demethylation of methylated mercury

A major mechanism that limits MeHg uptake by biota is photodemethylation, which is considered one of the major sinks of MeHg in surface waters (Chandan, Ghosh, & Bergquist, 2015). Although the estimates of MeHg photodemethylation are difficult to make, this process accounts up to 80% of the MeHg loss from natural waters (Hammerschmidt & Fitzgerald, 2006; Lehnherr & St. Louis, 2009; Y. Li et al., 2010). Light intensity and frequency, type and amount of dissolved organic matter, photo-reactive trace metals, and pH may affect MeHg photodegradation (Chandan et al., 2015); however, reaction mechanisms of this biogeochemical process are still largely unknown (Lehnherr & St. Louis, 2009).

Although biotic MeHg demethylation accounts for a relatively small part of MeHg loss from natural waters, it is directly connected to bacterial detoxification against the adverse

MeHg effects. Two biological mechanisms of MeHg demethylation are mostly studied: oxidative and reductive demethylation. During oxidative demethylation, MeHg is decomposed to Hg^{II} and CO_2, hence name oxidative, as methyl group is oxidized to CO_2 (Marvin-DiPasquale et al., 2000). Oxidative demethylation of MeHg occurs under non-specific mechanism and it is not induced by elevated environmental mercury concentrations. Instead, this pathway is pseudo-metabolic process that involves simple organic molecules (Hsu-Kim et al., 2013). Mechanisms of methanogenic degradation of methanamine (Barkay & Wagner-Döbler, 2005) and oxidation of acetate by sulfate-reducing bacteria (Oremland, Culbertson, & Winfrey, 1991) are similar to proposed pathways for oxidative demethylation of MeHg (Hsu-Kim et al., 2013). Even though oxidative demethylation can occur in both oxic and anoxic environments (Baldi et al., 2012; Oremland et al., 1991), it seems that oxidative pathway is more common under anoxic conditions characterized by greater abundance of heterotrophic bacteria (Marvin-DiPasquale & Oremland, 1998).

Contrary to oxidative demethylation, reductive demethylation is mercury-specific detoxification process. Bacteria which are capable for reductive demethylation possess *mer*-operon (Barkay, Miller, & Summers, 2003). The *mer*-operon contains several *mer*-genes, which encode for different enzymes associated with mercury resistance. The *merB* gene encodes for translation of organomercury lyase enzyme, which is responsible for reductive MeHg demethylation (Barkay et al., 2003). Organomercury lyase is a cytosolic enzyme that splits Hg–C bond and degrades MeHg (Marvin-DiPasquale et al., 2000). Contrary to oxidative demethylation, this pathway is dependent on intracellular mercury concentrations, and hence on its environmental concentrations. Estimated threshold Hg concentration that triggers the transcription of *mer*-operon is about 50 nmol L^{-1} (Barkay & Wagner-Döbler, 2005). As these concentrations are considerably higher than natural environmental concentrations in seawater, this pathway generally occurs in highly contaminated environments (e.g. near the industrial effluents of chlor-alkali plants, in rivers influenced by mercury mine or coal combustion plant). Reductive demethylation also depends on specific redox conditions that favor this pathway. Therefore, this pathway usually occurs near the oxygen depletion zone characterized with higher MeHg concentrations (Baldi et al., 2012; Fitzgerald et al., 2007; Hines et al., 2012; Hines, Faganeli, Adatto, & Horvat, 2006).

In addition to *merB*-dependent reductive demethylation, there is an additional pathway for reductive demethylation that is not *merB*-dependent (Baldi, Pepi, & Filippelli, 1993). This pathway was first observed in bacteria *Desulfovibrio desulfuricans*, which can degrade bis(methymercury)sulfide without involvement of organomercury lyase.

Although there are only few evidences of autotrophic mercury methylation, for autotrophic demethylation there are no direct evidences present. Monperrus et al. (2007) showed that autotrophic microorganisms might be partially responsible for MeHg degradation. They found that under dark conditions, the demethylation rates were systematically lower than those obtained under diurnal incubations (Table 1.1), indicating that MeHg demethylation was mainly controlled by photochemical reactions or photobiological activity. Water filtration before incubation led to lower demethylation rates suggesting that microorganisms (i.e. bacteria and phytoplankton) are partially involved in MeHg demethylation. As dark conditions inhibited MeHg demethylation, the contribution of photosensitive microorganisms (i.e. autotrophs) to MeHg demethylation in seawaters might be greater than expected (Monperrus, Tessier, Amouroux, et al., 2007). However, direct autotrophic MeHg demethylation under laboratory conditions has not been observed yet.

The knowledge described in this section influenced the experimental design of our work on possible biotic MeHg demethylation. In this work, we measured MeHg concentrations

and the abundance of heterotrophic bacteria and autotrophic picoplankton in the transect from the coastal to open sea. Using statistical analysis, we tried to relate MeHg concentrations with the abundance of autotrophic picoplankton and heterotrophic bacteria, bacterial cell production, and specific bacterial production under the presence and absence of probable phosphorus limitation conditions. The results are presented in the article "Relations Between Mercury Fractions and Microbial Community Components in Seawater Under the Presence and Absence of Probable Phosphorus Limitation Conditions" (Živković et al., 2018) (Section 3.4). Our results revealed that bacterial demethylation under $^{NO-P}$P-limitation conditions might be the main biotic transformation in the open waters during warmer times of the year. Alongside photodemethylation, metabolically dependent demethylation could be possible at station CJ007, as the correlation coefficient between MeHg and the specific bacterial production under $^{NO-P}$P-limitation is significantly different from that under ^{P}P-limitation conditions.

1.3 Open Issues in Microbial Mercury Transformations

Special attention must be given to microbial mercury transformations in the marine environment. Alongside abiotic mercury transformations, biologically mediated reactions are of the greatest importance for mercury biogeochemistry due to ubiquitous occurrence of biota in water. Even though inorganic and photochemical reactions play a considerable role in mercury biogeochemistry, biological mercury transformations are of the greatest importance for human because they are directly connected to mercury transfer up the food chain. As human exposure to mercury is mainly through fish consumption, marine food web is of great importance. Therefore, mercury transformations and accumulation in the lowest member of the marine food web consequently has a direct impact on human health.

1.3.1 Unidentified relations between methylated mercury and microbial food web

Even though Heimbürger et al. (2010) have identified picoplankton as the most important fraction for mercury methylation in the seawater column, it is still unknown which components of picoplankton community contribute the most. Picoplankton includes both prokaryotic heterotrophs and autotrophs, but also small eukaryotic cells (picoeukaryotes). Heterotrophic picoplankton (mostly heterotrophic bacteria) and autotrophic picoplankton (picoeukaryotes and cyanobacteria *Prochlorococcus* and *Synechococcus*) are the most important components of the marine picoplankton. The importance of these picoplankton fractions is greater in oligotrophic waters (such as the Adriatic Sea) than in the eutrophic environments, because they are well adjusted to low-nutrient environments (Grob et al., 2007; W. K. W. Li & Harrison, 2001; Magazzù & Decembrini, 1995). Autotrophic picoplankton plays an important role in oligotrophic waters where it accounts for the majority of primary production, and biomass and energy transfer (Šolić et al., 2016). In the Mediterranean Sea, autotrophic picoplankton accounts for 71% of primary production in oligotrophic waters, compared to only 44% in eutrophic waters (Magazzù & Decembrini, 1995). This emphasizes the importance of autotrophic picoplankton for mercury methylation under oligotrophic conditions.

Heterotrophic bacteria are known to be mercury methylator in seawater, but no study has tried to narrow down specific groups that are most important for methylation. Heterotrophic bacteria are a generic term for highly diverse components of the ocean system that utilize dissolved organic matter, which sustains their metabolism and produces new biomass (Landa et al., 2014). They play an important role in marine environments through

organic matter remineralization and nutrient recycling. Heterotrophic bacteria transform organic matter and nutrients into forms suitable for primary producers, which in return provide reduced carbon sources to bacteria (Šolić et al., 2016). These mutualistic feedback interactions in energy and matter transfer are the basis of the microbial food web.

MeHg is biomagnified along the food trophic levels, but the conditions of this transfer are still not well understood, especially in the microbial food web. Only recently, MeHg biomagnification from bacteria to heterotrophic nanoflagellates (HNF) has been experimentally demonstrated (Jonsson et al., 2017). HNF are eukaryotic organisms that are responsible for the most of grazing of both heterotrophic and autotrophic picoplankton. Phagotrophic protozoa are one of the most important grazers on bacteria that remove bacterial biomass from the aquatic environments (Fenchel, 1982; Sherr, Sherr, & Pedrós-Alió, 1989). Grazing rate on bacteria is essential for understanding the biomass transfer from the bacterial level to higher trophic levels. In the Adriatic Sea, small HNF (< 8 μm) are the most important grazers of bacteria, by controlling their abundance and production, as they account for 80% of total grazing on bacteria (Šolić & Krstulović, 1994).

The lack of identification of key microbes involved in Hg methylation and bioaccumulation/biomagnification in the open seawater influenced the experimental design of our research in the Adriatic Sea. In this work, we wanted to determine the main microorganisms involved in these processes. Therefore, our two-year research included the determinations of nanoflagellates and autotrophic picoplankton, along with the abundance of heterotrophic bacteria described in Section 1.2.2. We determined the possible relations between the abundances of these microorganisms and MeHg concentrations in seawater. The results presented in Section 3.4 showed that significant correlation between MeHg and *Prochlorococcus* under $^{NO-P}$P-limitation could be due to their direct Hg methylation in the water column or *Prochlorococcus* might present a source of organic matter that stimulates heterotrophic methylation. The biomagnification of MeHg from bacteria to HNF under $^{NO-P}$P-limitation conditions might be possible, although correlations were weak because HNF abundance had good correlation only with the abundance of high nucleic acid bacteria. Results indicated that MeHg might not be readily transferred along microbial food web under ^{P}P-limitation conditions which might have strong implications for Hg content in fish.

1.3.2 Importance of microorganisms in the production of dissolved gaseous mercury

Volatility of elemental mercury is the main reason for its ubiquitous occurrence in the environment. Mercury reduction from the aquatic and terrestrial systems causes its evaporation, transport through the atmosphere and consequently deposition to pristine environments (Mason et al., 2017). Although the greatest share of Hg reduction is associated with the abiotic photo-induced mechanisms (Ariya et al., 2015; Fitzgerald & Lamborg, 2007), mercury reduction by heterotrophic and autotrophic microorganisms represents an important link in the global mercury biogeochemical cycle (Mason, Morel, & Hemond, 1995).

Volatile dissolved Hg species (Hg^0 and DMHg) diffuse from deep ocean waters towards the surface and evaporate into the atmosphere (Gårdfeldt et al., 2003; Kotnik et al., 2007, 2017; Nerentorp Mastromonaco, Gårdfeldt, & Wängberg, 2017), representing one of the biggest Hg sinks for the ocean waters. Major DGM sources in the Mediterranean are biological processes in deep and surface waters, as well as geotectonic activity at the Mediterranean sea bottom (Horvat et al., 2003). Photoreduction and photooxidation in surface waters are two important driving forces of net DGM concentrations. The decrease of DGM in the surface Mediterranean waters is due to Hg^0 oxidation in a strong oxidizing

environment (Gårdfeldt, Sommar, Strömberg, & Feng, 2001; Horvat et al., 2003). Biological and photochemical transformations seem to be the most important driving forces at the surface, while at depth biologically mediated transformations are possible, alongside hydrodynamic movement of water masses and geotectonic activities (Kotnik et al., 2007, 2017). Geotectonic characteristics of the Mediterranean seafloor might represent the greatest natural source of DGM from the sea depths (Rajar, Četina, Horvat, & Žagar, 2007; Žagar et al., 2014).

Heterotrophic mercury reduction can trigger a specific energy-dependent detoxification mechanism associated with *mer*-operon (Barkay et al., 2003; Barkay & Wagner-Döbler, 2005; Freedman, Zhu, & Barkay, 2012). Mercury reductase is a bacterial enzyme coded by *merA* gene within the *mer*-operon, which can be found in different heterotrophic microorganisms, including gram-negative and gram-positive bacteria, and archaea (Barkay et al., 2003). The mechanism of this reduction pathway is well known and described in detail in literature (Barkay et al., 2011, 2003; Barkay & Wagner-Döbler, 2005; Daughney, Siciliano, Rencz, Lean, & Fortin, 2002; Ndu, Barkay, Schartup, Mason, & Reinfelder, 2016).

Because phototrophic organisms live in the photic zone, synergetic effects of photoreduction and biologically mediated reduction make assessing individual contributions rather difficult. Phytoplankton can directly affect mercury volatilization through enzymatic Hg reduction (Rolfhus & Fitzgerald, 2004). In addition, mercury reduction can occur through mechanisms associated with photosynthetic apparatus (Grégoire & Poulain, 2014), through reduction of inorganic mercury complexed with photoreactive compounds released by plankton (Deng, Fu, & Deng, 2009), or *via* glutaredoxin/*merA*-like enzyme pathway (Marteyn et al., 2013).

Mercury reduction by phototrophic microorganisms has been linked to a mixotrophic lifestyle, in which microbes gain energy photosynthetically but utilize diverse carbon compounds. Under phototrophic conditions, microbial growth rates can increase with increasing Hg^{II} concentrations, while alternative electron sinks can cause decreased Hg^0 production (Grégoire & Poulain, 2016). These microorganisms can reduce Hg only phototrophically, regardless of the organic carbon source supplied, while chemotrophically grown cells do not reduce Hg. Therefore, mercury can fulfil a physiological role in phototrophic cells that use Hg^{II} as an electron sink. These cells benefit from Hg reduction to maintain low intracellular Hg levels, but also from the reduction potential of Hg to maintain redox homeostasis (Grégoire & Poulain, 2016).

Biotic oxidation of Hg^0 has been rarely observed by either heterotrophs or autotrophs. Some bacteria are able to oxidize Hg^0 to Hg^{II} (Colombo, Ha, Reinfelder, Barkay, & Yee, 2014), while some can convert Hg^0 directly to MeHg (Hu et al., 2013). The oxidation mechanism probably occurs on the outer cell membrane through mercury complexation with thiol groups. Biological oxidation of Hg^0 can also occur through the activity of enzyme catalase, which can be induced by hydrogen peroxide in the environment and can oxidize Hg^0 to Hg^{II} through a non-specific reaction (Siciliano, O'Driscol, & Lean, 2002; Smith, Pitts, McGarvey, & Summers, 1998).

As described above and in our review paper (Živković, Kotnik, et al., 2017) (Section 3.1), the majority of DGM production can be attributed to photochemical reduction of inorganic Hg compounds, while the specific conditions that promote biotic DGM production are still not well understood. Therefore, in this dissertation we wanted to determine whether Adriatic microorganisms could contribute to biotic DGM production in seawater. Our research included the determination of DGM concentrations in seawater and the abundances of microbial community components (heterotrophic bacteria, HNF, and autotrophic picoplankton). Using statistical analysis, we related DGM concentrations with the abundances of these microorganisms under different conditions (sampling season, station, and the ^{P}P-limitation conditions). Results presented in Section 3.4 revealed that

under $^{NO-P}$P-limitation conditions, DGM was related with the abundances of these microbial community components and chlorophyll *a* concentrations, which could indicate possible biological DGM production (Živković et al., 2018). However, we observed that this possible biotic Hg reduction was probably not metabolism-dependent, as most of significant correlations were also observed under ^{P}P-limitation conditions (except with *Prochlorococcus*). A negative correlation between DGM and heterotrophic bacteria under ^{P}P-limitation indicated that DGM might be oxidized by heterotrophic bacteria in coastal waters, possibly through the activity of mercury oxidase/catalase.

1.4 Mercury Analysis in Seawater

The low environmental concentrations of THg in the Mediterranean seawater are on average about 1.5 pmol L^{-1} (Cossa, Durrieu de Madron, Schäfer, Guédron, et al., 2017; Horvat et al., 2003; Kotnik et al., 2007), which requires specific and precise instrumentation for accurate Hg determinations. The accurate Hg determination is a pre-requirement for any further analysis regarding mercury relations to microbiological transformations in the seawater.

Generally, mercury concentrations in natural water samples can be determined using various analytical techniques. The most commonly used techniques are cold vapor atomic absorbance spectrometry (CV-AAS) and cold vapor atomic fluorescence spectrometry (CV-AFS). However, analytical methods for the determination of mercury in seawaters at the picomolar levels are mostly based on CV-AFS, since the limit of detection (LOD) of the instrument is about an order of magnitude lower than that of CV-AAS. As CV-AFS is a simple, very sensitive and relatively inexpensive Hg detector, it can be easily used for Hg determination in seawater samples (Method 1631, US EPA, 1999). In general, problems arising in THg determinations in water samples are associated with possible contaminations during sampling and losses due to Hg volatilization from the solution, and adsorption on container walls (Creswell et al., 2016).

1.4.1 Mercury speciation/fractionation in seawater

Mercury speciation/fractionation in seawater samples can be achieved using a sample pre-concentration and a convenient separation technique coupled with a suitable detector (Amde, Yin, Zhang, & Liu, 2016; Horvat, 1996). The most commonly determined mercury fractions in seawater are DGM and MeHg.

DGM in seawater can be determined by a commonly used discrete method based on sample purging, gold amalgamation, thermal desorption and CV-AFS detection (Gårdfeldt et al., 2003; Horvat et al., 2003). This method has low LOD (25 fmol L^{-1}) and good repeatability (4%). The method is described in detail in Horvat et al. (2003) and was used in this dissertation for DGM determinations in seawater.

The most commonly used method for MeHg determinations in seawater is based on sample pre-concentration, aqueous-phase ethylation, gas chromatography (GC) coupled with CV-AFS detection (Bloom, 1989; Liang, Horvat, & Bloom, 1994). The major drawback of this method is associated with the clean-up/pre-concentration step (extraction or distillation) (Bloom, 1989; Horvat, Liang, & Bloom, 1993), which is tedious and time consuming. Variable spike recoveries consequently cause low repeatability and reproducibility of the results. The biggest problem represents multiple procedure steps that cause high and unrepeatable blanks, and consequently high LOD (15–150 fmol L^{-1}) (Bloom, 1989; Bratkič et al., 2016; Horvat et al., 2003). These values can be too high to determine accurately the MeHg concentrations in the Mediterranean seawater, which can be as low

as 20 fmol L^{-1} (Cossa, Durrieu de Madron, Schäfer, Lanceleur, et al., 2017). Furthermore, labware cleaning is long-lasting and utilizes strong acids.

Derivatization of mercury species by hydride generation, coupled with cryogenic trap, GC and CV-AFS detector represents a simple method for the determination of low femtomolar MeHg concentrations in seawater (Stoichev, Rodriguez Martin-Doimeadios, Tessier, Amouroux, & Donard, 2004). Hydride generation has low LOD due to low sample blanks. Unlike ethylation, hydride generation does not require undesirable preparation clean-up steps and its long-time consumption, and is therefore not as susceptible to contamination (Stoichev et al., 2004). Compared to extraction, this method does not require any labware cleaning. These characteristics provide lower LOD compared to ethylation method, good repeatability and reproducibility, and enable measurements of MeHg concentrations down to 5 fmol L^{-1} (Cossa, Durrieu de Madron, Schäfer, Lanceleur, et al., 2017).

Co-elution of two mercury species (Hg^0 and Hg^{II}) within the same peak represents a major problem in the hydride generation method. This can cause difficulties due to possible overlapping with MeHg peak and consequently underestimation of MeHg concentration in seawater. This is of great importance, especially for samples from coastal areas of the Adriatic Sea, which can have elevated concentrations of inorganic mercury compared to open sea (Bratkič et al., 2013; Kotnik et al., 2015). Quantitative separation of mercury peaks is a necessary condition for obtaining accurate MeHg concentrations in seawater.

The greatest concern when comparing data from different studies is that the used analytical procedures are not identical, making comparison difficult. Uncertainty estimation is a common procedure that allows direct comparison of the results obtained by different methods. Contributions to measurement uncertainties of the ethylation and hydride generation methods depend on all sources that can lead to errors, including sampling, method procedure, repeatability, reproducibility, recovery, and measurement itself (Ellison & Williams, 2012). This is especially important when determining very low MeHg concentrations (near LOD).

Our review paper (Živković, Kotnik, et al., 2017) (Section 3.1) has demonstrated that MeHg concentrations in the Adriatic waters can be very low and even reach LOD. Therefore, we needed to optimize and validate a method based on hydride generation in order to reliably measure low MeHg concentrations in the Adriatic seawater. Method optimization required lowering LOD and the uncertainty of the method. Based on previous knowledge, we designed a cryogenic trap/chromatographic column for the hydride generation method and compared the performance of the method with the commonly used ethylation method. The results of this research are published in paper "Optimization and Measurement Uncertainty Estimation of Hydride Generation–Cryogenic Trapping–Gas Chromatography–Cold Vapor Atomic Fluorescence Spectrometry for the Determination of Methylmercury in Seawater" (Živković, Fajon, et al., 2017) (Section 3.3). Results of this study showed that hydride generation had lower LOD (3.94–15.9 fmol L^{-1}) than ethylation, and acceptable expanded standard uncertainty (11.1–21.3%).

Chapter 2

Aims and Hypothesis

Microorganisms play an important role in mercury transformations. Interactions with marine biota enable specific mercury transformations and affect its transport between environmental compartments. In this dissertation, we wanted to determine the effect of microbial community on mercury speciation in the Adriatic Sea and show that different microbial communities affect changes in mercury dynamics during different seasons in coastal and open seawaters.

As the coastal and open waters differ in input of nutrients and mercury sources, they might also show different effects on mercury transformations and concentrations of mercury species. We hypothesized that the seasonal changes in the microbial communities (i.e. changes in total bacterial abundance, picoeukaryotes, cyanobacteria, nanoflagellates) are reflected in the concentration changes of Hg species in the water column. We assumed that biological processes within microbial and/or planktonic communities are connected to transformations of Hg species in the water column (e.g. methylation, reduction), as previously indicated in the western Mediterranean Sea (Heimbürger et al., 2010). We wanted to show that different environmental parameters (dissolved nitrogen and phosphorus salts, temperature, apparent oxygen utilization) during different seasons have an influence on mercury behavior in seawater, which is directly connected to the abundance of marine microorganisms (as a consequence of biologically mediated transformations).

Our intention was to demonstrate that parameters affecting mercury methylation in the water column depend on heterotrophic microbial activity, and that the seasonal changes in the abundances of the microbial community components coincide with the changes in MeHg concentrations within the water column. As the composition of the Adriatic microbial community changes due to changing environmental parameters, we wanted to show that these changes are also reflected in mercury (bio)transformations, providing an insight into biogeochemical processes in the Adriatic Sea under different environmental circumstances at the lowest levels of the marine food web.

To establish mercury interactions with marine biota we needed accurate analytical methods. Current methods for the determination of low MeHg concentrations in seawater are not accurate and reliable enough. Therefore, in this dissertation we described the optimization of the method for MeHg determination in seawater at femtomolar level based on hydride generation.

2.1 Research Goals and Hypothesis

Goals of the dissertation are:

i) To provide a detailed and critical review of literature about:
   - Mercury speciation in the Mediterranean and Adriatic Sea
   - Temporal and spatial changes of Hg in seawater, sediments and biota
   - Microbial transformations of mercury species in the seawater
   - Methods for the determination of MeHg in seawater

ii) Optimization and validation of hydride generation method for the determination of low MeHg concentrations in seawater

iii) Determination of seasonal variations of mercury species in seawater

iv) Determination of abundance and composition of microbial communities in the seawater

v) Determination of physical and chemical properties of the Adriatic seawater

vi) Sampling and the determination of mercury speciation in marine microseston and mesozooplankton

vii) Evaluation of data on mercury speciation, physico-chemical parameters of seawater, and abundance and composition of microbial communities

Hypothesis of the doctoral dissertation are:

i) Hydride generation can be readily used for the determination of low concentrations of methylmercury in seawater samples

ii) Different chemical processes and seasonal changes drive mercury speciation in the coastal and open marine environment

iii) Speciation of mercury in the Adriatic Sea depends on sea depth, season, location, marine biology and hydrometeorological parameters

iv) Mercury speciation in the photic zone depends on physico-chemical properties of seawater and characteristic structure of the marine food web

v) Mercury bioaccumulation in the marine food web depends on plankton dynamics and composition

Chapter 3

Scientific Publications

Publications included in this dissertation are listed in such a manner that their consecutive appearances follow general dissertation content. Candidate's contributions to individual scientific article are described before each article. In the dissertation, publications appear in the following order:

Živković, I., Kotnik, J., Šolić, M., & Horvat, M. (2017). The abundance, distribution and speciation of mercury in waters and sediments of the Adriatic Sea – a review. *Acta Adriatica*, *58*(1), 165–186.

Živković, I., Šolić, M., Kotnik, J., Žižek, S., & Horvat, M. (2017). The abundance and speciation of mercury in the Adriatic plankton, bivalves and fish – a review. *Acta Adriatica*, *58*(3), 371–399.

Živković, I., Fajon, V., Tulasi, D., Obu Vazner, K., & Horvat, M. (2017). Optimization and measurement uncertainty estimation of hydride generation–cryogenic trapping–gas chromatography–cold vapor atomic fluorescence spectrometry for the determination of methylmercury in seawater. *Marine Chemistry*, *193*, 3–7. https://doi.org/10.1016/j.marchem.2017.03.003

Živković, I., Fajon, V., Kotnik, J., Shlyapnikov, Y., Obu Vazner, K., Begu, E., … Horvat, M. (2018). Relations between mercury fractions and microbial community components in seawater under the presence and absence of probable phosphorus limitation conditions. *Journal of Environmental Sciences*. https://doi.org/10.1016/j.jes.2018.03.012

3.1 The Abundance, Distribution and Speciation of Mercury in Waters and Sediments of the Adriatic Sea – a Review

Živković, I., Kotnik, J., Šolić, M., & Horvat, M. (2017). *Acta Adriatica*, *58*(1), 165–186.

In this paper, the authors presented an overview of mercury research in the Adriatic seawater and sediments. Because the Adriatic Sea is a special biogeochemical subunit of the Mediterranean Sea, the authors first focused on mercury speciation in this sea. Alongside the reported concentrations of total mercury, they also discussed mercury speciation in seawater and sediments, and factors influencing mercury transport and transformation.

The authors then focused on mercury in the Adriatic waters and sediments. They summarized total mercury concentrations and mercury speciation data from relevant literature and presented spatial (northern, central and southern Adriatic) and temporal changes (from 1970s onward) of various mercury species/fractions. The authors presented vertical profiles of mercury fractions in seawater and discussed probable explanations for observed trends.

Mercury in the Adriatic sediments was presented through an overview of studies performed from 1970s. Even though the monomethylmercury data are not available in older literature, THg concentrations represented a good indicator of mercury contamination in the Adriatic sediments. The authors compared concentrations of different mercury fractions in sediments obtained from different parts of the Adriatic and from different years to assess the changes in mercury contamination. Mercury speciation in sediments and porewaters was also presented, as well as corresponding fluxes of mercury fractions, which were compared to those obtained in the Mediterranean. The authors also presented the transport of mercury species in the Adriatic Sea and discussed oceanographic and environmental parameters affecting these processes.

In this paper, special attention was addressed to the Adriatic case studies (Ravenna Lagoon, Venice Lagoon, Marano and Grado Lagoon, the Gulf of Trieste, Kaštela Bay and the Bay of Vlora) where mercury is frequently monitored due to anthropogenic hot-spot mercury sources. Mercury in the Krka river estuary was also presented, as an example of pristine environment. For each case study, the authors described the source(s) of mercury contamination in water and sediments, presented concentrations of mercury fractions in these coastal environments, discussed possible causes and effects of mercury transformation and transport and their overall impact on the Adriatic Sea. At the end, the role of plankton in mercury biogeochemistry was shortly presented, due to its importance in mercury transformations in oligotrophic waters.

The candidate's contribution to this paper consisted of literature overview, extraction of data from published papers and summarizing data by environmental compartment, geographical area, sampling period, and case study. The candidate drew figures and the table, and drafted the manuscript in collaboration with other co-authors.

ISSN: 0001-5113 AADRAY	ACTA ADRIAT., 58(1): 165 - 186, 2017	REVIEW PAPER

The abundance, distribution and speciation of mercury in waters and sediments of the Adriatic Sea – a review

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This review focuses on mercury speciation in the Adriatic Sea, a marginal sea of the Mediterranean, which represents its distinct biogeochemical subunit due to anthropogenic mercury loadings. The current knowledge about mercury cycling in the Adriatic is presented through an overview of the state of the art of research in this area: temporal and spatial distributions and occurrence of mercury species in seawater and sediment, and chemical transformations. We summarised research results of mercury speciation in order to describe its presence and fate in the Adriatic Sea. The Adriatic Sea represents a net source of mercury to the Mediterranean Sea due to the highest total mercury concentrations observed in the North Adriatic Sea and the highest methylmercury concentrations in the South Adriatic Pit. However, the biogeochemical cycle of mercury is not completely known and our understanding of mercury transport between compartments and its (bio)transformations is limited. Future research needs to focus on microbial and chemical processes of mercury transformations to improve our understanding of the impacts of mercury contamination on the environment and human health in the Adriatic Sea.

Key words: mercury, chemical speciation, methylmercury, Adriatic, Mediterranean

INTRODUCTION

Among trace metals, mercury (Hg) is one of the contaminants of most concern because of its high toxicity, persistence and accumulative behaviour in the environment and biota (UNEP, 2013). The atmosphere is the most important medium that transports Hg around the globe (SPROVIERI *et al.*, 2010). Gaseous mercury can reside in the atmosphere and be transported over large geographical distances and exchanged with land or surface waters. Hence, mercury contamination is a global environmental problem (AMAP/UNEP, 2013; UNEP, 2013). Due to transformation and transport processes, mercury can travel between environmental compartments: from atmosphere to water, soil and biota. The pathways and fate of mercury in the aquatic environment are important due to chemical and biological processes affecting speciation, partitioning and bioavailability of mercury (UNEP, 2013).

The chemical form of Hg in aquatic systems strongly depends on environmental conditions that determine its mobility and reactivity (AMAP/UNEP, 2013), and the concentration of inorganic

and organic complexing agents (FITZGERALD *et al*., 2007). In seawater, mercury has a very reactive chemistry. It can be present in different forms, whether dissolved or bound to particulates. The main dissolved Hg fractions are dissolved elemental mercury (DEM), Hg(II) complexes with organic and/or inorganic ligands, reactive mercury (RHg), and organomercury compounds (methylmercury (MeHg) and dimethylmercury (DMHg)) (HORVAT *et al*., 2003; O'DRISCOLL *et al*., 2005). Binding of mercury species onto particulate matter in water causes their settling and burying in sediments, which is a major pathway for removal of mercury from the biologically active environment (UNEP, 2013). Remobilisation from sediments is an important source of Hg to coastal and open marine systems and potentially to biota (ŽAGAR *et al*., 2013). Most mercury enters the marine environment by wet or dry deposition, with a significant fraction in oxidised form (SOERENSEN *et al*., 2010; ZHANG *et al*., 2009). Exceptions are small, semi-enclosed basins where river discharges, coastal erosion and ocean currents account for half of mercury input (UNEP, 2013). Rivers are the most important sources of mercury contamination from land. While riverine Hg inputs dominate in coastal watershed systems, combined atmospheric and riverine inputs are observed in "multiple-input" waters (CHEN *et al*., 2016). Major part of deposited mercury into the ocean is re-emitted to the atmosphere. Due to supersaturation, mercury emitted from open ocean to the atmosphere is mostly Hg^0, whereas the majority of deposited Hg is oxidised mercury (SPROVIERI *et al*., 2010). It is easily deposited to surface waters by wet deposition because of high solubility (HEDGECOCK *et al*., 2003; HOLMES *et al*., 2009; SPROVIERI *et al*., 2003).

Surface seawater, especially its euphotic zone, is an important compartment for the global Hg biogeochemical cycle (UNEP, 2013). Volatile Hg species (DGM; sum of DEM and DMHg) diffuse from deep ocean waters to the surface and evaporate into the atmosphere. The major sources of DGM are biological processes in deep and surface waters (DRISCOLL *et al*., 2013; LANZILLOTTA *et al*., 2002), as well as geothermal/hydrothermal fields at the sea bottom (FANTOZZI *et al*., 2009; FERRARA *et al*., 2000; HORVAT *et al*., 2003; PIRRONE *et al*., 2010). In surface waters, photoreduction and photooxidation are two important driving forces for the DEM cycle (COSTA & LISS, 1999; LANZILLOTTA *et al*., 2002; QURESHI *et al*., 2010). DMHg can be the dominant Hg specie in the deeper waters (MASON *et al*., 2012) but it readily evades from surface waters and is photochemically degraded (FITZGERALD *et al*., 2007; FITZGERALD & LAMBORG, 2003). Mercury chemistry in the open Mediterranean waters is very reactive, which results in elevated MeHg and DGM concentrations (MONPERRUS *et al*., 2007a). MeHg produced in the sediments by sulphate reducing bacteria can be released to the overlying water (EMILI *et al*., 2011, 2014; KORON & FAGANELI, 2012; MONPERRUS *et al*., 2007b) and could constitute an important source of the MeHg content in marine biota. However, the estimated methylated mercury fluxes in the ocean suggest that riverine input, coastal sources and atmospheric deposition are insufficient to explain observed methylated Hg sinks in the ocean (bioaccumulation, removal by fisheries, photochemical and biological degradation, and direct removal to the deep ocean and sediments) (MASON *et al*., 2012). Findings based on detailed MeHg profiles support the hypothesis that *in situ* sources of MeHg are dominant over advection (COSSA *et al.,* 2009). There are two zones of MeHg productivity connected to the re-mineralisation of organic carbon in the water column: first in the bottom of the euphotic layer (COSSA *et al*., 2009, 2011; HEIMBÜRGER *et al*., 2010) and the other at the oxygen minimum in the sub-thermocline (BOWMAN *et al*., 2015; HAMMERSCHMIDT & BOWMAN, 2012; HEIMBÜRGER *et al*., 2010). Conversely, photochemical and microbial demethylations of MeHg are the major removal processes of MeHg in the ocean (FITZGERALD *et al*., 2007; LEHNHERR *et al*., 2011).

Microbial activity can convert inorganic mercury into MeHg, which is rapidly incorporated into the food web and biomagnifies throughout trophic levels. MeHg is a powerful neurotoxin affecting fish and wildlife (UNEP, 2013). Due to its toxicological properties, pos-

sible presence in food, and transfer to humans, MeHg is the most important form of mercury; fish and aquatic biota are the predominant food sources involved in human exposure (AMAP/UNEP, 2013).

Sea represents a significant part of the mercury cycle in the environment. The Mediterranean and Adriatic Sea are especially important due to the presence of natural and anthropogenic sources of mercury contamination. Research results in the Mediterranean and Adriatic Sea have demonstrated that mercury is present in elevated concentrations in biota (BERTO *et al.*, 2006; CULLAJ *et al.*, 2004; GIANI *et al.*, 2012; HORVAT *et al.*, 2014; KLJAKOVIĆ-GAŠPIĆ *et al.*, 2006; MIKAC *et al.*, 1989; STORELLI *et al.*, 1998; TROMBINI *et al.*, 2003) compared to the Atlantic Ocean. The objective of this paper is to describe mercury contamination and emphasise the importance of its speciation in the Adriatic Sea as a biogeochemical subunit of the Mediterranean Sea. We evaluate the current state in the Adriatic Sea by presenting relevant spatial and temporal changes in mercury concentration in different compartments.

STUDY AREA

The Mediterranean Sea is an enclosed basin that includes large but diverse coastal and marine ecosystems. Strait of Sicily separates Western and Eastern basins, each comprised of four seas (Fig. 1). The Mediterranean Sea connects to the Atlantic Ocean through the Strait of Gibraltar and to the Black Sea through the Bosporus-Marmara-Dardanelles system. The Adriatic Sea is an elongated basin and the northernmost part of the Mediterranean Sea with which water is exchanged through the Strait of Otranto (Fig. 1). The Adriatic Sea is 783 km long and about 200 km wide (UNEP, 2012). It is divided into three regional basins (north, central and south)

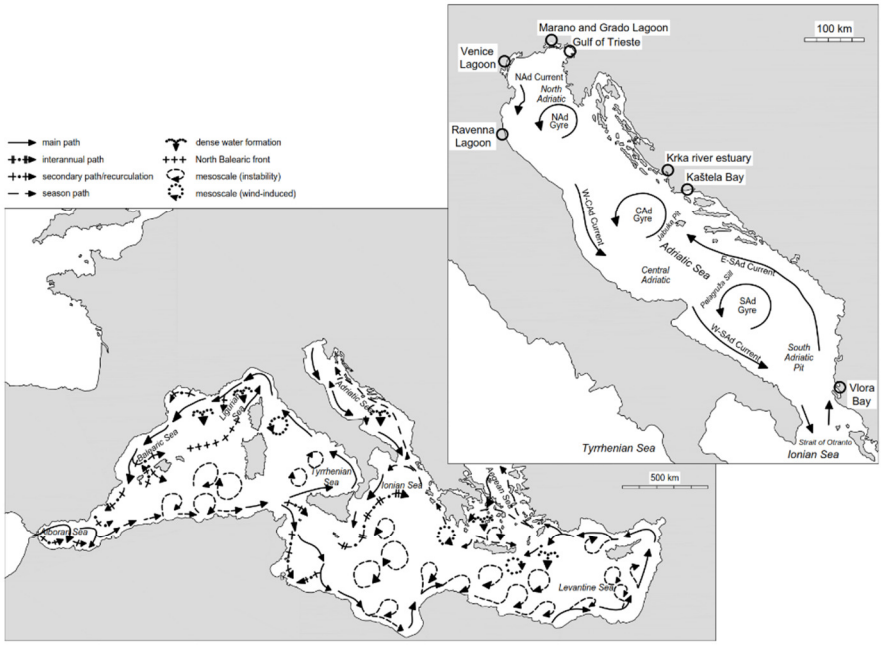


Fig. 1. Surface water circulation flows and geographical divisions of the Mediterranean and the Adriatic Sea. Redrawn from KOTNIK et al. (2015) and MILLOT & TAUPIER-LETAGE (2005)

which differ in latitude, bathymetry, physiography and biogeochemical characteristics (UNEP, 2012). Northern Adriatic is very shallow with an average bottom depth of 35 m. The central part is on average 140 m deep while the southern end is characterised by a wide depression (~1250 m) (BENSI *et al*., 2014). The Adriatic water circulation (Fig. 1) is dominated by North Adriatic current (N-Ad current), cyclonic Central and Southern Adriatic gyres (CAd and SAd), eastern South Adriatic current (E-SAd current) and western South Adriatic current (W-SAd current). Three water masses (surface water, Levantine intermediate water and deep water) were identified in three sub-basins of the Adriatic Sea by their physical properties (ARTEGIANI *et al*., 1997). Thermocline is present in spring and summer down to 30 m, 50 m and 75 m in the North, Central and South Adriatic Sea, respectively (ARTEGIANI *et al*., 1997). The Po River is the most important river regarding freshwater discharge (1550 m^3 s^{-1}) (ŽAGAR *et al*., 2013) and contributes to 46.5% of the overall freshwater input (UNEP, 2012). Most of the freshwater input is in the north-western Adriatic, while only 27% is discharged in the eastern side (UNEP, 2012). Sediments input from the Po River is ~15×10^6 t a^{-1} (NITTROUER *et al*., 2004) while the overall input to the northern and central Adriatic is 46.6×10^6 t a^{-1} (FRIGNANI *et al*., 2005). Sediments originating from the Po River consist mainly of silt and clay (COMBI *et al*., 2016; NITTROUER *et al*., 2004). The sea bottom in the eastern Adriatic is rocky with corallogenic concretions along the islands (UNEP, 2012).

MERCURY IN THE MEDITERRANEAN SEA

The Mediterranean Sea is subject to mercury contamination from natural and anthropogenic sources. Although it covers only about 1 % of the world oceans, almost 65 % of the world mercury reserves are situated in the cinnabar belt under the Mediterranean area (ŽAGAR *et al*., 2013). The most important Hg sources in the Mediterranean (in t a^{-1}) are atmospheric deposition (38), river inflow (13) and sediments (19), while the main outputs are evasion to atmosphere (68)

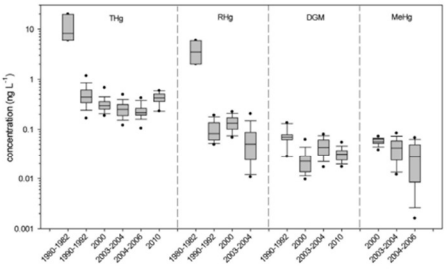


Fig. 2. Box-and-whisker plots of THg, RHg, DGM and MeHg concentrations in the Mediterranean Sea from 1980-2010 (median with 5, 10, 25, 75, 90 and 95th percentiles). Data from COSSA et al., 1997, 2009; FANTOZZI et al., 2013; FERRARA et al., 1986, 2003; HORVAT et al., 2003; KOTNIK et al., 2007

and burial in the sediments (25) (ŽAGAR *et al*., 2013). The Mediterranean Sea is slowly coping with mercury contamination because Hg output exceeds the input by about 5 t a^{-1}.

In the 1970s, THg values in some Mediterranean pelagic fish were two fold higher than those in the same species living in the Atlantic Ocean (COSSA & COQUERY, 2005), which raised questions about Hg contamination in the Mediterranean. High THg concentrations were also observed in water. Even though BERNHARD (1988) reported THg concentrations higher than 10 ng L^{-1}, it was stated that the validity of this data is doubtful. Since then, measured concentrations have been decreasing in the last decades (Fig. 2), mainly because more attention has been given to sample contamination during sampling, storage and analysis (BERNHARD, 1988; HORVAT, 2005), and because of using more sensitive instrumentation, mainly atomic fluorescence spectrometry (HORVAT, 2005).

Average THg concentration in the entire open Mediterranean Sea is 0.29±0.12 ng L^{-1} and is comparable in all measured water masses in the basin (KOTNIK *et al*., 2013). The highest concentrations are always found in coastal areas (FANTOZZI *et al*., 2013; KOTNIK *et al*., 2015). The highest open sea THg (0.71 ng L^{-1}) is found in the surface water in the Strait of Sicily, probably due to elevated geotectonic activity in this area (KOTNIK *et al*., 2007). Most of mercury in the open waters is present in dissolved form (avg.

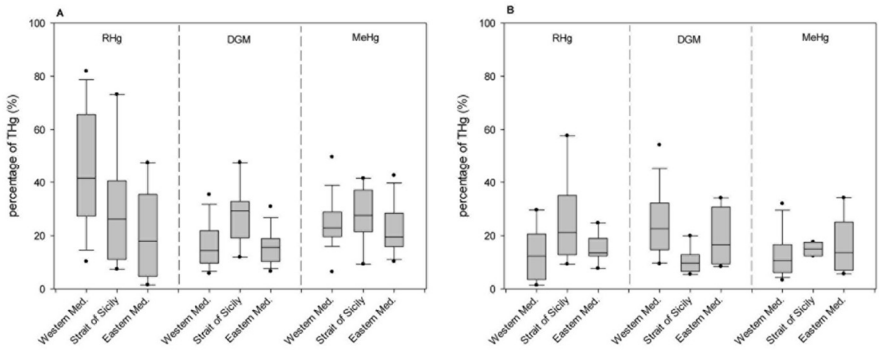


Fig. 3. Box-and-whisker plots of the geographical distribution of RHg, DGM and MeHg percentage of THg during spring (A) and summer (B) in the Mediterranean Sea. Data from KOTNIK et al., 2007

70%), due to the low abundance of particulate matter (HORVAT *et al.*, 2003). The concentrations of various Hg compounds are lower than those in world oceans, despite the geological mercury anomaly (KOTNIK *et al.*, 2013). Major factors influencing the distribution of RHg in the Mediterranean Sea are the presence of volcanic, geotectonic and biological activity, and photochemical reactions (KOTNIK *et al.*, 2007). RHg is lower in the summer than in the spring (KOTNIK *et al.*, 2007), as is its percentage of THg (Fig. 3). RHg vertical profiles are mostly the opposite of DGM, as inorganic Hg is a substrate for DGM production (KOTNIK *et al.*, 2013). Most of DGM is DEM, indicating its geotectonic origin. DGM generally increases with depth; this suggests the existence of volatile Hg sources in deeper waters (KOTNIK *et al.*, 2013), bacterial activity in the oxygen-depleted waters (KOTNIK *et al.*, 2007), or the oxidation of Hg^0 in the presence of UV light and chlorine/bromine (HORVAT *et al.*, 2003) or hydroxyl radical (GÅRDFELDT *et al.*, 2001) in the surface waters. The average MeHg concentration is higher during the spring (56.2±24.1 pg L^{-1}) than during the summer (32.1±20.1 pg L^{-1}) (KOTNIK *et al.*, 2007). The highest concentrations are usually found in the bottom water layer, indicating input from sediment (OGRINC *et al.*, 2007), microbially mediated methylation in deeper waters, or photochemical/microbial remineralisation of sinking organic matter from the surface waters (KOTNIK *et al.*, 2007). In addition, particulate scavenging can remove MeHg from surface waters and release it into deeper waters through particulate dissolution (COSSA *et al.*, 2009). MeHg/THg ratio (Fig. 3) follows the variability of MeHg concentration; it is higher in spring than in summer. This could be due to the higher biological activity (significantly higher chlorophyll levels) occurring in spring; however, MeHg concentrations are only slightly elevated, not statistically significant (KOTNIK *et al.*, 2007). The vertical distribution of DMHg depends on the presence of the thermocline. Levels below limit of detection are found in the surface layer, while the highest concentrations (2.43 pg L^{-1}) are measured in the deep waters (COSSA *et al.*, 1997; HORVAT *et al.*, 2003; KOTNIK *et al.*, 2007). Similar to MeHg, DMHg/THg ratio is higher during spring than summer. Since the Mediterranean deep waters are ~10°C warmer than the Atlantic waters of the same depth, more microbially mediated methylation could occur due to its temperature dependence (COSSA *et al.*, 1997; COSSA & COQUERY, 2005).

Mercury accumulation rates in the Mediterranean sediments range from 10.3-30.9 ng m^{-2} d^{-1} and 2.59-4.93 ng m^{-2} d^{-1} in the Western and Eastern basins, respectively (OGRINC *et al.*, 2007). THg concentrations in the sediments of the western basin decrease from the surface (78.2-90.3 ng g^{-1}) and at a depth of 10 cm the concentrations are within the range of the earth crust values (OGRINC *et al.*, 2007; COSSA &

COQUERY, 2005). THg concentrations in the Eastern Mediterranean sediment are lower (COSSA & COQUERY, 2005). THg distribution in deeper layers may reflect redistribution during diagenetic processes or changes in the sedimentation conditions (OGRINC *et al.*, 2007). The highest MeHg concentrations (up to 2.97 ng g^{-1}) are generally found in the uppermost sediment layer and decrease with sediment depth. Methylation potentials for surface sediments are in the range 0.16-0.71% d^{-1} and generally follow the MeHg depth profile (OGRINC *et al.*, 2007).

MERCURY IN THE ADRIATIC SEA

Distribution of mercury species in seawaters

Total mercury in the seawater exhibits a concentration gradient: the lowest values are found in the southern area and increase in the central part, while the highest values are found in the Northern Adriatic (Fig. 4A) (FERRARA & MASERTI, 1992; KOTNIK *et al.*, 2015). As in the Mediterranean Sea, there is a decrease in measured THg concentrations with time in the Adriatic Sea (Fig. 5), probably due to questionable validity of older data and possible sample contamination. Another possibility is a real decrease of Hg concentrations due to the closure of the Idrija mine in 1994 (FAGANELI *et al.*, 2003).

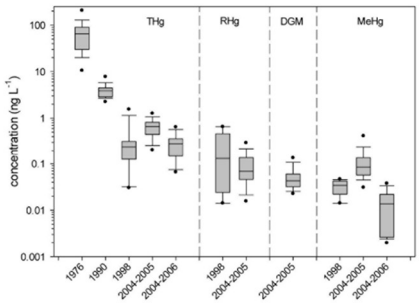


Fig. 5. Box-and-whisker plots of THg, RHg, DGM and MeHg concentrations in the Adriatic Sea from 1976-2005 (median with 5, 10, 25, 75, 90 and 95th percentiles). Data from COSSA et al., 2009; FERRARA & MASERTI, 1992; HORVAT et al., 2003; KOSTA et al., 1978; KOTNIK et al., 2015

Generally, average THg concentrations in the open Adriatic Sea are low (up to 0.32 ng L^{-1} in uncontaminated sites), which is similar to the values found in the open Mediterranean Sea (HORVAT *et al.*, 2003). The highest THg values are observed at the Otranto Strait at a depth of 40 m in summer (0.381 ng L^{-1}) and at 600 m in spring (0.379 ng L^{-1}) (KOTNIK *et al.*, 2007). Vertical salinity gradient indicates downwelling of surface Adriatic seawater; anthropogenic mercury pollution could be the reason for observed Hg peaks within this gradient (KOTNIK *et al.*, 2007). Elevat-

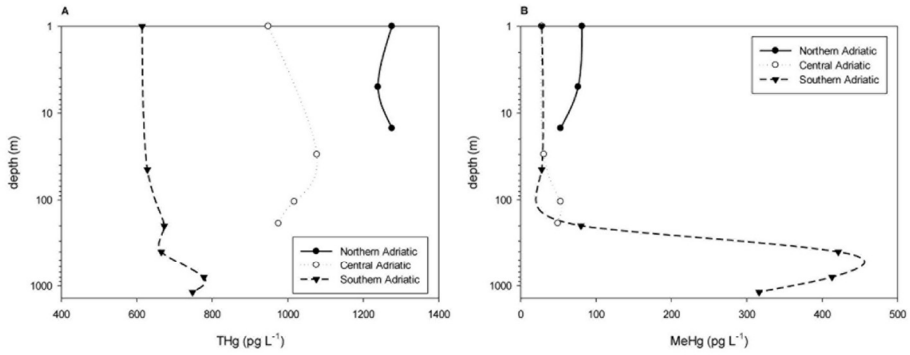


Fig. 4. Vertical seawater profiles of THg (A) and MeHg (B) in the Northern, Central and Southern Adriatic Sea. Data from KOTNIK et al., 2015

ed THg concentrations in the bottom water layer of the southern Adriatic Sea (Fig. 4A) could be due to southward transfer of the downwelling surface waters from the contaminated northern Adriatic Sea. Wide range in the THg associated with suspended particulate matter (SPM) can be observed in the three parts of the Adriatic Sea, with the highest values found in the northern Adriatic. The major sources of mercury bound to SPM are the Isonzo River (microcrystalline cinnabar), the Marano and Grado lagoon and the Po river delta. Generally, the amount of Hg associated with the SPM is often comparable or higher than that of the dissolved fraction (COVELLI *et al.*, 2006b, 2007; FERRARA & MASERTI, 1992).

COSSA *et al.* (2009) made vertical profiles of methylated mercury species (MeHg+DMHg) in the Mediterranean Sea, which were the first high-resolution profiles showing consistent features. All the vertical profiles, including Adriatic, exhibit similar patterns: very low concentrations of methylated mercury species in the surface waters, a sharp gradient within the biologically most active zone (0-600 m), an intermediate maximum (200-1000 m) and lower concentrations in the deep waters (COSSA *et al.* 2009; HEIMBÜRGER *et al.* 2010). KOTNIK *et al.* (2015) obtained a similar vertical profile in the southern Adriatic (Fig. 4B). The highest MeHg concentrations (0.50 ng L^{-1}) and the highest percentage of THg as MeHg (Fig. 6) were measured in the bottom water of the eastern South Adriatic shore. These concentrations were considerably higher than values reported in the Mediterranean Sea (COSSA *et al.*, 2009; HORVAT *et al.*, 2003; KOTNIK *et al.*, 2007). Even in the most contaminated northern Adriatic, no significant increase in MeHg concentration along the water column is observed. The highest concentrations of methylated mercury species are not found near the shelf edge, suggesting that the sediments of the continental shelf are not the main MeHg source for the water column in the open sea (COSSA *et al.*, 2009). MeHg production is the greatest in the deeper central and southern Adriatic waters (southern Adriatic Pit), probably due to biological or chemical processes, an unknown benthic source or slower removal of MeHg from the water column (KOTNIK *et al.*, 2015). KOTNIK *et al.* (2007) measured DMHg below the detection limit (80 fg L^{-1}) at the Strait of Otranto. The average concentration of DMHg in Adriatic waters is 1.1 pg L^{-1}, while significantly lower values are found in the northern Adriatic (KOTNIK *et al.*, 2015). However, elevated concentrations of DMHg (up to 4.7 pg L^{-1}) are found near the island of Vis above the sea bottom, and in deep waters of the Southern Adriatic Sea. On average, DMHg represents 1.7% of THg. Differences between concentrations in the Adriatic and Mediterranean Sea are probably due to various anthropogenic sources (e.g. chlor-alkali plants, the Idrija mercury mine) (KOTNIK *et al.*, 2015) which provide sufficient substrate for methylation in this semi-enclosed sub-basin.

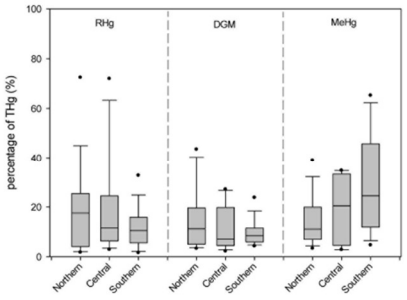


Fig. 6. Box-and-whisker plots of the geographical distribution of RHg, DGM and MeHg percentage of THg in the Adriatic Sea. Data from KOTNIK et al., 2015

DGM is relatively low in surface waters and usually shows a steady increase towards the bottom during summer and spring (KOTNIK *et al.*, 2007), which reflects the importance of evasion and photochemical reactions at the surface (HORVAT *et al.*, 2003). In deeper waters, DGM distribution indicates the importance of redox processes, microbial production, diffusion from sediment and/or geothermal/hydrothermal fields, especially at locations in the southern Adriatic Pit, which is tectonically very active (KOTNIK *et al.*, 2013). DGM spatial distribution mostly follows THg distribution; the highest concentrations and percentages are found in the northern Adriatic (Fig. 6), reflecting anthropogenic sources (KOTNIK *et al.*, 2015). Correlations between maximum

DGM and RHg, and the oxygen-depleted zone are usually observed (KOTNIK *et al*., 2013). Spatial distribution of RHg usually follows that of THg; the highest concentrations are found in the most contaminated parts of the northern Adriatic (up to 0.37 ng L^{-1}) (KOTNIK *et al*., 2015).

Mercury in the Adriatic sediment

Mercury distribution in the open Adriatic sediments was investigated in the 1970s and 1980s, but these research mostly covered the open northern Adriatic. We will present results from these studies, and although some values can be compared with recent results, it cannot be systematically performed due to lack of data, mainly MeHg concentrations. Mercury in the sediment of the Gulf of Trieste mainly originates from the discharge of the Soča/Isonzo River, which has been draining cinnabar-rich deposits of the Idrija mine for centuries (COVELLI *et al*., 2001, 2007; FAGANELI *et al.,* 2003). The effect of fluvial contribution, sediment re-suspension and the presence of localized natural and anthropogenic mercury sources can explain the observed highest THg levels in the northern Adriatic Sea (Fig. 7). The largest anthropogenic Hg sources are chlor-alkali/PVC factories that had used mercury in their technological processes (ACQUAVITA *et al*., 2012; CULLAJ *et al*., 2004; GUÉDRON *et al*., 2012; KWOKAL *et al*., 2002).

Previous studies found that THg in the sediments of the open northern Adriatic range from <0.1-16.9 µg g^{-1} (DONAZZOLO *et al*., 1984) with an average of 0.78 µg g^{-1} (MANTOVAN *et al*., 1985). Frequency histogram shows that 64% of samples have <0.4 µg g^{-1} (DONAZZOLO *et al*., 1984). The same percentage was reported by GUERZONI *et al*. (1984) for the open Adriatic sediments between Ancona and the Po River mouth. Decreasing THg gradient from the Po River mouth (0.97 µg g^{-1}) to Pesaro (0.06 µg g^{-1}) indicates that the Po River has an outstanding role in the trace metal pollution of this area (FRIGNANI *et al*., 1976, 1978). The average THg concentrations in the Gulf of Venice (0.56 µg g^{-1}) (DONAZZOLO *et al*., 1981), in surface sediments offshore the Po River mouth (0.4 µg g^{-1}) (FRASCARI *et al*., 1988; GUERZONI *et al*., 1985), and at the dredge disposal site outside the Ravenna harbour (0.28 µg g^{-1}) (GIANI *et al*., 1994; PELLEGRINI *et al*., 1992) are in the range observed for the open northern Adri-

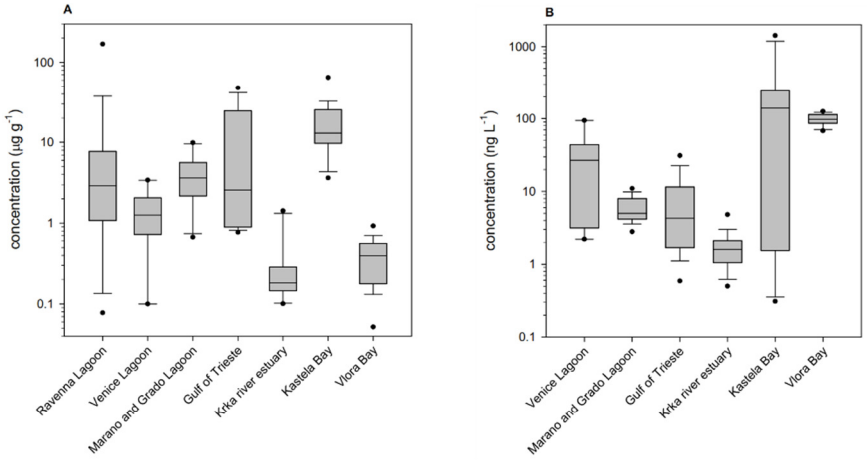


Fig. 7. Box-and-whisker plots of THg in sediments (A) and water (B) of the case studies. Data obtained from: ACQUAVITA et al., 2012; ANCONELLI et al., 1980; BERTO et al., 2006; BILINSKI et al., 2000; BLOOM et al., 2004; CULLAJ et al., 2004; FAGANELI et al., 2003; HINES et al., 2000; HORVAT et al., 1999, 2003; KOTNIK et al., 2015; KWOKAL et al., 2002; KWOKAL & LOVRIĆ, 2006; LAZO & CULLAJ, 2002; MELAKU CANU et al., 2015; MIKAC et al., 2006; QARRI et al., 2014; TROMBINI et al., 2003

atic sediments. In the southern Adriatic, THg in sediments range from 0.07-0.97 μg g^{-1}, with a median of 0.1 μg g^{-1} (SELLI *et al.*, 1973). FERRARA & MASERTI (1992) measured THg concentrations in the sediments of all three sub-basin of the Adriatic Sea. The concentration ranges are 0.05-8.63, 0.02-0.13 and 0.03-0.07 μg g^{-1} in the northern, central and southern Adriatic sediments, respectively. This is in a good agreement with the results from KOSTA *et al.* (1978) (0.01-15.1, 0.02-0.07, and 0.01-0.11 μg g^{-1} in the northern, central and southern Adriatic sediments, respectively). These concentrations exhibit the north-south gradient and emphasize the influence of localised natural and anthropogenic sources on Hg distribution. Mercury is mostly (95%) accumulated in the fine fraction of the sediment (DONAZZOLO *et al.*, 1984). The average THg concentration in offshore surface muds (0.34 μg g^{-1}) is higher than in offshore relict of the northern Adriatic (mostly sand; 0.10 μg g^{-1}) which reflects the observed positive correlation between THg and clay/mud (DONAZZOLO *et al.*, 1981; GUERZONI *et al.*, 1984).

Recent research shows that THg concentrations in the Adriatic sediments can vary more than 150-fold among locations, depending on the loading sources (KOTNIK *et al.*, 2015). In the offshore sediments, THg concentrations (0.02-0.26 μg g^{-1}) are in range with the values observed in the Mediterranean. The average concentration in unpolluted areas of the Central and South Adriatic is 0.12 μg g^{-1} (KOTNIK *et al.*, 2015) and is comparable with the previous measurements. In the coastal sediments (not including industrial hot-spots), THg values range from 0.10 μg g^{-1} in the Krka river estuary (KWOKAL *et al.*, 2002) to 47.8 μg g^{-1} in the Gulf of Trieste (HINES *et al.*, 2000). In the sediments contaminated by industrial activity, THg range from 0.05-250 μg g^{-1} (Fig. 7A). Based on the sedimentation rates, sediment porosity and density, calculated mercury accumulation rates in the Adriatic sediments range from 12.0-148 ng m^{-2} d^{-1} (KOTNIK *et al.*, 2015). These values are up to 14 and 57 times higher than in the Western and Eastern Mediterranean, respectively.

In front of the Po river plume, THg concentrations in sediment are characterised by a two-fold decrease from the top layer to the deep core (FOWLER *et al.*, 1999; GUERZONI *et al.*, 1985). Similarly, THg vertical profile is characterised by 2 to 6-fold surface enrichment with respect to background levels outside the Ravenna harbour (GIANI *et al.*, 1994), and 3 to 11-fold in the northern Adriatic sediment (FABBRI *et al.*, 2001). The background THg levels in the northern Adriatic cores are estimated to 0.10-0.12 μg g^{-1} (DONAZZOLO *et al.*, 1984; GUERZONI *et al.*, 1984, 1985; FRASCARI *et al.*, 1988). The maximum concentration of THg and MeHg at 22 cm depth is probably related to the year of maximum Hg production (i.e. 1913) when the Idrija mine produced double the annual Hg amount (FOWLER *et al.*, 1999). THg concentrations in the southern Adriatic sediment decrease from the surface (12.2 ng g^{-1}) to the deeper levels. Similarly, vertical profile of MeHg concentrations at the Strait of Otranto shows maximum in the surface sediment (2.94 ng g^{-1}) and decreases with depth to the range of the crust values (OGRINC *et al.*, 2007). Elevated MeHg concentrations at the Strait of Otranto are possibly a consequence of relatively high content of MeHg in the SPM, which is transported by water currents exiting the Adriatic Sea. During winter, sinking of surface water could cause the deposition of these particles onto the sediments (OGRINC *et al.*, 2007).

In the uppermost two centimetres, MeHg in porewaters has the highest average value found for the whole Mediterranean (8.78 ng L^{-1}) (OGRINC *et al.*, 2007). Vertical distribution of RHg and MeHg in porewaters are different; RHg is mostly constant while MeHg is constant in the uppermost four centimetres and then decreases with depth. Methylation potential for surface sediment is 0.14% d^{-1}; this is one of the lowest values for the whole Mediterranean, which is unusual when taking into account high MeHg concentration in the sediment and porewaters. Sedimentation of SPM rich in MeHg at the Strait of Otranto could explain this observation (OGRINC *et al.*, 2007). Estimated diffusive fluxes at the Strait of Otranto are 35.1 ng m^{-2} d^{-1} and 6.0 ng m^{-2} d^{-1} for RHg and MeHg, respectively. These values are 3 to 5-fold higher than corresponding fluxes in the western Mediterra-

nean Sea (OGRINC *et al.*, 2007). KOTNIK *et al.* (2015) calculated lower MeHg diffusive fluxes in the Adriatic (up to 3.35 ng m^{-2} d^{-1}) indicating local restriction on input of MeHg from the sediment.

Transport of mercury species in the Adriatic

KOTNIK *et al.* (2015) estimated mercury transport and mass balance in the Adriatic Sea based on several contributions (Table 1). They calculated overall deficit of more than 5 t a^{-1} of THg and 2.3 t a^{-1} of MeHg, and attributed it to additional Hg sources such as tectonic activity, dry deposition, and evasion overestimation. The greatest input fluxes are inflow from the Mediterranean Sea, terrestrial freshwater contributions (mainly Isonzo and Po rivers), and atmospheric deposition. Considering only seawater masses, the mass balance shows net import of inorganic Hg (0.6 t a^{-1}) and net export of MeHg (2.4 t a^{-1}) to the Mediterranean Sea. As the South Adriatic Deep Water flows out to the Mediterranean Sea at ~800 m depth (CIVITARESE & GAČIĆ, 2001), it is carrying Hg to the Mediterranean deep waters. Considering that the highest MeHg concentrations are found in the South Adriatic Deep Water (KOTNIK *et al.*, 2015), the Adriatic Sea exports methylated mercury into the Mediterranean Sea. The highest THg concentrations are usually found in the North Adriatic Surface Water. During cold and dry winters in the North Adriatic, this water mass cools, sinks and forms North Adriatic Deep Water (ARTEGIANI *et al.*, 1997) resulting in southward transfer of mercury. Due to the marine current system in the Adriatic Sea (Fig. 1) that carries particulate matter from the river Po delta in anticlockwise direction, the western coast of the central Adriatic Sea exhibits elevated Hg concentrations in SPM (FERRARA & MASERTI, 1992). Similarly, the greatest fraction of THg as RHg is found in the northern and central Adriatic (Fig. 6), but mostly along the western shore, indicating that inflowing water is not as reactive as outflowing water (KOTNIK *et al.*, 2015). Alongside the outflow to the Mediterranean, evasion to the atmosphere is the most important route for Hg removal from the marine environment. Due to supersaturation of surface water with DEM, it easily escapes to the atmosphere (GÅRDFELDT *et al.*, 2003), exceeding atmospheric deposition by a factor of 8. DGM in surface water shows higher values in the summer than in spring due to higher water temperatures and higher evaporation rates (KOTNIK *et al.*, 2007). Based on the sedimentation rates, it appears that sediment is slowly removing both THg and MeHg from the water column.

Case studies in the Adriatic Sea

Coastal environments are areas of great ecological and economic importance, but also some of the most affected environments due to human activities. In the Adriatic Sea, coastal sites that bear the greatest Hg contamination are Ravenna Lagoon, Venice Lagoon, Marano and Grado Lagoon, the Gulf of Trieste, the Bay of Kaštela and the Bay of Vlora (Fig. 1). Pristine environment of the Krka river estuary is also a subject of mercury research. Different conditions in these coastal areas (redox potential, temperature, salinity, presence of methylating bacteria,

Table 1. Mercury mass balance in the Adriatic Sea. Data from KOTNIK et al., 2015. Negative values represent output fluxes from seawater

Source	THg (t a^{-1})	MeHg (t a^{-1})
Terrestrial freshwater contributions	9.1	0.014
"Hot spots"	1.1	0.006
Atmospheric deposition	2.2	
Evasion to atmosphere	-16.2 (as DGM)	
Sedimentation	-2.0	-0.62
Resuspension from sediment	0.5	0.114
Inflow from the Mediterranean	11.2	2.8
Outflow to the Mediterranean	-13.0	-5.2
Present is seawater	22.9 t	4.2 t

etc.) cause specific biogeochemical transformations of mercury.

Ravenna Lagoon (Pialassa Baiona)

Pialassa Baiona Lagoon mostly consists of shallow ponds. This lagoon received ~38 t of the estimated 100-200 t of Hg discharged by an acetaldehyde plant (1958-1978) (COVELLI *et al.*, 2011). THg concentrations in sediments are variable and follow pollution gradient caused by northward water transport (TROMBINI *et al.*, 2003). The highest THg concentrations (250 μg g^{-1}) are found in the channel connected to the discharge of the plant, indicating the presence of dissolvable Hg in the surface sediment (FABBRI *et al.*, 1998). Deep sediment profiles show an Hg concentration peak (40 μg g^{-1}) at 3-7 cm depth, which matches the time of industrial site conversion (MATTEUCCI *et al.*, 2005). COVELLI *et al.* (2011) found that Hg^0 represents the dominant fraction (87.5%) in the sediment. Differences in mercury speciation across the lagoon might reflect different biogeochemical cycling (TROMBINI *et al.*, 2003). MeHg concentrations in sediments (0.13-44.6 ng g^{-1}) represent a small fraction of THg (up to 0.07% in surface sediments) which TROMBINI *et al.* (2003) attributed to bacterial detoxification; this value is smaller than the characteristic ~0.5% for estuarine environments (COVELLI *et al.*, 2011). MeHg porewaters concentrations show the highest values in the first centimetre (COVELLI *et al.*, 2011), suggesting that methylation occurs in a thin layer, close to sediment-water interface. Calculated distribution coefficient K_D for THg (log K_D 6.27) is almost constant with depth, indicating a steady-state equilibrium between two phases that controls THg distribution in the presence of sulphide (COVELLI *et al.*, 2011). Mercury is still present in surface sediments and can pose a "time bomb" for the ecosystem (FABBRI *et al.*, 1998).

Venice Lagoon

Venice Lagoon is the largest lagoon in the Mediterranean. Two chlor-alkali plants (1951-1988) were the main contributors to Hg contamination (GUÉDRON *et al.*, 2012) with an estimated discharge of 100-300 t (BLOOM *et al.*, 2004). In the most contaminated areas, THg in water is up to 201 ng L^{-1}. Dissolved THg accounts for 4-39% of THg, while dissolved MeHg represents up to 2.8% of THg (BLOOM *et al.*, 2004). Mercury distribution in SPM shows weak concentration gradient from the northern (1.17 μg g^{-1}) to the southern part (0.76 μg g^{-1}) of the lagoon. The highest value (3.26 μg g^{-1}) is found in the industrial canal (BLOOM *et al.*, 2004). In surface sediments, THg has slightly higher values, while MeHg shows 4-10 times higher values in SPM (log K_D 6.89) (BLOOM *et al.*, 2004). Therefore, tidal flushing may enhance dispersal of particulate MeHg throughout the lagoon (GUÉDRON *et al.*, 2012). MeHg in sediments never excided 0.45% of THg; it acts as a net source of MeHg for SPM and a sink for particulate inorganic Hg (GUÉDRON *et al.*, 2012). MeHg is highly correlated to THg and organic matter, suggesting their influence on methylation in the sediments (GUÉDRON *et al.*, 2012). Specific methylation rates are usually the highest in the surface layers (up to 5.5% d^{-1}) (KIM *et al.*, 2011). GUÉDRON *et al.* (2012) found three MeHg peaks in sediment porewaters at the depths corresponding to the reduction of Fe oxides, oxidation of fresh OM, and sulphate reduction. Their results indicate that the Fe reduction zone is the most important source of MeHg for the water column. High molecular diffusion fluxes (9.46 and 1.09 ng m^{-2} d^{-1} for Hg(II) and MeHg, respectively) (GUÉDRON *et al.*, 2012) indicate that open lagoon sediments may represent an important source of dissolved MeHg (GUÉDRON *et al.*, 2012). BLOOM *et al.* (2004) estimated 1.1 t a^{-1} Hg flux into the Adriatic Sea, which makes the Venice Lagoon one of the biggest exporters of Hg to the Adriatic Sea.

Marano and Grado Lagoon

The Grado-Marano wetland system consists of the western, shallow Grado sector, and eastern Marano sector, which is largely covered in saltmarshes (FONTOLAN *et al.*, 2012). Source of Hg pollution was a chlor-alkali plant (1949-1984) and 186 t of Hg were deposited

into the lagoons (ACQUAVITA *et al*., 2012). Hg^0 from chlor-alkali plant is the main fraction in the Marano sector and sulphide-bound Hg from Isonzo River in the Grado sector (ACQUAVITA *et al*., 2012). Presence of Hg from the Isonzo River is observed through the THg gradient in surface sediments; from 11 μg g^{-1} in Grado to 1 μg g^{-1} in Marano (COVELLI *et al*., 2011). The highest THg concentrations are usually found in the top core sections with almost exponential decrease from about 30 cm depth to the basal core where background values are found (average 0.13 μg g^{-1}), indicating recent contamination (COVELLI *et al*., 2012). MeHg in sediments accounts for up to 0.2% of THg (COVELLI *et al*., 2008). MeHg is the highest in surface sediments (up to 21.9 ng g^{-1}) and decreases with depth almost exponentially, suggesting that MeHg accumulation and production occur just below the sediment-water interface (COVELLI *et al*., 2008). MeHg accounts for only 0.03% of THg in SPM, and therefore additional MeHg must be produced by microbial activity (COVELLI *et al*., 2008). The average concentrations of THg and MeHg in porewaters are 99.1 ng L^{-1} and 0.84 ng L^{-1}, respectively (EMILI *et al*., 2012). COVELLI *et al*. (2008) found that THg in porewaters of the Grado sector increased with depth which could be due to Hg scavenging by soluble Fe and Mn (oxy)hydroxides at low oxygen and sulphide conditions. The log K_D for MeHg is ~3 (COVELLI *et al*., 2008; HINES *et al*., 2012), indicating low affinity for sediment matter. Diffusive fluxes range from -37-570 ng m^{-2} d^{-1} for THg and from -5.5-16.3 ng m^{-2} d^{-1} for MeHg (EMILI *et al*., 2012). High benthic fluxes (2.54-41.1 μg m^{-2} d^{-1} for THg and 0.08-2.29 μg m^{-2} d^{-1} for MeHg) (EMILI *et al*., 2012) suggest that sediments in the lagoon are an important source of mercury for the water column (COVELLI *et al*., 2008, 2011). Sulphate reducing bacteria (SRB) are mostly responsible for Hg methylation and the oxidative demethylation of MeHg (HINES *et al*., 2012). Demethylation rates are ~10 times higher than methylation rates (HINES *et al*., 2012), which might decrease the rate of MeHg accumulation.

The Gulf of Trieste

The Gulf of Trieste is a shallow water basin (up to ~25 m) located in the northeastern part of the Adriatic Sea. Mercury contamination originates from the Isonzo River, which drains the cinnabar deposits from the world's second largest Hg mine in Idrija, Slovenia (HORVAT *et al*., 1999). Over 5×10^9 kg of Hg ore was excavated and 73% (estimation) of the mercury was recovered (COVELLI *et al*., 1999). THg in the surface water layer of the central part of the Gulf is relatively low, but higher concentrations (up to 64.5 ng L^{-1}) are found in the area in front of the Isonzo River mouth as a consequence of sediment re-suspension (Fig. 7B) (FAGANELI *et al*., 2003). MeHg shows low surface values and higher bottom values (up to 126 pg L^{-1}) (FAGANELI *et al*., 2003). In the Gulf waters, THg is highly associated with SPM (log K_D 5-6). Particulate Hg accounts for <10% to >90% of THg present in the seawater, with the highest values found in the surface layer near the Isonzo River outflow (FAGANELI *et al*., 2003). During high river floods, concentrations of particulate Hg in front of the river outflow are 5-40 times higher than during low river discharge (COVELLI *et al*., 2007). Low log K_D for MeHg (<1) indicates much weaker binding of MeHg to SPM (FAGANELI *et al*., 2003; HORVAT *et al*., 1999). THg in sediments and short cores (0-10 cm) shows homogeneous distribution of mercury (HORVAT *et al*., 1999). On the contrary, cores below 100 cm depth have THg concentrations around the background values (0.17 μg g^{-1}) while the sudden increase is observed toward the surface layers (COVELLI *et al*., 2001, 2006a). MeHg concentrations in surface sediments (0.2-60.1 ng g^{-1}) correspond to 0.3-1.0% of THg. This value increases with the distance from the river mouth (HORVAT *et al*., 1999), suggesting that coastal areas where methylation occurs are not necessarily areas with the highest THg concentrations (COVELLI *et al*., 2001). Methylation activity in sediments (up to 2.0% d^{-1}) decreases with depth, indicating that recent sediment could represent a source of MeHg for the water column (COVELLI *et al*., 1999; HORVAT *et al*., 1999). SRB mediated oxidative demethylation is mostly active in the

uppermost layer of sediment, while reductive demethylation is negligible (<10%) (HINES *et al.*, 2000). THg concentration in porewaters is relatively high (107 ng L^{-1}). This elevated value could be related to the solubility changes of Fe and Mn (oxy)hydroxides (COVELLI *et al.*, 1999), similarly as in the Marano and Grado Lagoon. Diffusive fluxes range from -2.12-41.3 ng m^{-2} d^{-1} and from -1.77-4.39 ng m^{-2} d^{-1} for THg and MeHg, respectively (COVELLI *et al.*, 1999). Benthic fluxes (71-665 ng m^{-2} d^{-1} for THg and 238-577 ng m^{-2} d^{-1} for MeHg) (EMILI *et al.*, 2014) are much lower than those observed in the Marano and Grado Lagoon. Dissolved Hg in the benthic chamber water is dominantly in the methylated form during anoxic phase (EMILI *et al.*, 2011, 2014). About 75% of THg is buried in the sediment and the rest is recycled at the sediment-water interface. Methylated form represents ~23% of recycled THg (COVELLI *et al.*, 1999), making the Gulf of Trieste an efficient trap for THg and a source of MeHg to the bottom waters (HINES *et al.*, 2000).

Kaštela Bay

The Bay of Kaštela is the largest bay in the central Adriatic. It is an area of high primary production due to the weathering of nutrients from surrounding fields (ZVONARIĆ, 1991). The Bay was contaminated with at least 150 t of Hg (estimated) originating from a chlor-alkali plant (1950-1990) (KWOKAL *et al.*, 2002; ZVONARIĆ, 1991). Concentrations of THg in surface waters close to the chlor-alkali plant are high (up to 145 ng L^{-1}). Even higher concentrations in bottom waters suggest re-suspension from sediments or partial dissolution (KWOKAL *et al.*, 2002). The lowest THg concentrations are found at the exit of the bay (Fig. 7B) (HORVAT *et al.*, 2003). Approximately 70-100% of THg in water column is associated with SPM (KWOKAL *et al.*, 2002). MeHg concentrations in waters are in the range 0.01-0.04 ng L^{-1} (HORVAT *et al.*, 2003). Percentages of dissolved MeHg vary, but the highest value (46.8%) is found near the chlor-alkali plant (HORVAT *et al.*, 2003). Elevated THg concentration in the sediment (Fig. 7A) can be explained by the fact that the chlor-alkali plant discharged ~1 t a^{-1} of Hg^0 until 1990 (KWOKAL *et al.*, 2002). Very high concentrations are also found at up to 0.5 m depth (MIKAC *et al.*, 2006). Depth distribution of THg is irregular near the chlor-alkali plant due to human perturbation of sediments, which are a mixture of coal ash and natural sediments (MIKAC *et al.*, 2006). MeHg concentrations are typical for slightly to highly contaminated estuarine sediments (KWOKAL *et al.*, 2002) but the MeHg/THg ratio in sediments is rather low (<0.18%). Even though the sediments are highly anoxic, this indicates that requirements for *in situ* methylation do not exist (KWOKAL *et al.*, 2002). Therefore, most of Hg remains in the discharged form. Hg^0 is probably the only stable phase or perhaps Hg is bound to sulphide or sulphydryl groups (KWOKAL *et al.*, 2002).

Vlora Bay

The Bay of Vlora represents the natural boundary between the Ionian and the Adriatic Sea. Pollution arises from the use of metallic Hg for chlor-alkali electrolysis. During factory operation (1976-1992), liquid wastewaters containing 1.1 mg L^{-1} of mercury was discharged directly into the sea (CULLAJ *et al.*, 2004). It has been estimated that about 65 t of mercury had been discharged in the environment from 1977-1983 (CULLAJ *et al.*, 2004). In the sediment, mercury can reach up to 0.92 μg g^{-1} (Fig. 7A). Pollution zone is not restricted to the plant area: CULLAJ *et al.* (2004) observed Hg relocation to the SW part of the Bay of Vlora and attributed it to hydrological factors. These factors do not favour Hg transportation to the open sea due to restricted communication. The biggest contributor to Hg transport is the fine sediment fraction. Mercury is mostly present in the fine fraction of the sediment (<63 μm; 0.60 μg g^{-1}), contrary to the coarse fraction (<250 μm) that contains only 0.23 μg g^{-1} of mercury (CULLAJ *et al.*, 2004). THg concentrations in seawaters are high (Fig. 7B) and the highest values are found in front of the chlor-alkali plant (QARRI *et al.*, 2014; LAZO & CULLAJ, 2002). Organic mercury fraction represents 50-59% of THg (LAZO & CULLAJ, 2002). Although the mercury-polluted soil was encap-

sulated in 2011, previous emissions of metallic mercury can still be observed in the sediments of the Bay of Vlora due to soil erosion (QARRI *et al*., 2014).

Krka River estuary

The Krka river estuary is a stratified estuary located in the central part of the Eastern Adriatic coast. The biggest contamination sources are industrial and municipal wastewaters from the town of Šibenik. In comparison with other sites, this region can be characterised as relatively pristine (KWOKAL *et al*., 2002). THg concentrations in the sediment of Krka river estuary are generally low (Fig. 7A). Slightly elevated values are found in the vicinity of urbanized areas near the city of Šibenik. MeHg has the lowest values among all case studies (0.50-2.96 ng g^{-1}) (KWOKAL *et al*., 2002). In the water, MeHg values are in the range of 0.03-0.14 ng L^{-1}. The highest values are found within the freshwater-seawater interface (FSI) which can be explained by the association of Hg with mineral particles and dissolved organic compounds in the FSI (KWOKAL & LOVRIĆ, 2006). Presence of hydrophobic heterodispersed substances provides evidence that Hg is bound to hydrophobic particulate matter (BILINSKI *et al*., 2000). THg concentrations in the water column (up to 12.5 ng L^{-1}) vary with depth and within each layer. THg maximum can be found in freshwater, at the FSI, below the FSI, or not at all (BILINSKI *et al*., 2000). Input of atmospheric mercury into the estuary *via* precipitation can be observed through the Hg maximum in the freshwater layer. Strong winds can affect mercury concentration in the water column. During Bura wind (NE), mercury is transported to the sediments, resulting in low water column concentrations (<1 ng L^{-1}). On the contrary, during Jugo wind (SE) the outflow of brackish water decreases and water current in the marine layer goes from the sediment to the FSI (BILINSKI *et al*., 2000; KWOKAL & LOVRIĆ, 2006).

ROLE OF PLANKTON IN MERCURY BIOGEOCHEMISTRY

In the Mediterranean Sea, several fish species (STORELLI *et al*., 1998) show elevated mercury levels compared to the same species in the Atlantic Ocean, although THg concentrations in these two water masses are comparable (ŽAGAR *et al*., 2013). The hypothesis that above average natural environmental levels in the Mediterranean are responsible conflicts with measured concentrations and distribution of mercury in the Mediterranean Sea (HORVAT *et al*., 2003). In the open sea, oligotrophic environment plays an important role in mercury biogeochemistry. With relatively few nutrients, low primary productivity and low phytoplankton biomass, the Mediterranean Sea is classified as oligotrophic (UNEP, 2012). Oligotrophic conditions are characterised by higher proportions of dissolved rather than particulate carbon (ŠOLIĆ *et al*., 2010). In oligotrophic systems, picoplankton dominates the biomass and the production (ŠOLIĆ *et al*., 2010). Heterotrophic picoplankton (mostly heterotrophic bacteria) and autotrophic picoplankton (*Prochlorococcus*, *Synechococcus* and picoeukaryotes) represent the major components of the marine picoplankton community, especially in oligotrophic areas such as the Adriatic Sea (ŠOLIĆ *et al*., 2010, 2016). Vertical profiles of methylated mercury species show nutrient-like patterns within the biologically active zone (COSSA *et al*., 2009). HEIMBÜRGER *et al*. (2010) showed that Hg methylation was promoted during oligotrophic periods in the hypoxic water layer when primary production was dominated by nano- and picophytoplankton. These microorganisms may favour Hg methylation due to their great surface/volume ratio, which enables efficient accumulation of particle-reactive trace metals. Two MeHg maxima exist in the water column in which phytoplankton-derived dissolved organic carbon and particulate organic carbon are the main bacterial carbon sources in the photic and aphotic zone, respectively (HEIMBÜRGER *et al*., 2010). Elevated MeHg concentrations in the surface and bottom water in the southern Adriatic Sea are probably the result of similar biological processes (KOT-

NIK *et al.*, 2015). Concentrations of MeHg in the seawater are correlated with chlorophyll *a* and oxygen concentrations, emphasising the role of planktonic production and mercury regeneration through methylation/demethylation processes (KOTNIK *et al.*, 2013, 2015). Also higher temperature of the deep waters may promote microbiologically mediated formation of MeHg in the water and sediment (COSSA & COQUERY, 2005). The structure of the microbial food web and the microbial role in biogeochemical processes in aquatic environments can vary considerably and depends on environmental trophic status (ŠOLIĆ *et al.*, 2010).

CONCLUSIONS

The Adriatic Sea exhibits the complexity of mercury cycling and the importance of the anthropogenic influence, especially in coastal areas. Mercury in the Adriatic seawaters and sediments shows a concentration gradient from the north to the south; the highest THg concentrations in the whole Mediterranean Sea are found in the northern Adriatic Sea. Changes in concentrations of mercury species with time can be observed in both the Mediterranean and the Adriatic Sea, especially for THg and RHg concentrations in seawater. These changes are probably connected to the closure of the Idrija mine and chlor-alkali plants, or possible sample contamination in the past. The trend cannot be accurately assessed for other Hg fractions in seawater because most of them were not measured prior to 1990s. In the sediments, THg concentrations are comparable with the newer results. However, THg values in the central and southern Adriatic sediments, and Hg speciation in sediments are too scarce to comprehensively assess Hg biogeochemistry in the Adriatic sediments. From the available data, it is evident that Hg contamination is mostly limited to the surficial sediments, while concentrations in the deeper cores are near background values.

Although the waters of the northern Adriatic Sea, along with the coastal lagoons, have the highest THg values in the whole of Adriatic, this does not reflect the distribution of MeHg. Recent data indicate that MeHg production in the water column is the major source of MeHg in the Adriatic waters (especially in the southern Adriatic Pit), and that diffusion from the sediments cannot account for all observed MeHg. This indicates the important role of plankton in the methylation processes. However, in the coastal areas, bacterial methylation in the sediments plays an important role. Mercury methylation rates in the sediments of the Gulf of Trieste and the Venice lagoon are the highest in the whole Adriatic Sea. Water circulation can transfer produced MeHg and disperse it into the open Adriatic.

The spatial distribution of Hg in the marine environment depends on the seawater circulation, and biological and geological factors. These factors influence the transformations of Hg, leading to production of substantial amounts of MeHg, which can represent a major ecological burden, especially to the marine biota in the Adriatic Sea. In order to fully assess the impact of contamination in the Adriatic Sea, future research needs to focus on parameters that control Hg transformations in the environment. Thorough determination and speciation of Hg in all environmental compartments, together with the identification of specific biological (microbial methylation/demethylation), physical (diffusion, solubility, volatility) and chemical processes (redox, radical and photoreactions, methylation/demethylation, stable isotopes fractionation), could provide a better insight into Hg biogeochemistry and the susceptibility of the Adriatic Sea to contamination.

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Zastupljenost, raspodjela i specijacija žive u vodama i sedimentima Jadranskog mora – pregled

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SAŽETAK

Ovaj pregledni članak usredotočuje se na specijaciju žive u Jadranskom moru, rubnom moru Mediterana, koje predstavlja njegovu posebnu biogeokemijsku podjedinicu zbog antropoloških izvora žive. Trenutno znanje o kruženju žive u Jadranu je predstavljeno kroz pregled vrhunskih istraživanja na ovom području o vremenskim i prostornim raspodjelama i pojavnostima živinih specija u vodi i sedimentu te kemijskim transformacijama. Sumirani su rezultati istraživanja o specijaciji žive kako bi se opisala njena prisutnost i sudbina u Jadranskom moru. Jadransko more predstavlja neto izvor žive u Sredozemlju zbog najviših koncentracija ukupne žive uočenih u sjevernom Jadranu te najviših koncentracija metilžive u južnojadranskom ponoru. Međutim, biogeokemijski ciklus žive nije u potpunosti poznat te je ograničeno naše razumijevanje živinog transporta između odjeljaka i njenih (bio)transformacija. Buduće istraživanje se treba usredotočiti na mikrobne i kemijske procese živinih transformacija kako bi se bolje razumio utjecaj kontaminacije živom na okoliš i zdravlje ljudi u Jadranskom moru.

Ključne riječi: živa, kemijska specijacija, metilživa, Jadran, Sredozemlje

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3.2 The Abundance and Speciation of Mercury in the Adriatic Plankton, Bivalves and Fish – a Review

Živković, I., Šolić, M., Kotnik, J., Žižek, S., & Horvat, M. (2017). *Acta Adriatica*, *58*(3), 371–399.

In this paper, the authors presented an overview of published research about mercury abundance and speciation in plankton, bivalves and fish from the Adriatic Sea. General information about mercury transformation, uptake and bioaccumulation in these marine organisms were presented. Microbial role in trophic transfer of contaminants was discussed as autotrophic and heterotrophic picoplankton play an important role in marine biogeochemistry under oligotrophic and nutrient-limited conditions.

Mercury abundances in the Adriatic phytoplankton/microseston and zooplankton were compared to values obtained in other world seas, while special attention throughout this paper was given to the comparison with the Mediterranean Sea. The authors presented statistical analysis of spatial and temporal trends in mercury abundance and speciation in zooplankton from the Adriatic Sea.

Similarly, the abundances of mercury species in Mediterranean mussel from the Adriatic Sea were compared to those from other world seas. In addition, the authors presented mercury speciation data in other bivalves and discussed possible reasons for observed differences in mercury bioaccumulation between species. Importance of coastal lagoons in mercury transformations and bioaccumulation in bivalves was also estimated. Variations of mercury content in different mussel's tissues and the role of metallothioneins in mercury detoxification processes in mussels were briefly presented.

The authors gave particular attention to the comparison between three most important fish species from the Mediterranean and Adriatic Sea to assess spatial and temporal changes in THg concentrations. The authors collected, summarized and sorted mercury concentrations data in most studied Adriatic fish species by increasing median values to estimate the effect of feeding habits on mercury bioaccumulation. Influence of different environmental and biological parameters on mercury bioaccumulation in various fish species was also discussed. At the end, the authors presented mercury distribution in different fish tissues and the role of selenium on mercury detoxification processes in fish.

The candidate's contribution to this paper consisted of a thorough literature overview regarding mercury speciation in plankton, bivalves and fish. The candidate extracted data from published papers and summarized data in Excel sheets. As data were reported in various measurement units and were referred to both dry weight and wet weight basis, he transformed data to a common measurement unit based on wet weight using literature transformation factors. The candidate sorted data by marine organism, geographical area, and sampling period (where applicable). He performed statistical data analysis, drew figures and tables, and drafted the manuscript in cooperation with co-authors.

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The abundance and speciation of mercury in the Adriatic plankton, bivalves and fish – a review

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This article presents an overview of available research on mercury speciation in the most studied biota of the Adriatic Sea as a specific biogeochemical subunit of the Mediterranean. We present current knowledge about mercury concentration, speciation, spatial distribution and temporal changes in plankton, bivalves and fish from the Adriatic Sea. Results from mercury speciation studies in marine organisms are used to describe the extent of mercury bioaccumulation in the Adriatic Sea. Mercury biogeochemical cycle in the Adriatic is characterised by increased mercury concentrations from the water column, through plankton, bivalves and smaller fish to predator fish species. Although the Adriatic Sea has the highest mercury concentration in the whole Mediterranean Sea, fish species at the higher trophic levels have similar mercury concentrations everywhere in the Mediterranean, indicating incomplete understanding of the transfer mechanisms of mercury from seawater to upper trophic levels. As consumption of (contaminated) food is the main route of human exposure to mercury, it is of great importance to understand the influence of mercury contamination in the Adriatic Sea.

Key words: mercury, methylmercury, plankton, fish, mussel

INTRODUCTION

Mercury (Hg) is a toxic environmental pollutant due to its transport and transformation properties (DRISCOLL *et al.*, 2013; UNEP, 2013). Reactive chemistry of mercury drives its cycling through atmospheric, terrestrial, marine and biological compartment (FITZGERALD *et al.* 2007; MASON *et al.*, 2012). Methylmercury (MeHg) is a mercury species of the greatest concern because it readily bioaccumulates and biomagnifies through marine food web (MASON *et al.*, 2012; UNEP, 2013).

Phytoplankton and zooplankton

Although plankton represents the link between Hg marine cycle and higher trophic levels, this point of Hg entry into ocean food webs has not been extensively investigated (GOSNELL & MASON, 2015). This is due to low oceanic MeHg concentrations which impede measurements of MeHg uptake and trophic transfer under natural conditions (LAMBORG *et al.,* 2014; MASON *et al.,* 2012). Trace metal speciation in the marine environment is affected by microbial sequestration, composition and abundance of microorganisms, presence of natural complexing agents and remineralisation of organic matter (GOSNELL & MASON, 2015). Bioaccumulation factor (BAF) for MeHg ranges between 10^4-10^6 for phytoplankton (FITZGERALD *et al.,* 2007; LEE & FISHER, 2016; PICKHARDT & FISHER, 2007). BAF for MeHg increases by approximately $10^{0.5}$ units with the next trophic level (WATRAS & BLOOM, 1992), suggesting that each higher trophic level derives MeHg from the previous trophic level. MeHg bioaccumulation mechanisms in plankton and subsequent trophic transfer are still not well understood. Previous studies reported passive diffusion (LEE & FISHER, 2016; MASON *et al.,* 1996) and active transport of MeHg across the cell membrane (PICKHARDT & FISHER, 2007; WATRAS *et al.,* 1998). MeHg could be also co-transferred alongside the accumulation of strong MeHg ligands (e.g. cysteine, thiols) (MOYE *et al.,* 2002), or act as a surrogate for actively transported compounds (CHEN *et al.,* 2008). It seems that deep-sea sediments are not an important source of MeHg in the ocean plankton; HAMMERSCHMIDT & BOWMAN (2012) suggest that most of MeHg in phytoplankton (and subsequently in higher trophic levels) originates from production in the mixed layer of the open ocean.

Bivalves

Molluscs are one of the most important types of commercial shellfish with a variety of species cultured globally (STANKOVIĆ *et al.,* 2012). Bivalve molluscs are suspension feeders that filter phytoplankton, zooplankton and inorganic matter from the surrounding water through their gills (BARKER JØRGENSEN, 1990). The presence of trace metals in bivalves depends on environmental (location, metal concentrations in surrounding water, solubility, temperature, dissolved oxygen, pH, salinity) and biological factors (size, age, sex, species, feeding habits) (LEE *et al*., 1998; LOBEL *et al*., 1991; PHILLIPS, 1990). Mussels and oysters are convenient bioindicators of toxic metals in the marine environment due to their wide geographical distribution, sedentary filter-feeding and ability to accumulate high concentrations of contaminants relative to seawater (RAINBOW, 1995). An important mussel species for biomonitoring of environmental pollution in coastal areas is the Mediterranean mussel (*Mytilus galloprovincialis*). Mediterranean mussels are more extensively used than any other mollusc species such as oyster and clam, although their feeding habits are similar. In general, all shellfish are reliable pollution indicators due to their ability to bioaccumulate contaminants in their tissues, which can be used for the estimation of pollution with toxic metals in marine environment (EISLER, 1981; RAINBOW, 1995).

Fish

Fish are an important component of a well-balanced diet, providing a healthy source of energy, high-quality proteins, vitamins, essential metals and ω-3 polyunsaturated fatty acids (GRIBBLE *et al.,* 2016). However, consumption of carnivorous fish is the main pathway for human exposure to MeHg that can cause adverse health effect. Despite extensive research, exact reasons for elevated Hg concentrations in some fish species are still unknown (CHOY *et al.,* 2009). Nutrition is the dominant Hg uptake pathway (HALL *et al.,* 1997); fish at higher trophic levels are more likely to accumulate more Hg concentrations (STORELLI *et al.* 2002a). Benthic species have higher Hg concentrations due to their predatory lifestyle and longer lifespan (CHOY *et al.,* 2009; KOENIG *et al.,* 2013). Generally, Hg levels in fish increase with increasing size and age (ENDO *et al.,* 2008; HORVAT *et al.,* 2014). MeHg accounts for 75–100% of total Hg present in most fish species (BOSCH *et al.,* 2016; STORELLI *et al.,* 1998, 2005a). In

the fish muscles, it is bound to the protein fraction through sulfhydryl complexes (MASON *et al.,* 2006). Mercury distribution in fish tissues is governed by the uptake route connected with its chemical speciation (HORVAT *et al.,* 2014). Combined effects of external (physical and chemical properties of the marine environment) and internal factors (physiological and biochemical properties of fish) influence Hg uptake through biological membranes and its accumulation in tissues (CHOY *et al.,* 2009). These effects can lead to variations in metal accumulation within and among fish species, locations and seasons. This makes fish a good bioindicators for measuring Hg bioaccumulation in the marine environment (HORVAT *et al.,* 2014).

Objective

The objective of this paper is to present Hg bioaccumulation and biomagnification in selected marine organisms (plankton, bivalves and fish) and to emphasise the importance of Hg speciation in the Adriatic Sea as a specific biogeochemical subunit of the Mediterranean Sea. We present Hg concentrations in these organisms and statistically compare them to values obtained in different parts of the Adriatic and Mediterranean Sea in order to evaluate the current state of spatial Hg distribution. When possible, we also present temporal changes in Hg concentrations and corresponding statistical analysis in order to assess the effects of reduced anthropogenic Hg releases on its bioaccumulation in these organisms.

MATERIALS AND METHODS

Study area

The Mediterranean Sea is an elongated basin situated between Africa, Asia and Europe. The length of the basin along the east-west axis is 4000 km with a maximum width of 800 km and an average water depth of 1.5 km. The Strait of Sicily separates the Eastern and Western basins. The Mediterranean Sea exchanges water with the Atlantic Ocean through the Strait of Gibraltar, and with the Black Sea through the Bosporus



Fig. 1. Surface water circulation and geographical division of the Adriatic Sea. Adapted from KOTNIK et al. (2015).

Strait. Cinnabar deposits are the main natural Hg source – 65% of world's Hg reserves are located under the Mediterranean (ŽAGAR *et al.,* 2014). Main anthropogenic Hg sources are mining, cement production, coal combustion, and chlor-alkali plants (KOCMAN *et al.,* 2013; ŽAGAR *et al.,* 2014).

The Adriatic Sea (Fig. 1) is an elongated basin situated in the northernmost part of the Mediterranean Sea. It is connected with the rest of the Mediterranean Sea through the Strait of Otranto. Based on different biogeochemistry, latitude and bathymetry, it is divided into northern, central and southern Adriatic (UNEP/MAP, 2012). The southern Adriatic is the deepest (~1250 m) and the northern is the shallowest with an average depth of 35 m. Central Adriatic is on average 140 m deep. Eastern and western South Adriatic currents (E-SAd and W-SAd), Central and Southern Adriatic gyres (CAd and SAd), and North Adriatic current (N-Ad) are the main water currents in the Adriatic Sea (Fig. 1).

Conversion of measurement units, analytical methods and statistical analysis

Mercury concentrations in different marine biota cover a wide range and are reported in lit-

erature on either wet weight (ww) or dry weight basis (dw). We converted all concentrations to ng g^{-1} or µg g^{-1} ww so that we could compare values with the EU maximal allowed levels in foodstuffs (EUROPEAN COMMISSION, 2006). We used literature data for transformations of dw to ww values, as follows. Water content in phytoplankton/microseston and zooplankton fractions is assumed to be 90 and 95%, respectively (KNAUER & MARTIN, 1972). Hg concentrations in bivalves and fish are converted using the average dw/ww conversion factors of six and five, respectively (COSSA *et al.,* 2012; CRESSON *et al.,* 2014; JOVIĆ & STANKOVIĆ, 2014; KLJAKOVIĆ-GAŠPIĆ *et al.,* 2007; NASCI *et al.,* 1998). We used these conversion factors throughout this review, unless otherwise stated in the literature.

The choice of analytical methods for the determination of total mercury (THg) and MeHg in environmental samples depends on sample matrix and expected mercury concentrations. The most currently used method for the determination of THg in biological samples is acid digestion (or microwave digestion) followed by reduction and suitable detection (atomic absorbance spectrometry, atomic fluorescence spectrometry, inductively coupled plasma mass spectrometry) (e.g. ANDRAL *et al.,* 2011; BOGDANOVIĆ *et al.,* 2014; MIEDICO *et al.,* 2013). Determination of MeHg requires previous separation from inorganic Hg compounds. From the sample, MeHg can be extracted with suitable organic solvent or leached with diluted nitric acid. Derivatisation of extracted/leached MeHg (usually ethylation) is followed by MeHg adsorption on trap, separation from other Hg compounds by gas chromatography and suitable detection after pyrolysis (e.g. STORELLI *et al*., 2002b; HAMMERSCHMIDT *et al.,* 2013).

Statistical analysis was performed using Stata 12.0 software. Differences between groups were determined using Mann-Whitney U test (two groups) and Kruskal-Wallis test (three or more groups), after testing normality of original and transformed data (Wilk-Shapiro test). In figures, the distribution of Hg concentrations is presented with box-and-whisker plot (median with 5, 10, 25, 75, 90 and 95th percentiles).

RESULTS AND DISCUSSION

Microbial role in trophic transfer of contaminants

The microbial community plays a critical role in the biogeochemical Hg cycling, influencing its chemical speciation, reactivity, and bioavailability through mediation of processes such as methylation, demethylation, oxidation, and reduction. The key process that drives Hg bioaccumulation is methylation. HINES *et al.* (2017) found high Hg methylation potential in the sediments from the Gulf of Trieste, where sulphate-reducing bacteria are often involved in the transformation of inorganic Hg compounds. PARKS *et al.* (2013) showed that the number of bacteria capable of methylating Hg is much higher. Oxic ocean water can also host biotic Hg methylation (HEIMBÜRGER *et al.,* 2010). The highest seawater MeHg concentrations are related to the presence of small algae (nano- and picophytoplankton), as shown for Western Mediterranean (HEIMBÜRGER *et al.,* 2010). Dissolved MeHg, which is the most biologically available organic Hg form, is bioaccumulated up to a million times in microscopic particles, including phytoplankton and bacteria, via adsorption to cell surfaces in the water column (MASON *et al.,* 1996). These MeHg-enriched particles are then consumed by zooplankton, which in turn are a primary food source for larval, juvenile, and some adult fish (HALL *et al.,* 1997).

Heterotrophic bacteria and autotrophic picoplankton (APP) (*Prochlorococcus*, *Synechococcus* and picoeukaryotes) represent the major components of the marine picoplankton community, especially in oligotrophic areas such as the Adriatic Sea (ŠOLIĆ *et al.,* 2010, 2015, 2016). These organisms are consumed by heterotrophic nanoflagellate grazers, which are consumed in turn by larger ciliated protozoa, forming a link ('microbial loop') to higher trophic levels (AZAM *et al.,* 1983) (Fig. 2). The reason for domination of these microorganisms in oligotrophic conditions is due to the number of advantages these organisms have for living in nutrient-poor environments: lower energetic costs (because of simple biomass composition), rapid metabolism and

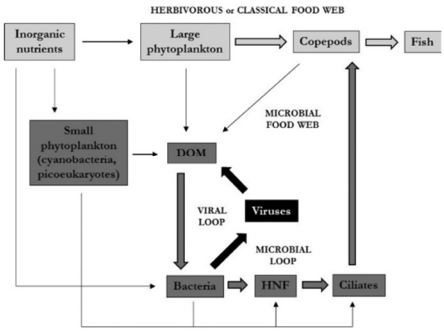


Fig. 2. Herbivorous (classical) food web versus microbial food web and role of "microbial loop" and "viral loop" within microbial food web. Redrawn from ŠOLIĆ et al. (2016).

small size which means less internal machinery and large surface/volume ratio (higher efficiency in nutrients uptake). The same factors that enable scavenging nutrients at low concentrations also facilitate contaminant accumulation in their biomass for subsequent transfer to other components in the food web. Prokaryotic heterotrophs accumulate contaminants more efficiently than phytoplankton (AXELMAN *et al.,* 1997), suggesting that prokaryotes may be a critical component of the food web that can promote our understanding of the behaviour of contaminants in marine systems.

FENCHEL (2008) suggested that autotrophic and heterotrophic organisms in the microbial loop play key roles in Hg transfer into marine food webs, influencing its biogeochemical cycling, as well as being major contributors to elemental cycling and vertical fluxes. One important impact of the microbial loop on Hg cycling in the water column is the acceleration of organic matter mineralization and the regeneration of nutrients to fuel primary production (FENCHEL, 2008). Furthermore, in comparison to classical herbivorous food web (large phytoplankton – large zooplankton – fish), microbial loop involves a higher number of trophic steps (bacteria – heterotrophic nanoplankton – microzooplankton – large zooplankton – fish), which implies greater concentration of contaminants along the food chain through biomagnification. Moreover, in eutrophic areas, where algal biomass increases, the concentration of MeHg per cell decreases, resulting in a lower dietary input to the herbivorous food web and actually reducing bioaccumulation in algal-rich eutrophic systems (PICKHARDT *et al.,* 2002; CHEN & FOLT, 2005). Further strengthening of the role of microbial food web could be expected in mainly oligotrophic Adriatic Sea if predicted rise of temperature in the near future results with increase of bacterial production (ŠOLIĆ *et al.,* 2017).

MERCURY IN PLANKTON

Mercury in phytoplankton/microseston

Mercury content in phytoplankton/microseston fraction is not often measured in the Adriatic or Mediterranean Sea. Therefore, available data do not allow systematic analysis of spatial and temporal trends. Seston can sometimes consist mainly of biological material other than phytoplankton. Analysis of material obtained by towing net (pore size 20 μm) in the Venice Lagoon showed that it was mostly composed of seagrass debris (DOMINIK *et al.,* 2014).

THg in phytoplankton (mostly dinoflagellates, diatoms and coccolithophorids) in Kaštela Bay (Fig. 1) was on average 315 ng g^{-1} (ZVONARIĆ *et al*., 1991). However, these samples were obtained during the operation of chlor-alkali plant that was releasing Hg-containing effluents directly into nearby sea. In the 1970s, FOWLER (1986) performed the most comprehensive analysis of THg in Mediterranean microseston. The overall average THg concentration was 5.40 ng g^{-1}. Similar to THg in zooplankton, western sub-basin had slightly lower THg content in microseston than eastern sub-basin (4.41 and 6.62 ng g^{-1}, respectively). To our knowledge, Hg speciation data in the Adriatic and Mediterranean phytoplankton are not available.

Average THg concentration in the Mediterranean seston is higher than in other world seas: 3.88 ng g^{-1} in NW Atlantic (HAMMERSCHMIDT *et al.,* 2013), 0.77 ng g^{-1} in Long Island Sound (GOSNELL *et al.,* 2017), 3.00 ng g^{-1} in the Baffin Bay (CAMPBELL *et al.,* 2005), 2.00 ng g^{-1} in the Baltic Sea (NFON *et al.,* 2009), and 1.95 ng g^{-1} in

the Ross Sea (BARGAGLI *et al.,* 1998). The higher average value found in the central Pacific Ocean (6.89 ng g^{-1}) shows that phytoplankton community concentrates more Hg from the water in the oligotrophic open ocean (GOSNELL & MASON, 2015). Direct comparison of these values should be taken with reserve however, due to differences in sampling methods, sampling years, and environmental conditions that influence Hg distribution in the sea. Furthermore, differences could also be attributed to changes in dominant organisms within the phytoplankton community (GOSNELL *et al.,* 2017).

Mercury concentrations in zooplankton from different seas

Mercury concentrations in marine plankton have been determined in the Mediterranean Sea since 1960s to provide an insight in Hg contamination at the lowest marine trophic levels. Comparison of recent data for THg in zooplankton from the Adriatic, Mediterranean, Atlantic and Pacific shows inverse relations (Fig. 3A) with statistically significant differences among locations (Kruskal-Wallis, $P < 0.001$). Although the highest THg concentrations in seawater are found in the Adriatic Sea (KOTNIK *et al.,* 2015), it seems that specific conditions that influence Hg uptake in biota are considerably different. Copepods account on average for 77% of mesozooplankton from the Adriatic and Mediterranean Sea, while long-lived copepods and euphausiids are the major zooplankton genera in the Atlantic Ocean (HAMMERSCHMIDT *et al.,* 2013). Even though the composition of zooplankton community is similar, the observed differences in THg concentrations in zooplankton from different seas (Fig. 3A) are probably due to different

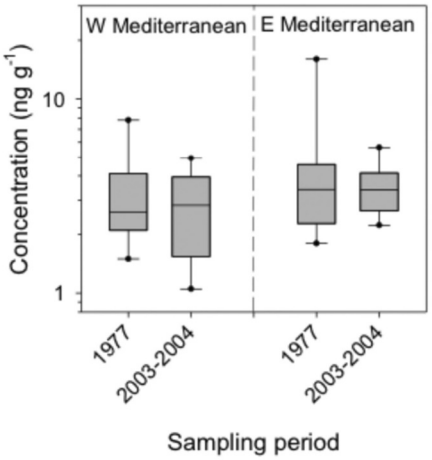


Fig. 4. Box-and-whiskers plots of the geographical distribution of THg in zooplankton from the Mediterranean Sea. Data from FOWLER, 1986; and M. Horvat (unpublished data).

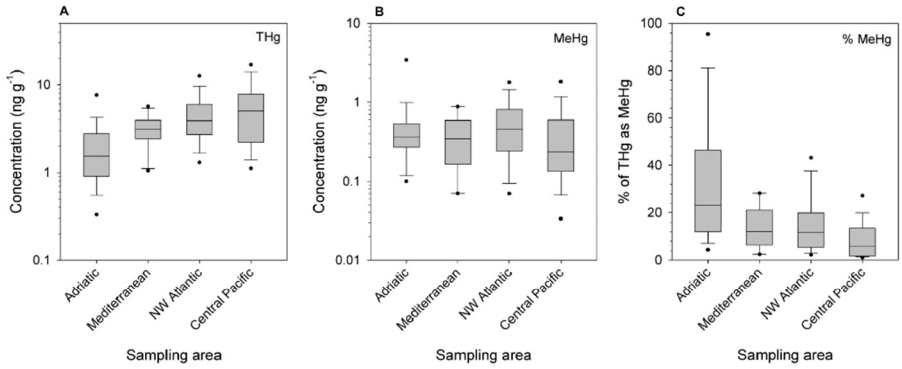


Fig. 3. Comparison of recent THg and MeHg concentrations and percentage of THg as MeHg in zooplankton from different world seas. Data from GOSNELL & MASON, 2015; GOSNELL et al., 2017; HAMMERSCHMIDT et al., 2013; and M. Horvat (unpublished data).

sampling methods and/or sampling periods. Another explanation might be significant difference in zooplankton water content, which causes error during transformation of concentrations expressed on a dry weight basis.

For MeHg, there are no significant differences between the Adriatic, Mediterranean and Atlantic zooplankton (Kruskal-Wallis, $P = 0.335$). Lower MeHg concentrations are found in the Pacific Ocean (Fig. 3B). Due to lower THg content, percentage of MeHg in zooplankton is the highest in the Adriatic Sea (33.1% on average; Fig. 3C).

In the Mediterranean Sea, the western sub-basin has slightly lower THg content in zooplankton than the eastern sub-basin (2.85 and 3.51 ng g^{-1}, respectively). It seems that these concentrations have not changed in the past 30 years (Fig. 4) indicating that localised Hg inputs do not have a considerable influence on THg concentrations in zooplankton in the open Mediterranean Sea as they do in the small enclosed basin such as Adriatic Sea (Fig. 5). Mercury speciation is rarely performed in zooplankton. Average MeHg concentrations in zooplankton are 0.25 and 0.28 ng g^{-1} in the western and eastern Mediterranean, which account for 9.16 and 9.68% of THg in Mediterranean zooplankton, respectively (M. Horvat, unpublished data).

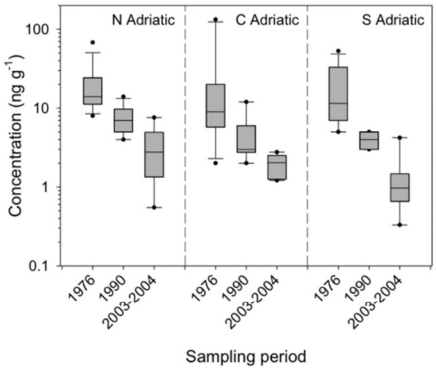


Fig. 5. Box-and-whiskers plots of the geographical distribution of THg in zooplankton from the Adriatic Sea. Data from FERRARA & MASERTI, 1992; KOSTA et al., 1978; and M. Horvat (unpublished data).

Mercury in Adriatic zooplankton

Changes in THg concentrations with time in the Adriatic zooplankton are significant (Fig. 5) (Kruskal-Wallis, $P < 0.01$ for all three sub-basins), contrary to trend observed in the Mediterranean Sea. In the Adriatic Sea, decrease in localised Hg input (mainly due to closure of Idrija mine and chlor-alkali plants in the northern Adriatic) is followed by up to 10-fold decrease in zooplankton THg. The highest natural THg concentrations (up to 53 ng g^{-1}) are found in the Gulf of Trieste, which have been affected by the polluted Soča/Isonzo River and its centuries-long draining of cinnabar-rich deposits from the Idrija mine (FAGANELI *et al.*, 2003). Temporal variability of zooplankton Hg is the consequence of its biomass, species composition and grazing. The speed of this variability is observed through a 25-fold decrease during a nine month period (HORVAT *et al.*, 1999). The highest THg concentrations in zooplankton are related to industrial mercury sources; VUČETIĆ *et al.* (1974) measured up to 1.63 µg g^{-1} of THg in zooplankton at the nearshore station in the central Adriatic Sea, and attributed this value to industrial pollution originating near Split. As in the Mediterranean Sea, MeHg measurements in the Adriatic zooplankton are scarce. In the Gulf of Trieste, MeHg ranges from 0.21-6.57 ng g^{-1}, with an average of 2.16 ng g^{-1} (HORVAT *et al.*, 1999) which represents 12.4% of THg in zooplankton. In the open Adriatic waters, MeHg in zooplankton shows a north-south concentration gradient (0.40, 0.41 and 0.80 ng g^{-1} in the northern, central and southern Adriatic, respectively; M. Horvat, unpublished data). The highest values coincide with the area of the highest MeHg in the Adriatic seawater (KOTNIK *et al.*, 2015), which indicates possible methylation of inorganic Hg by Adriatic microorganisms.

MERCURY IN BIVALVES

Mercury in mussels from different seas

The MEDPOL Programme recommended use of Mediterranean mussels as biomonitors of chemical contaminants in the Mediterranean Sea

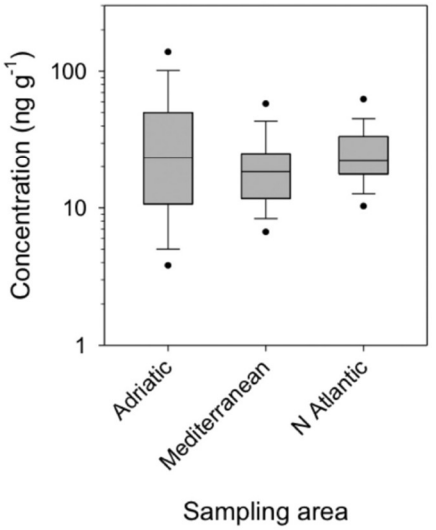


Fig. 6. Comparison of THg concentrations in mussels (Mytilus galloprovincialis) from different world seas. Data from: BENEDICTO et al., 2011; BILANDŽIĆ et al., 2016; BRIANT et al., 2017; CASTELLANI et al., 2015; CHEN et al., 2009; CLAISSE et al., 2001; CUBADDA et al., 2006; CULLAJ et al., 2006; FABBRI et al., 2006; FATTORINI et al., 2008; GALGANI et al., 2014; GORBI et al., 2008; KLJAKOVIĆ-GAŠPIĆ et al., 2006; KWON et al., 2014; LOCATELLI, 2003; LOCATELLI & MELUCCI, 2010; MARKOVIĆ et al., 2012; MOSCHINO et al., 2016; MZOUGHI & CHOUBA, 2012; SPADA et al., 2013; STANKOVIĆ et al., 2011; TSANGARIS et al., 2013.

(BENEDICTO *et al.*, 2011). Since this project was conducted in 1970s, several other projects (e.g. MYTILOS and MYTIOR) have been established to assess the state of trace metal contamination in the marine environment (ANDRAL *et al.*, 2011; GALGANI *et al.*, 2014). Recent data show that THg values in mussels are slightly, but significantly lower (Kruskal-Wallis, $P < 0.01$) in the Mediterranean Sea than in the Adriatic Sea and Atlantic Ocean (Fig. 6). The Adriatic THg values are obtained from both low-Hg and contaminated locations (BILANDŽIĆ *et al.*, 2016; KLJAKOVIĆ-GAŠPIĆ *et al.*, 2006) resulting in a wider box-and-whiskers plot. In the Mediterranean Sea, THg concentrations are characterised by approximately 10-fold decrease in 30 years period (Fig. 7). On average, THg in mussels from the western Mediterranean is higher than in the eastern sub-basin (23.0±19.2 and 17.9±11.5 ng g^{-1}, respectively), but without statistically significant difference (Mann-Whitney, $P = 0.124$). Mercury speciation data in the Mediterranean mussels are scarce; therefore, only THg concentrations are available as a proxy for environmental contamination. The most comprehensive Hg speciation in mussels from the western Mediterranean (French coast) was recently published by BRIANT *et al.* (2017). MeHg concentrations in these samples range from 3.00-28.7 ng g^{-1} (average 10.5 ng g^{-1}) and account for 11.4-57.0% of THg (average 30.3%). Almost the same percentage (29.0%) is found in the Ionian Sea (SPADA *et al.*, 2012). A similar mussel species (Blue mussel, *Mytilus edulis*) from the NW Atlantic, has an average MeHg concentration of 20.6 ng g^{-1} which accounts for 37.4% of THg (CHEN *et al.*, 2009). Slightly higher percentage (40.8%) is found in the same species from the French

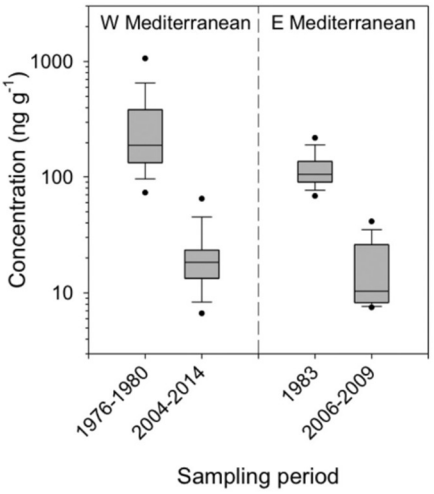


Fig. 7. Box-and-whiskers plots of the geographical distribution of THg in mussels (Mytilus galloprovincialis) from the Mediterranean Sea. Data from BENEDICTO et al., 2011; BRIANT et al., 2017; CASTAGNA et al., 1985; GALGANI et al., 2014; LEONZIO et al., 1981; MZOUGHI & CHOUBA, 2012; TSANGARIS et al., 2013.

Atlantic coast, but with almost 50% lower average MeHg concentration (11.4 ng g^{-1}) (BRIANT *et al.,* 2017). Bioaccumulation of MeHg in mussels can be observed through the 3-fold higher percentage of MeHg compared to Mediterranean zooplankton. However, filter-feeding bivalves accumulate less MeHg than fish and present a lower risk to humans (STANKOVIĆ *et al.,* 2012).

Mercury in Adriatic mussels

In the Adriatic Sea, relative changes in median THg concentrations with time (Fig. 8) are not as great as in the Mediterranean Sea. In the northern Adriatic Sea, an approximately 3-fold decrease in median mussel THg can be observed. This is probably due to decrease in environmental concentrations caused by closure of Idrija mine and chlor-alkali plants in the northern Adriatic (FAGANELI *et al.,* 2014). Interesting to note is the increase in mussel THg in the southern Adriatic, but this might be due to limited data prior to 1990s. When low-Hg environment and contaminated locations are separately compared (Fig. 9A), statistically significant decreases in mussel THg values with time are observed in both environments (Kruskal-Wallis, $P < 0.001$ for Kaštela Bay; Mann-Whitney, $0.002 < P < 0.05$ for Krka River estuary, Venice Lagoon and Ravenna Lagoon). Kaštela Bay, which has been contaminated by industrial effluents from a chlor-alkali plant (KLJAKOVIĆ-GAŠPIĆ *et al.,* 2010), has a 70-fold decrease in average mussel THg after plant closure in 1990. On the contrary, in the low-Hg environment of the Krka River estuary (Fig. 1), only a slight decrease can be observed (~26% during five years). Although water currents along the eastern Adriatic flow northwards (Fig. 1), it seems that anthropogenic Hg contamination is localised at its source. This effect is best observed in the shallow northern Adriatic Sea where counter-clockwise water currents carry cinnabar-rich particles from the Soča/Isonzo River delta to the adjacent Marano and Grado Lagoon. The average THg concentration in mussels from this lagoon (220±60 ng g^{-1}; MAJORI *et al*., 1991) was higher than in other parts of the northern Adriatic Sea (Fig. 8). The uncontrolled Hg discharges from the Torviscosa

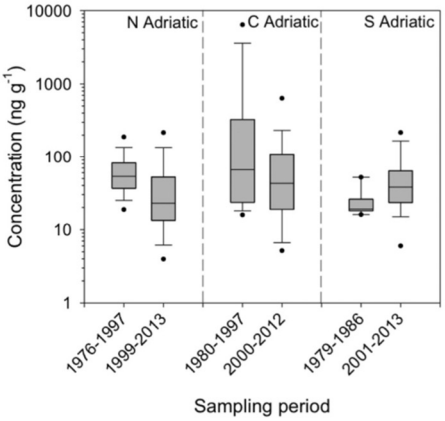


Fig. 8. Box-and-whiskers plots of the geographical distribution of THg in mussels (Mytilus galloprovincialis) from the Adriatic Sea. Data from BILANDŽIĆ et al., 2016; BLOOM et al., 2004; BOGDANOVIĆ et al., 2014; CASTELLANI et al., 2015; CATTANI et al., 1999; CORSI et al., 2011; CUBADDA et al., 2006; CULLAJ et al., 2006; DA ROS et al., 2000; DOMINIK et al., 2014; FABBRI et al., 2006; FATTORINI et al., 2008; FAVRETTO et al., 1997; GIORDANO et al., 1991; GORBI et al., 2008; JOVIĆ & STANKOVIĆ, 2014; KLJAKOVIĆ-GAŠPIĆ et al., 2006; KOSTA et al., 1978; LOCATELLI, 2003; LOCATELLI & MELUCCI, 2010; LOWE et al., 1995; MAJORI et al., 1991; MARKOVIĆ et al., 2012; MARTINČIĆ et al., 1987; MIEDICO et al., 2013; MIKAC et al., 1985, 1989, 1996; MOSCHINO et al., 2016; NAJDEK & SAPUNAR, 1987; NASCI et al., 1998; ODŽAK et al., 2000; RAMŠAK et al., 2012; SPADA et al., 2013; STANKOVIĆ et al., 2011; STORELLI & MARCOTRIGIANO, 2001; TURK et al., 2007; WIDDOWS et al., 1997; ZATTA et al., 1992; ZVONARIĆ, 1991.

chlor-alkali plant (1949-1984) were additional Hg sources in this lagoon (PIANI *et al*., 2005). Elemental Hg from chlor-alkali plant is the main sediment fraction in the Marano sector and sulphide-bound Hg from Soča/Isonzo River in the Grado sector (ACQUAVITA *et al*., 2012). Venice and Ravenna lagoons were also contaminated by Hg discharges from chlor-alkali and acetaldehyde plants in the Marghera industrial zone and Ravenna, respectively (BLOOM *et al*., 2004; COVELLI *et al*., 2011). Following the closure of these plants, average THg concentrations in mussels

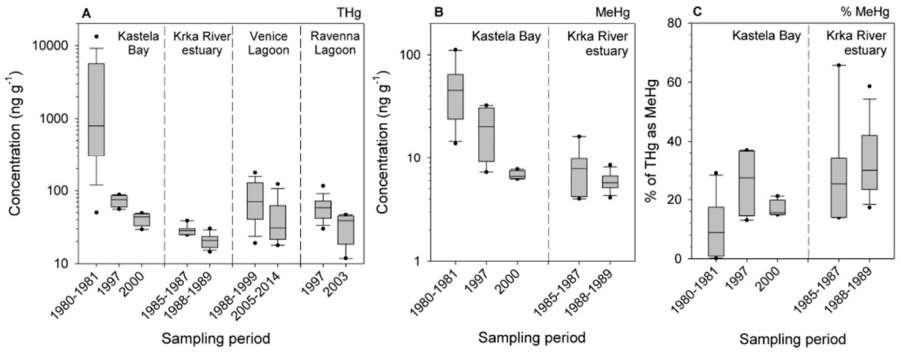


Fig. 9. Temporal changes of THg and MeHg concentrations and percentage of THg as MeHg in mussels from case studies in the Adriatic Sea. Data from BILLE et al., 2015; CASTELLANI et al., 2015; CATTANI et al., 1999; CUBADDA et al., 2006; DA ROS et al., 2000; DOMINIK et al., 2014; FABBRI et al., 2006; KLJAKOVIĆ-GAŠPIĆ et al., 2006; LOWE et al., 1995; MIKAC et al., 1985, 1989, 1996; NASCI et al., 1998; ODŽAK et al., 2000; WIDDOWS et al., 1997; ZATTA et al., 1992.

are characterised by an approximate 2-fold decrease (Fig. 9A). In addition, there is no statistically significant differences in THg concentrations in mussels after 2000 from Kaštela Bay, Venice and Ravenna Lagoons (Kruskal-Wallis, $P = 0.778$).

Mercury speciation data in mussels are scarce. However, some locations are more studied providing an insight in MeHg bioaccumulation in the Adriatic mussels. In both Kaštela Bay and Krka River estuary, we can observe a decrease in MeHg concentrations with time (Fig. 9B), but this decrease is not as large as for THg. The average MeHg values are similar (6.79 ng g^{-1} in Kaštela Bay in 2000, and 5.91 ng g^{-1} in Krka River estuary in 1988-1989), but are lower than observed in the western Mediterranean during 1987-2014 (10.5 ng g^{-1}; BRIANT *et al.,* 2017). Percentage of mercury as MeHg increases with time, to average values of 16.8 and 32.7% in Kaštela Bay and Krka River estuary, respectively (Fig. 9C). The percentage in the Krka River estuary is similar to that in the Mediterranean, and the low value in Kaštela Bay is in agreement with the observation that in this region conditions do not promote Hg methylation (KWOKAL *et al.,* 2002). In the Venice Lagoon, average MeHg concentration (52.2 ng g^{-1}) accounts for 57.4% of THg (DOMINIK *et al.,* 2014). Previous researches in this lagoon found lower percentage (37.9%), but exceptionally higher average MeHg concentration (138 ng g^{-1}) (BLOOM *et al.,* 2004).

Mercury speciation in tissues from Adriatic mussels

Only a few papers describe Hg speciation in mussel organs and tissues. In Kaštela Bay, average THg concentrations are the highest in the digestive gland (189 ng g^{-1}), followed by gills (109 ng g^{-1}), while the whole soft tissue has the lowest THg concentrations (70.6 ng g^{-1}) (ODŽAK *et al.,* 2000). In the Venice Lagoon, average THg concentration in digestive gland is much lower (105 ng g^{-1}) (LOWE & FOSSATO, 2000). THg values in digestive gland are approximately four times higher than in the gills in mussels from the Gulf of Trieste (ŽNIDARIČ *et al.,* 2006). Contrary to THg, MeHg has a different distribution in tissues: the highest average values are found in the whole soft tissue (20.0 ng g^{-1}), while gills and digestive gland have similar MeHg values (11.7 ng g^{-1}). KLJAKOVIĆ-GAŠPIĆ *et al.* (2006) found similar THg distribution, but almost uniform MeHg concentrations in all tissues. The lowest percentage of THg as MeHg was found in the digestive gland (8.31%), compared to whole soft tissue (21.3%) (KLJAKOVIĆ-GAŠPIĆ *et al.,* 2006).

Mercury speciation in tissues indicates different distribution pathways in the mussels. It appears that MeHg is transported to other tissues after uptake through digestive gland and gills (ODŽAK *et al.*, 2000), while inorganic Hg is accumulated in the digestive gland where it is probably transformed to some less toxic form before its excretion or storage (KLJAKOVIĆ-GAŠPIĆ *et al.*, 2006).

Mercury in other bivalves

Although mussels are usually used as bioindicators, THg concentrations in other bivalves can also be used to assess Hg contamination and distribution in the Adriatic Sea. Table 1 shows THg concentrations in whole shellfish tissue of Adriatic bivalves from recent studies. A decreasing southward THg gradient can be observed in European flat oyster (*Ostrea edulis*) and Manila clam (*Ruditapes philippinarum*) (Table 1). Observed average THg concentrations in these bivalves generally follow THg gradient in sediments and seawater, which is a consequence of either natural or anthropogenic Hg contamination (ŽIVKOVIĆ *et al.*, 2017).

Table 1. Total mercury concentrations (ng g^{-1} wet weight) in Adriatic bivalves.

Bivalve specie	Location	Range	Average	Reference
Callista chione	Veneto region	2.00-60.0	30.0	BILLE *et al.*, 2015
Cerastoderma edule	Veneto region	2.00-230	60.0	BILLE *et al.*, 2015
Cerastoderma glaucum	Venice Lagoon		166	DOMINIK *et al.*, 2014
Chamelea gallina	Apulia	6.00-25.0	17.0	MIEDICO *et al.*, 2013
Chamelea gallina	Veneto region	2.00-250	30.0	BILLE *et al.*, 2015
Mimachlamys varia	Istria/Gulf of Trieste	26.0-512	252	BOGDANOVIĆ *et al.*, 2014
Mimachlamys varia	Veneto region	2.00-120	20.0	BILLE *et al.*, 2015
Crassostrea gigas	Veneto region	2.00-120	60.0	BILLE *et al.*, 2015
Crassostrea gigas	Venice Lagoon		105	DOMINIK *et al.*, 2014
Crassostrea gigas	northern Adriatic	10.0-340	92.5	BURIOLI *et al.*, 2017
Donax trunculus	Veneto region	2.00-80.0	30.0	BILLE *et al.*, 2015
Ensis minor	Veneto region	10.0-130	60.0	BILLE *et al.*, 2015
Mactra stultorum	Veneto region	10.0-60.0	30.0	BILLE *et al.*, 2015
Ostrea edulis	Apulia	6.00-64.0	15.3	MIEDICO *et al.*, 2013
Ostrea edulis	Istria	30.0-100	60.0	BILANDŽIĆ *et al.*, 2015
Ostrea edulis	northern Adriatic	15.0-109	60.0	BILANDŽIĆ *et al.*, 2016
Ostrea edulis	southern Adriatic	13.0-70.0	39.0	BILANDŽIĆ *et al.*, 2016
Ostrea edulis	Veneto region	30.0-60.0	40.0	BILLE *et al.*, 2015
Pecten jacobaeus	Istria	10.0-30.0	20.0	BILANDŽIĆ *et al.*, 2015
Pecten jacobaeus	Veneto region	2.00-30.0	10.0	BILLE *et al.*, 2015
Ruditapes decussatus	Veneto region	20.0-190	70.0	BILLE *et al.*, 2015
Ruditapes philippinarum	Marano Grado Lagoon	54.0-985	308	GIANI *et al.*, 2012
Ruditapes philippinarum	Marano Grado Lagoon	225-338	272	SFRISO *et al.*, 2008
Ruditapes philippinarum	Ravenna Lagoon	42.0-92.0	77.4	TROMBINI *et al.*, 2003
Ruditapes philippinarum	Veneto region	2.00-190	40.0	BILLE *et al.*, 2015
Ruditapes philippinarum	Venice Lagoon		121	DOMINIK *et al.*, 2014
Ruditapes philippinarum	Venice Lagoon	30.0-70.0	52.2	SFRISO *et al.*, 2008
Venus verrucosa	Istria	0.00-50.0	20.0	BILANDŽIĆ *et al.*, 2015
Venus verrucosa	Kaštela Bay	49.0-378	169	BOGDANOVIĆ *et al.*, 2014
Venus verrucosa	Veneto region	40.0-80.0	60.0	BILLE *et al.*, 2015

Therefore, these organisms could also be used as environmental bioindicators of Hg contamination. Besides ambient THg concentrations, differences in bivalve THg also arise due to biological diversity between the species. For example, European flat oyster in the Adriatic has 2-3 times higher average THg concentrations than mussels (BILANDŽIĆ *et al.,* 2015, 2016). This could be due to a larger gill area and two-fold greater filtration rate in *Ostrea* sp. than in *Mytilus* sp. (WALNE, 1972).

Mercury speciation data in the other Adriatic bivalves are limited. However, these data show that MeHg accounts for approximately 40-50% of THg in *Cerastoderma glaucum*, *Ruditapes philippinarum* and *Crassostrea gigas* (DOMINIK *et al.,* 2014; GIANI *et al.,* 2012). These values are higher than in mussels, showing that biological parameters, alongside environmental ones, have significant influences on Hg bioaccumulation in bivalves. The highest average percentage of MeHg (81.2%) and the highest average of MeHg concentrations (85.7 ng g^{-1}) are found in *Ruditapes philippinarum* from Ravenna Lagoon (TROMBINI *et al.,* 2003).

Importance of coastal lagoons in mercury transformations and bioaccumulation

Shallow coastal lagoons are particularly complex environments, often subjected to strong anthropogenic impacts. Lagoon environments are often ideal for mariculture due to their high productivity and their shallow and protected surroundings (COVELLI *et al*., 2008). However, these coastal lagoons accumulate labile organic matter in anoxic sediments that drives microbial processes responsible for rapid Hg methylation (HINES *et al*., 2012). In addition, the redox potential has values that are usually more negative along the coast due to reduced hydrodynamics (BRAMBATI, 2001). For example, Venice lagoon is a shallow lagoon bordered by highly productive intertidal wetlands, contains high nutrient loading from agricultural runoff and urban sewage, and therefore represents an ideal site for Hg methylation (BLOOM *et al*., 2004). The water flow through the Venice lagoon represents the net source of THg and MeHg to the open Adriatic Sea (BLOOM *et al*., 2004), which could be later accumulated by the Adriatic biota.

In addition to bacterial methylation processes in the sediments, MeHg transfer to organisms in the Marano and Grado Lagoon depends on intake of already enriched organic substances (micro and macro epiphytes, bits of plant tissue, etc.) originating from the lagoon and/or the Isonzo River (BRAMBATI, 2001). The abundance of plant and animal species in lagoons increases the risk of Hg remobilization from sediments (by bioturbation) to the water column and subsequent bioaccumulation (COVELLI *et al*., 2008), especially in benthic species like bivalves. The increased bioaccumulation is observed through the higher THg values in mussels from the Venice Lagoon and the Marano and Grado Lagoon, compared to those from low-Hg environment of the Krka River estuary (Fig. 9A). In addition, high THg and MeHg values in clams from the northern Adriatic lagoons (e.g. *Ruditapes philippinarum*) could be due to high bioaccumulation and methylation potentials in these ecosystems.

Role of metallothioneins in mussels in mercury detoxification

The synthesis of cysteine-rich metallothioneins (MTs) is induced in mussels when exposed to certain metals, including Hg. They are biomarkers of response to metal contamination and have important roles in the regulation, storage and detoxification of metals in mussels (ŽNIDARIČ *et al.,* 2006). Concentrations of MTs in mussels from the Hg contaminated Vlora Bay (Fig. 1) are up to 50% higher than in mussels from the background station (CULLAJ *et al*., 2006). Field study in the Gulf of Trieste showed that MTs and metal content in mussels are not well correlated (RAMŠAK *et al.,* 2012). However, other biological and environmental factors may influence MTs concentrations. Although Hg is not a strong inducer of MTs synthesis, it has a strong binding affinity to MTs and can displace other metals bound to MTs (RAMŠAK *et al.,* 2012). ŽNIDARIČ *et al.* (2006) showed that Hg binds to MTs in gills and digestive glands in mussels exposed to inorganic Hg, but only in digestive glands in wild mussels from industrial site. Hg

binding to MTs in digestive gland could explain the observed higher THg values in digestive gland than in other tissues.

MERCURY IN FISH

Comparison of mercury in fish from different seas

In the 1970s, THg concentrations in Mediterranean fish were higher than in the same fish species caught in the adjacent Atlantic Ocean (BERNHARD & RENZONI, 1977). Although this trend can also be observed today by comparing literature data, only few recent studies have simultaneously measured THg concentrations in both basins. Average THg concentrations in European hake are up to 5.5 times higher in the Mediterranean Sea than in the Atlantic Ocean (Table 2). These observations raise questions about specificity of Mediterranean mercury biogeochemistry, but definite conclusions have not yet been found.

Due to their ubiquitous distribution in the Mediterranean, red mullets (*Mullus barbatus*

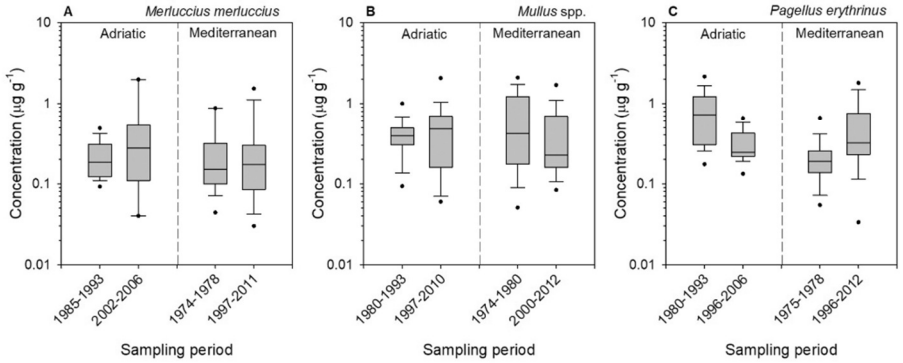


Fig. 10. Box-and-whiskers plots of the geographical distribution of THg in the three most studied fish species (A – Merluccius merluccius, B – Mullus spp., C – Pagellus erythrinus) from the Adriatic and Mediterranean Sea. Data obtained from BERNHARD & RENZONI, 1977; BILANDŽIĆ et al., 2011; BONSIGNORE et al., 2013; BRAMBILLA et al., 2013; BUZINA et al., 1989, 1995; COPAT et al., 2012; COSSA et al., 2012; ČULIN & ZVONARIĆ, 1995; DURAL et al., 2010; FALCÓ et al., 2006; HARAKEH et al., 1985; HARMELIN-VIVIEN et al., 2009, 2012; HORNUNG et al., 1980; HORVAT et al., 1999; JUREŠA & BLANUŠA, 2003; KONTAS, 2006; LAHAYE et al., 2006; LEONZIO et al., 1981; MIKAC et al., 1985; MINIERO et al., 2014; NAUEN et al., 1980; PERUGINI et al., 2009; STORELLI, 2008; STORELLI & MARCOTRIGIANO, 2000, 2005; STORELLI et al., 2003, 2005a, 2007; STORELLI & BARONE, 2013; TUNÇEL et al., 1980; ULUTURHAN & KUCUKSEZGIN, 2007; VUKADIN et al., 1995; YANNAI & SACHS, 1978; ZVONARIĆ, 1991.

Table 2. Comparison of THg concentrations expressed as µg g^{-1} wet weight in various fish species from the Mediterranean Sea and the Atlantic Ocean.

Fish specie	Mediterranean		Atlantic		Reference
	Range	Average	Range	Average	
Merluccius merluccius	0.034-0.058	0.045	0.018-0.046	0.033	LAHAYE *et al.*, 2006
Merluccius merluccius	0.022-2.862	0.334	0.008-0.132	0.060	COSSA *et al.*, 2012
Sardina pilchardus	0.067-0.127	0.094	0.019-0.034	0.023	LAHAYE *et al.*, 2006
Engraulis encrasicolus	0.043-0.061	0.050	0.026-0.032	0.028	LAHAYE *et al.*, 2006
Micromesistius poutassou	0.020-0.023	0.021	0.016-0.017	0.016	LAHAYE *et al.*, 2006
Xiphias gladius		1.678		0.773	DAMIANO *et al.*, 2011

and *Mullus surmuletus*) are probably the most analysed fish species, beside the commercially important species European hake (*Merluccius merluccius*) and common pandora (*Pagellus erythrinus*). Fig. 10 shows the comparison of THg in these fish species from the Adriatic and Mediterranean Seas through time. Recent data shows no statistically significant differences in fish THg concentrations from the two seas (Mann-Whitney, $P = 0.493$ for European hake and $P = 0.430$ for red mullets). In addition, there are no significant changes in THg concentrations in Adriatic fish with time (Mann-Whitney, $P = 0.358$ for European hake and $P = 0.565$ for red mullets). For common pandora, there is a significant decrease in Adriatic THg with time and significant difference between concentrations in two basins during past measurements (Mann-Whitney, $P < 0.001$). Specific reasons for these observations in common pandora are unknown, especially when taking into account the similar habitats, eating habits and trophic position of red mullets and common pandora in food web. However, recent results (Fig. 10C) show no statistically significant difference between THg concentrations in common pandora from the Adriatic and Mediterranean Seas (Mann-Whitney, $P = 0.167$).

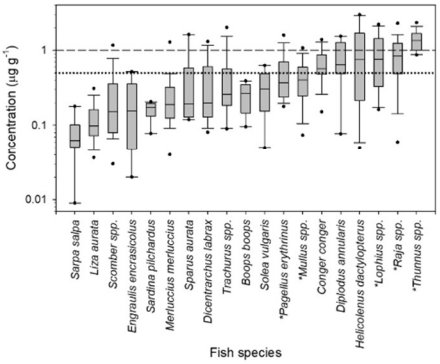


Fig. 11. Box-and-whiskers plots of THg concentrations in the most studied fish species from the Adriatic Sea. Dotted and dashed lines correspond to the EU maximal allowed THg levels of 0.5 and 1.0 µg g^{-1} (on graph), respectively. Data obtained from: BRAMBATI, 2001; BILANDŽIĆ et al., 2011; BUZINA et al., 1989, 1995; ČULIN & ZVONARIĆ, 1995; DELLA TORRE et al., 2010; DOMINIK et al., 2014; HORVAT et al., 1999, 2014; JUREŠA & BLANUŠA, 2003; MIKAC et al., 1985; NAJDEK & BAŽULIĆ, 1983; PERUGINI et al., 2009; STORELLI, 2008; STORELLI & BARONE, 2013; STORELLI & MARCOTRIGIANO, 2000, 2004; STORELLI et al., 1998, 2002b, 2003, 2005a, 2006, 2007; VUKADIN et al., 1995; ZVONARIĆ, 1991; ŽVAB ROŽIČ et al., 2014.*

Total mercury in fish

Mercury content in the Adriatic fish was determined to observe effects of industrial effluents (from chlor-alkali plants) and elevated natural seawater levels (from the Idrija River) to marine biota. THg concentrations in Adriatic fish span several orders of magnitude. THg in Adriatic fish for which box-and-whisker graphs could be constructed from literature data, are presented in Fig. 11. This figure emphasises the importance of diet for mercury biomagnification; Hg in fish generally increases with increasing trophic position in food web of its prey (COSSA *et al.*, 2012; HORVAT *et al.*, 2014). The lowest THg concentrations are found in demersal herbivorous fish like salema porgy (*Sarpa salpa*) (STORELLI *et al.*, 1998), followed by planktivorous fish (e.g. European anchovy, *Engraulis encrasicolus*) (BILANDŽIĆ *et al.*, 2011), omnivorous (e.g. bogue, *Boops boops*) and carnivorous fish (e.g. annular seabream, *Diplodus annularis*) (BUZINA *et al.*, 1989). The highest THg values are found in top predator species (HORVAT *et al.*, 2014). For example, STORELLI *et al.* (2002a) measured 18.3 µg g^{-1} in hammerhead (*Sphyrna zygaena*) from the Ionian Sea. Exceptionally high average THg concentrations were also determined in gulper shark (*Centrophorus granulosus*) from the Adriatic Sea (9.66 µg g^{-1}) (STORELLI *et al.*, 2002a). Average THg concentrations higher than 2.0 µg g^{-1} in the top predator species can be found in ghostshark (*Chimaera monstrosa*), blackmouth catshark (*Galeus melastomus*), Mediterranean moray (*Muraena helena*), electric ray (*Torpedo nobiliana*) and European barracuda (*Sphyraena sphyraena*). In addition, high THg values can be found in European seabass (*Dicentrarchus labrax*) from the Artalina and Francamela

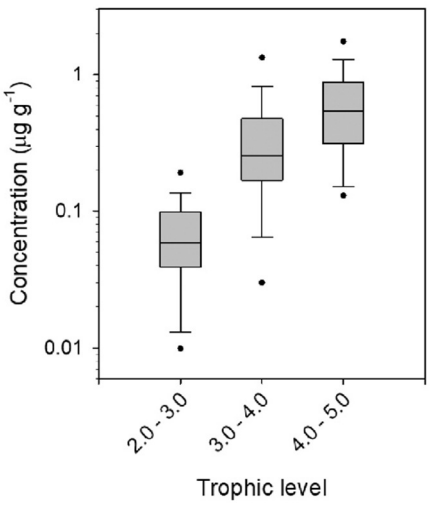


Fig. 12. Box-and-whisker plots of MeHg concentrations in 41 Adriatic fish species sorted in three trophic level ranges. Data obtained from: BRAMBATI, 2001; BUZINA et al., 1989, 1995; ČULIN & ZVONARIĆ, 1995; DOMINIK et al., 2014; HORVAT et al., 1999, 2014; MIKAC & PICER, 1985; NAJDEK & BAŽULIĆ, 1983; STORELLI et al., 2002a, 2002b, 2002c, 2003, 2005a, 2005b; VUKADIN et al., 1995; ZVONARIĆ, 1991.

fish farms (Grado Lagoon). These THg values indicate that mercury is readily biomagnified through the Mediterranean and Adriatic trophic food webs.

Methylmercury in fish

Methylmercury concentrations in the Adriatic fish are not as extensively measured as THg. MeHg concentrations were summarised from available papers by trophic position of fish species in food web (Fig. 12). Trophic level data were obtained from BRAMBILLA *et al.* (2013) or from internet database (www.fishbase.org). These values differ at most by 0.3 trophic level from the values calculated by DOMINIK *et al.* (2014) based on $\delta^{15}N$ measurements in the Venice Lagoon, and can therefore be used approximately as Adriatic trophic levels. From MeHg concentrations in 41 Adriatic fish species sorted in three trophic level ranges, it is evident that the lowest and highest MeHg concentrations are found at the lowest and highest trophic levels, respectively (Fig. 12). Differences in MeHg concentrations between trophic levels are statistically significant (Kruskal-Wallis, $P < 0.001$). MeHg biomagnifies in organisms, as indicated by its concentration increase with each subsequent trophic level (COSSA *et al.*, 2012; HORVAT *et al.*, 2014). MeHg can account for up to 100% of THg in the Adriatic fish. In the Gulf of Trieste, the percentage of THg present as MeHg increases from 57-77 % in small benthic fish to 38-71% in small pelagic fish and 90-100% in larger benthic fish to 100% in larger pelagic fish (FAGANELI *et al.*, 2014; HORVAT *et al.*, 2014). However, these percentages depend on specific biological and environmental factors. For example, MeHg percentage in fish species from Kaštela Bay was on average 30% lower than in the same species from the Island of Vis (BUZINA *et al.*, 1995). It seems that Hg originating from the chlor-alkali plant in Kaštela Bay is not readily available for methylation. Specific environmental conditions drive transformation of inorganic Hg to MeHg in the Adriatic sediment (BRATKIČ *et al.*, 2017) and water column (KOTNIK *et al.*, 2015), and therefore result in different MeHg content in fish.

Influence of environmental and biological parameters on mercury concentrations

Besides trophic position of the fish in food web, higher Hg concentrations have also been related to the living environment. Demersal fish usually have comparatively higher THg levels than pelagic fish (STORELLI *et al.*, 1998). Furthermore, fish that feed on benthic organisms accumulate Hg to a higher degree that other species (STORELLI *et al.*, 2002a). This is probably due to mercury absorption from Hg-rich porewaters and solid phase by benthic algae, and its consequent transfer to the benthic fauna and food web (FAGANELI *et al.*, 2014). Another possibility is active Hg-release mechanism from sediments to the water column and its transfer to benthic biota (BONSIGNORE *et al.*, 2013). Depth is also an important factor influencing Hg concentrations in fish.

Deep-sea species usually have higher THg values than fish inhabiting shallow waters (CHOY *et al.,* 2009; KOENIG *et al.,* 2013). Values above 1.0 μg g^{-1} have been reported for various fish species from the western Mediterranean waters deeper than 1000 m, even for the small planktivorous Mediterranean spiderfish (*Bathypterois mediterraneus*) (KOENIG *et al.,* 2013). To explain these observations, CRESSON *et al.* (2014) have recently proposed that food quality and quantity decreases with depth, affecting organisms by slowing their growth. Deep-water fish would appear older and would be able to accumulate Hg during a longer time. Second, most MeHg in water column is produced in oxygen-depleted waters (COSSA *et al.,* 2009), and can be readily adsorbed on sinking organic particles which represent food for deep-water animals (CRESSON *et al.,* 2014).

In the Adriatic Sea, variation of Hg with fish length or weight can be observed, as well. Various authors have described linear and exponential correlations between THg (MeHg) in fish muscle and length (STORELLI *et al.,* 2007; STORELLI & BARONE, 2013; HORVAT *et al.,* 2014) or weight (STORELLI *et al.,* 2002a, 2006; HORVAT *et al.,* 2014). This shows that the highest Hg concentrations are found in large older fish, which are usually predators. The positive correlation between fish size (which is a proxy for fish age) and Hg concentrations suggests that consumers who eat larger fish might have higher exposure to mercury than those who eat smaller fish (STORELLI *et al.,* 2006, 2007). Frequent consumption of fish with high Hg content can exceed the maximum tolerable weekly intake (1.6 μg kg^{-1} body weight). They should therefore be eaten in moderation (BOSCH *et al.,* 2016; STORELLI & BARONE, 2013). In the European Union, maximum levels for Hg in foodstuffs are set to 0.5 μg g^{-1} for most fish species, and 1.0 μg g^{-1} for benthic fish and top predator species (Fig. 11) (EUROPEAN COMMISSION, 2006). Although most of the Adriatic fish species have some values above these limits, some fish species exceed this limit in about 75% cases, such as European conger (*Conger conger*), annular seabream (*Diplodus annularis*) and tuna (*Thunnus* spp.). Although MeHg represents the greatest part of THg, EU legislation does not set maximum MeHg levels in fish.

Mercury in different tissues of Adriatic fish

Mercury concentrations are usually determined only in fish muscle used for human consumption. However, Hg content in other fish tissues shows variability, similarly as in mussels. Biomagnification studies should measure Hg concentrations in different organs and tissues of fish body, and not only in muscle, due to different concentrations in various organs (HAMMERSCHMIDT & FITZGERALD, 2006). In rays from the Gulf of Trieste HORVAT *et al.* (2014) found the highest THg and MeHg in muscle tissue followed by gills and liver. It seems that high values in the gills suggest the capability to accumulate high quantities of Hg from water, especially in inorganic form, as seen by the lowest percentage of THg present as MeHg. Negative correlation between the percentage of MeHg in the liver and weight of bull ray (*Pteromylaeus bovinus*) indicates a demethylation processes in the liver of older specimens. Demethylated MeHg in the liver shows weak mobility, probably due to formation of weakly soluble salts of inorganic Hg, such as mercury(II) selenide (HORVAT *et al.,* 2014). Contrary to rays, albacore (*Thunnus alalunga*) and European eel (*Anguilla anguilla*) from the Adriatic Sea have up to 2.5-fold and 6-fold higher THg concentrations in liver than in muscle, respectively (RENZI *et al.,* 2012; STORELLI & MARCOTRIGIANO, 2004). Different bioaccumulation in various tissues among species is due to their feeding behaviour (HORVAT *et al.,* 2014), while different accumulation in the liver is probably due to different metabolic activities (STORELLI & MARCOTRIGIANO, 2004).

Role of selenium in fish on mercury detoxification

Selenium can increase Hg elimination from organisms, and growing evidence suggests Se-mediated mercury toxicokinetics (GRIBBLE *et al.,* 2016). Several studies measured Se:Hg molar ratios in various fish species from the Adriatic Sea (MIKAC *et al.,* 1985; PERUGINI *et al.,* 2014;

STORELLI & MARCOTRIGIANO, 2002). This ratio is usually higher than 1.0 in Adriatic fish, and the highest ratios (>20) are found in smaller fish (PERUGINI *et al.,* 2014). Equimolar Se:Hg ratios, which can be found in marine animals with high THg concentrations (above 1 μg g^{-1}) (STORELLI & MARCOTRIGIANO, 2002), represent a threshold associated with Hg toxicity. Generally, Se:Hg molar ratios show stronger correlation with Hg concentrations than with Se concentrations, which means that Hg has greater biomagnification potential than Se (GRIBBLE *et al.,* 2016). Metallothioneins are rarely significantly correlated with Hg in the Mediterranean fish (SISCAR *et al.,* 2014), indicating that they are not as important as Se regarding Hg detoxification. Generally, glutathiones represent the primary defence against Hg toxicity, followed by metallothionein induction, and as the most important formation of Hg-Se-protein complexes (CUVIN-ARALAR & FURNESS, 1991; HORVAT *et al.,* 2014).

CONCLUSIONS

Complexity of biogeochemical Hg cycle in the Adriatic Sea is observed through variability of mercury concentrations along trophic levels. Although the highest Hg concentrations are found in the Adriatic Sea, THg in zooplankton does not appear to follow this pattern and has significantly lower values in Adriatic than in the Mediterranean and other world seas. However, methylation potential in the Adriatic Sea appears to be the highest, as observed from the highest percentage of THg present as MeHg. THg in zooplankton from all three Adriatic sub-basins shows considerable decrease with time.

THg concentrations in the Adriatic mussels are slightly higher compared to ones from the Mediterranean Sea, but content of THg in mussels from both seas declines by approximately 10-fold in the last 30 years. Mussels highly reflect localised influence of anthropogenic contamination. Percentage of mercury as MeHg is similar in the Mediterranean and the Adriatic low-Hg environments. However, previously contaminated locations can have lower MeHg percentages. This indicates low methylation potential and/or detoxification mechanisms.

European hake and red mullet have similar THg concentrations in the Adriatic and Mediterranean seas without clear seasonal variability. THg and MeHg increase with the position of fish in the food web from herbivorous organisms to the top predator species. Percentage of mercury present as MeHg also increases in the same manner through the trophic level. Mercury concentrations are usually positively correlated with length (age) and weight of fish.

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Zastupljenost i specijacija žive u jadranskom planktonu, školjkašima i ribi – pregled

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SAŽETAK

Ovaj članak predstavlja pregled dostupnih istraživanja o specijaciji žive u najproučavanijoj bioti Jadranskog mora kao posebne biogeokemijske podjedinice Mediterana. Predstavljamo postojeće znanje o koncentraciji žive, specijaciji, prostornoj raspodjeli i vremenskim promjenama u planktonu, školjkašima i ribi Jadranskog mora. Rezultati studija o specijaciji žive u morskim organizmima su korišteni za opis razdiobe bioakumulacije žive u Jadranskom moru. Biogeokemijski ciklus žive u Jadranu karakterizira povećanje koncentracije žive od vodenog stupca, preko planktona, školjkaša i manjih riba do predatorskih ribljih vrsta. Iako Jadransko more sadrži najviše koncentracije žive u cijelom Mediteranu, riblje vrste na višim trofičkim razinama imaju slične koncentracije žive posvuda u Mediteranu, ukazujući na nepotpuno razumijevanje prijenosnih mehanizama žive iz morske vode na više trofičke razine. Budući da je konzumiranje (kontaminirane) hrane glavni put ljudske izloženosti živi, od iznimne je važnosti razumijevanje utjecaja kontaminacije živom u Jadranskom moru.

Ključne riječi: živa, metilživa, plankton, riba, dagnja

3.3 Optimization and Measurement Uncertainty Estimation of Hydride Generation–Cryogenic Trapping–Gas Chromatography–Cold Vapor Atomic Fluorescence Spectrometry for the Determination of Methylmercury in Seawater

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In this paper, the authors presented optimization of the hydride generation method for the determination of low methylmercury concentrations in seawater. Optimized system parameters of the homemade system for hydride generation coupled with cryogenic trapping, gas chromatography and cold vapor atomic fluorescence spectrometry were compared to a reference method (ethylation–gas chromatography–cold vapor atomic fluorescence spectrometry).

In addition, the authors compared optimized system parameters of both methods with those from a recent paper. They compared effects of soda lime drying on analytical performance, LOD, analytical precisions, spike recoveries, calibration curves, method sensitivities, peak retention times and peak asymmetries.

Uncertainty of measurement results was estimated using a modelling approach described in GUM/Eurachem guidelines. Based on mathematical models, all uncertainty sources were accounted for and presented using fish-bone diagrams. Every uncertainty source from the model was quantified using ISO-GUM approach. Additional uncertainty sources were also estimated (recovery, repeatability and reproducibility). Relative combined standard uncertainties were calculated from individual uncertainty sources.

The expanded relative combined standard uncertainties ($U_{ex,r}$) were calculated from relative combined standard uncertainties by applying the coverage factor of two. The authors compared two analytical methods by their $U_{ex,r}$ values and by individual uncertainty contributions to expanded relative combined standard uncertainties. $U_{ex,r}$ values were 11.1%, 15.0% and 21.3% for hydride generation, and 15.8%, 18.2% and 19.3% for ethylation, at the highest, middle and lowest concentration levels, respectively. Sample repeatability had the biggest contribution to $U_{ex,r}$ in hydride generation, together with recovery in the ethylation method.

The candidate modified the system for hydride generation previously used in the laboratory. He co-designed the quartz column and designed the general outlook of a new homemade manual system for hydride generation from previously used components (heating units, atomic fluorescence spectrometer, Dewar flask). The candidate optimized most of system's parameters for the determination of methylmercury in seawater. He optimized its performance and compared it to the previously optimized performance of the reference ethylation method. After assessing possible uncertainty contributions, he created fish-bone diagrams for hydride generation and ethylation, and calculated expanded relative combined standard uncertainties and uncertainty contribution indexes for both methods. The candidate drew figures and the table, and drafted the manuscript.

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Optimization and measurement uncertainty estimation of hydride generation–cryogenic trapping–gas chromatography–cold vapor atomic fluorescence spectrometry for the determination of methylmercury in seawater

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ABSTRACT

This technical note presents optimization results of hydride generation volatilization coupled with cryogenic trapping, separation by gas chromatography and detection by cold vapor atomic fluorescence spectrometer after pyrolysis. Method performance was compared with the reference ethylation method and results from a previous study. Using GUM/Eurachem guidelines, we estimated expanded relative standard uncertainty ($k = 2$) of both methods. Expanded relative standard uncertainty was 11.1%, 15.0% and 21.3% for hydride generation, and 15.8%, 18.2% and 19.3% for ethylation, at the highest (>400 fM), middle (100–150 fM) and lowest (<50 fM) methylmercury concentration levels, respectively. Sample repeatability represents the biggest single contribution to the expanded standard uncertainty in hydride generation, together with recovery in ethylation method.
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1. Introduction

Methylmercury (MeHg) is a toxic form of mercury present in the marine environment due to its biomagnification and bioaccumulation properties (Mason et al., 2012). Consequently, a lot of attention has been given to the determination of MeHg in environmental samples.

Derivatization of mercury species by hydride generation (HG) using $NaBH_4$, coupled with cryogenic trapping (CT), gas chromatography (GC) and suitable detector can be used for MeHg determination in environmental samples. Hydride generation using $NaBH_4$ has been used for MeHg determination in sediments (Craig et al., 1999; Tseng et al., 1998), biological samples (Mališová et al., 2015; Sarıca and Türker, 2012) and water samples (Bravo et al., 2013; Cossa et al., 2009, 2011; Heimbürger et al., 2010, 2015; Regier et al., 2012). GC-AFS is a common technique for Hg analysis due to its high sensitivity and selectivity. Combined with hydride generation and cryogenic trapping, it represents a simple technique for determination of low MeHg concentrations in seawater.

This technical note reports the results of optimized method parameters for the hyphenated system of hydride generation, cryogenic trapping/gas chromatography with cold vapor atomic fluorescence spectrometry (HG-CT-GC-CVAFS) for determination of MeHg concentrations in seawater. We compared our method performance with those from recent literature (Stoichev et al., 2004). Measurement uncertainty was estimated for HG-CT-GC-CVAFS and compared with uncertainty of the reference method (ethylation–gas chromatography–cold vapor atomic fluorescence spectrometry, Eth-GC-CVAFS).

2. Materials and methods

All used chemicals were analytical or GC-ECD&FID grade. Solutions were prepared using Milli-Q water. To ensure low blanks, all labware was acid washed.

Using a home-made manual system for HG-CT-GC-CVAFS, a seawater sample is analyzed directly without any pre-treatment. Quartz column (0.3 cm internal diameter, packed with 11 cm length of 15% OV-3 on Chromosorb WAW-DMCS 60/80 mesh) is immersed into liquid nitrogen to cool while the sample is being prepared (minimum 0.5 min). Bubbler (65 mL) is rinsed with sample before it is filled to the 40 mL mark. Samples which had been previously stabilized with acid (to final concentration of 15 mM) are analyzed directly, while un-acidified samples, calibration blanks and standard solutions are treated with HCl (to the same final concentration) to obtain pH of about 1–2. For derivatization, $NaBH_4$ solution is added to the sample (to final concentration of 13.0 mM). Inorganic mercury and methylmercury react with $NaBH_4$, yielding volatile Hg^0 and MeHgH. These species are pre-concentrated by sparging with helium gas (550 mL min^{-1} for 5 min) onto a cooled quartz column. Argon carrier gas (23 mL min^{-1}) is released

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through the column after its removal from liquid nitrogen, mercury species are thermally desorbed, separated on the column and detected by AFS after pyrolysis at 650 °C. Signal output from the AFS is processed by a personal computer using Mercury Guru Software. This method has been recently validated using isotopic dilution–gas chromatography–sector field–inductively coupled plasma mass spectrometry (Heimbürger et al., 2015).

In the ethylation procedure, 70 mL of water sample are weighed in a 125 mL PTFE bottle. After addition of 5 mL of concentrated HCl and 30 mL of CH_2Cl_2, the bottle is closed and shaken overnight. The aqueous phase is separated from the CH_2Cl_2 layer, to which 40 mL of Milli-Q water are added. Organic phase is evaporated in a water bath at 80 °C. Aqueous samples are transferred into amber glass vials and pH is adjusted to 4.6 with 300 μL of 2 M citrate buffer. Then, 50 μL of 66.6 mM $NaBEt_4$ solution are added in the vials, which are capped and placed into the automated MERX system by Brooks Rand. Mercury species are sparged onto Tenax traps and thermally desorbed (180 °C) onto an isothermal GC column (36 °C). They are converted to Hg^0 by pyrolysis and their signals are determined by AFS. Although the reference method uses sample pre-concentration, recent modifications enable direct MeHg derivatization in seawater (Bowman and Hammerschmidt, 2011).

3. Results & discussion

Optimized method performance of Eth-GC-CVAFS and home-made system for HG-CT-GC-CVAFS are presented in Table 1, and compared with results from Stoichev et al. (2004).

Using both methods, MeHg concentration is calculated from the ratio of detector signals for MeHg in sample and standard MeHg solution in Milli-Q water (external calibration). These values are corrected using corresponding spike recovery factors (Table 1). The average spike recovery is lower in ethylation due to possible losses and incomplete extraction. Therefore, HG represents better quantitative method for MeHg determination in seawater. Ethylation is more sensitive than HG when determining MeHg in Milli-Q water, as seen by the higher values of calibration slope and relative sensitivity in Milli-Q water. When following method procedure, relative sensitivity of HG in spiked seawater samples is 0.94 (Table 1). This shows that ethylation is only slightly more sensitive than HG for MeHg determination in seawater. In addition, it seems that matrix plays an important role in MeHg determination (Horvat et al., 1993), as seen from differences in relative sensitivities between Milli-Q and seawater. Detection limit is lower for hydride generation than for ethylation, which is possibly due to impurities in the extraction reagent or in Milli-Q water. Additionally, higher detection limits in ethylation due to unreproducible blanks can also be related to contaminated PTFE labware (Horvat et al., 1993). Our detection limit for HG-CT-GC-CVAFS is lower than reported by Stoichev et al. (2004); however, it was recently lowered to 15 fM for 200 mL sample (Cossa et al., 2009). Method precision is practically the same for both methods, and slightly better than literature values. Overall performance of both HG systems is the same, as seen from identical MeHg relative retention times. The automated ethylation method still offers the best MeHg relative retention. MeHg peaks are symmetrical when soda lime is not used for drying gaseous reaction products during HG. Stoichev et al. (2004) also found that the asymmetry of MeHg peak during HG is lower without moisture trap, although their peaks are more asymmetrical.

Table 1
Optimized method performance of Eth-GC-CVAFS and home-made system for HG-CT-GC-CVAFS, and their comparison with results from Stoichev et al. (2004).

Parameter	Present study		Stoichev et al. (2004)	
	HG	Ethylation	HG	Ethylation
Soda lime drying	Without	Without	With	With
Detection limit (fM)	3.94–15.9[a]	7.73–25.3[a]	49.9[b]	99.7[bc]
Precision (RSD)	3.2[d]	2.9[d]	5[e]	6[f]
Spike recovery (%)[g]	98.5	79.1	n.a.	n.a.
External calibration slope[h]	164054	218641	n.a.	n.a.
Relative sensitivity in Milli-Q[i]	0.74	1.00	0.60	1.00
Relative sensitivity in seawater[j]	0.94	1.00	n.a.	n.a.
MeHg relative retention[k]	1.44	2.30	1.45	1.98
MeHg peak asymmetry	1.09 ± 0.17[d]	1.14 ± 0.10[d]	1.90 ± 0.39[l]	1.70 ± 0.18[l]

[n.a.] Not available.
[a] Calculated on 40 mL basis for hydride generation and 70 mL for ethylation.
[b] Calculated on 100 mL basis for hydride generation and 1 g for ethylation.
[c] fmol g^{-1}. Value for dry sediment sample.
[d] Calculated for 1.25 pM MeHg from 7 parallel measurements.
[e] Calculated for 24.9 pM MeHg from 10 parallel measurements.
[f] Calculated for 4.99 pmol MeHg in sediment from 11 parallel measurements.
[g] Calculated for 1.25 pM MeHg spike in seawater from 36 parallel measurements for HG and 21 parallel measurements for ethylation.
[h] Expressed in peak area (arbitrary units) per pM of MeHg.
[i] Expressed as a ratio of MeHg signal for HG and ethylation at 1.25 pM MeHg in Milli-Q and following method procedure (present study), and for 2.49 pmol in Stoichev et al. (2004).
[j] Expressed as a ratio of MeHg signal for HG and ethylation at 1.25 pM MeHg spike in seawater sample and following method procedure.
[k] Unadjusted relative retention; relative to retention time of the Hg^0 peak.
[l] Calculated for cases without the use of soda lime trap and for 2.49 pmol MeHg.

Uncertainty of measurement results was estimated using modelling approach described in GUM/Eurachem guidelines (Ellison and Williams, 2012; JCGM, 2008). Following these guidelines, MeHg concentration in seawater was determined by HG-CT-GC-CVAFS and Eth-GC-CVAFS using a mathematical model expressed by the following equation:

$$c(\mathrm{MeHg}) = \frac{A'(\mathrm{S})}{A'(\mathrm{Std})} * \frac{n(\mathrm{Std})}{V(\mathrm{S})} \quad (1)$$

where $A'(\mathrm{S})$ and $A'(\mathrm{Std})$ are fluorescence signal intensities of sample and MeHg standard (corrected for corresponding blanks), $n(\mathrm{Std})$ is known amount of the working MeHg standard, and $V(\mathrm{S})$ is volume of the sample. Contributions of the uncertainty sources based on this mathematical model are shown as fishbone diagrams in Fig. 1. Additional uncertainty sources include spike recovery corrections, $u(R_m)$, and uncertainties due to repeatability of sample and standard peaks, $u(\mathrm{rep.(S)})$ and $u(\mathrm{rep.(Std)})$. Uncertainty due to spike recovery corrections is expressed by Eq. (2):

$$u(R_m) = R_m * \sqrt{\frac{s^2_{n(obs)}}{N * n^2_{obs}} + \left(\frac{u(n_{spike})}{n_{spike}}\right)^2} \quad (2)$$

where R_m is the mean spike recovery, $s_{n(obs)}$ is the standard deviation of the mean observed amount of standard spike n_{obs}, N is the number of spiked samples, and $u(n_{spike})$ is the uncertainty in the added amount of standard spike n_{spike} (Barwick and Ellison, 1999). Decomposition of MeHgH was not included into the uncertainty estimation budget due to uncertain decomposition mechanism and unknown uncertainty of MeHgH half-life (Craig et al., 1999). Complying with the general relationship between the combined standard uncertainty and the uncertainty of the individual parameters for the mathematical model expressed in product (quotient) form (Ellison and Williams, 2012), we calculated the relative combined standard uncertainty ($u_{r,c}$) of MeHg measurements in seawater by HG and ethylation using Eq. (3). Relative standard uncertainties of individual uncertainty sources are denoted by u_r.

$$u_{r,c}(c(\mathrm{MeHg})) = \sqrt{\begin{array}{c} u_r(A'(\mathrm{S}))^2 + u_r(V(\mathrm{S}))^2 + u_r(A'(\mathrm{Std}))^2 + u_r(n(\mathrm{Std}))^2 \\ + \\ u_r(R_m)^2 + u_r(\mathrm{rep.(S)})^2 + u_r(\mathrm{rep.(Std)})^2 \end{array}} \quad (3)$$

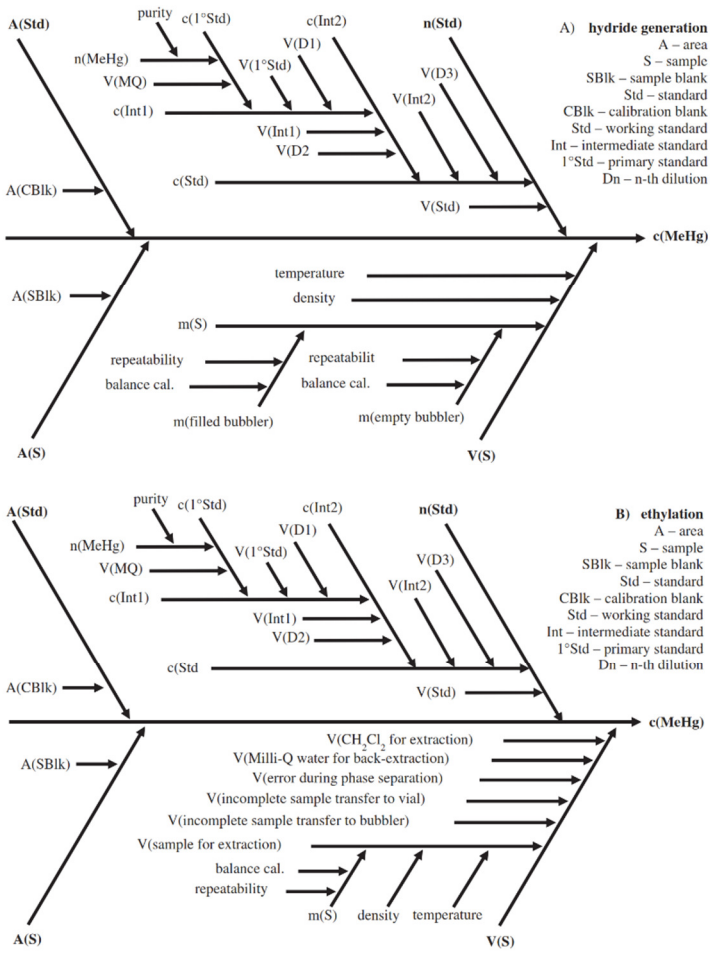


Fig. 1. Fishbone diagrams for the identification of uncertainty sources based on the model equation for hydride generation (A) and ethylation (B). [All volumes in the diagram have their own uncertainty sources (temperature, repeatability, calibration), but these were omitted from diagrams for clarity.]

Expanded relative standard uncertainty ($U_{ex,r}$) at a 95% confidence interval is obtained by applying a coverage factor k = 2. At the highest MeHg concentration level (>400 fM), middle (100–150 fM) and the lowest (<50 fM), $U_{ex,r}$ has a value of 11.1%, 15.0% and 21.3% for hydride generation, and 15.8%, 18.2% and 19.3% for ethylation, respectively. Hydride generation has better (lower) expanded standard uncertainty at higher MeHg concentrations than ethylation. However, ethylation has a narrower range of $U_{ex,r}$ values. Explanation for observed $U_{ex,r}$ values can be assessed by comparing relative contributions to combined uncertainty. Relative contributions (indexes) of the individual uncertainty sources to each $U_{ex,r}$ are presented in Fig. 2.

For HG, the highest relative contribution to the expanded relative standard uncertainty is from sample repeatability, at all concentration levels. This is best seen at the lowest concentration level (near detection limit), where repeatability of sample accounts for 47% of $U_{ex,r}$. This percentage slightly decreases with increasing MeHg concentrations. Similar trend is also observed in ethylation, but the relative contributions are lower. As ethylation is an automated system, it is expected that sample repeatability would be better than in a manual system such is hydride generation.

Relative uncertainty contributions related to MeHg standard repeatability grow from lower to higher concentration level, although their relative standard uncertainties are almost the same (about 3% in both methods at all MeHg concentration levels). Therefore, this increase is only proportional to the $U_{ex,r}$ decrease.

This observation is also valid for recovery, but for each method separately. Relative standard uncertainties for recovery at all MeHg concentration levels are about 1.4% and 5% for hydride generation and

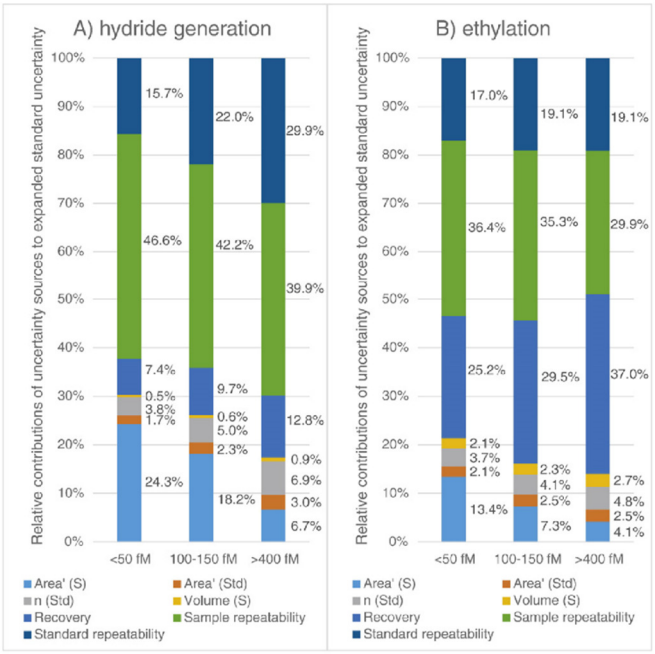


Fig. 2. Relative contributions (indexes) of the individual uncertainty sources to the expanded relative standard uncertainty for hydride generation (A) and ethylation (B) at different environmental MeHg concentrations.

ethylation, respectively. Higher values for the ethylation method are due to relatively low recoveries (79.1 ± 15.7%) compared to HG (98.5 ± 3.95%). Multiple procedure steps and incomplete solvent extraction, followed by back-extraction into Milli-Q by solvent evaporation are the main reasons for low and unreproducible recoveries (Horvat et al., 1993). At the highest MeHg concentration level (>400 fM), recovery has the highest contribution index for the ethylation method (37.0%). For HG, there is no pre-concentration step, resulting in almost quantitative recovery of the standard solution added to the sample.

Sample areas have a big contribution to $U_{ex,r}$ at lower MeHg concentrations for HG. This is mainly due to readability of small peaks which are the consequence of lower sensitivity of this method; slope of the external calibration curve for ethylation is about 35% higher than for hydride generation. Small peaks are sometimes differently integrated on different computers and operating systems. This observation was also included into the uncertainty estimation.

Although sample volume does not have a large contribution index, it is important to emphasize the reason for approximately three-fold greater value in ethylation. Possible sample losses during phase separation, back-extraction of sample from dichloromethane to Milli-Q water, incomplete sample transfer to the vial and instrument cause much higher uncertainties than direct derivatization in a single vessel during hydride generation. Other uncertainty sources do not represent a significant contribution to $U_{ex,r}$.

4. Conclusions

The coupled hydride generation–cryogenic trapping–gas chromatography–cold vapor atomic fluorescence spectrometry is a simple method for determination of MeHg in seawater. Low detection limits enable method application for MeHg determination in various environmental matrixes, especially for determination of low MeHg levels in seawater. Comparison with a previous study shows slight improvement in optimized method performance, most noticeably in detection limit and MeHg peak symmetry. Estimation and quantification of possible uncertainty sources for MeHg determination in seawater by HG-CT-GC-CVAFS revealed that sample repeatability has the highest relative contribution to the expanded uncertainty, especially at MeHg concentrations near detection limit (47%). Along with sample repeatability, MeHg recovery has the highest contribution index for Eth-GC-CVAFS, mainly due to unreproducible recoveries.

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3.4 Relations Between Mercury Fractions and Microbial Community Components in Seawater Under the Presence and Absence of Probable Phosphorus Limitation Conditions

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In this paper, the authors presented relations between mercury fractions and microbial community components in the seawater of the central Adriatic Sea under probable phosphorus limitation conditions (^{P}P-limitation) and their absence ($^{NO–P}$P-limitation). The research was conducted along the transect between the pristine environment of the Island of Vis and the Kaštela Bay, which has been previously contaminated with mercury-containing effluents from the chlor-alkali plant.

Seawater sampling for the determination of mercury fractions (THg, DGM and MeHg) and microbial community structure was mostly on a monthly basis during 2014–2015. Mercury fractions were determined at the Jožef Stefan Institute, while the physico-chemical characteristics of seawater and the structure of microbial communities were determined at the Institute of Oceanography and Fisheries.

The authors performed univariate, bivariate and multivariate analysis of obtained data. Dataset was divided into two groups based on the presence or absence of probable phosphorus limitation conditions, which were determined by the stoichiometric nutrient ratios from the literature. Statistical analysis showed significant differences in correlations between mercury fractions and some microbial community composition under the presence and absence of probable phosphorus limitation conditions.

Under probable phosphorus limitation, the relations between microbial community components and mercury fractions were not pronounced. The only significant relation was between DGM, THg, seawater temperature, and *Synechococcus*. The absence of significant relations with other microorganisms under probable phosphorus limitation possibly indicates that phosphorus limitation might produce metabolic constrains on microbial mercury transformations.

On the contrary, when conditions were not limited by phosphorus, DGM was related with numerous parameters, such as the abundances of high- and low-nucleic acid bacteria, *Prochlorococcus*, *Synechococcus*, HNF, chlorophyll *a* concentration, and seawater temperature. MeHg was related with the abundance of cyanobacteria at the neritic station in the Kaštela Bay, and with picoeukaryotes and *Prochlorococcus* at open sea stations. These results possibly indicate that non-limiting nutrient conditions might favor mercury transformations at the lowest marine trophic level.

The candidate participated in monthly samplings aboard the research vessel *Bios Dva*. He prepared bottles and equipment needed for seawater sampling and mercury analysis. After sampling, the candidate determined THg and MeHg concentrations in most of seawater samples. In addition, he determined THg and MeHg in freeze-dried microseston and zooplankton samples. Following the experimental part of the research, he performed univariate and bivariate data analysis. Based on previous research on phosphorus limitation

in seawater, he divided data into two corresponding groups and statistically compared them. The candidate drew maps, bar charts, box-and-whiskers diagrams and Ocean Data View plots. He summarized data in tables and drafted the manuscript in collaboration with other co-authors.

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Relations between mercury fractions and microbial community components in seawater under the presence and absence of probable phosphorus limitation conditions

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ABSTRACT

Microbial transformations of toxic monomethylmercury (MMHg) and dissolved gaseous mercury (DGM) at the lower levels of the marine food web are not well understood, especially in oligotrophic and phosphorus-limited seas. To examine the effects of probable phosphorus limitation (^{P}P-limitation) on relations between mercury (Hg) fractions and microorganisms, we determined the total mercury (THg), total methylated mercury (MeHg), DGM, and microbiological and chemical parameters in the Central Adriatic Sea. Using statistical analysis, we assessed the potential microbial effects on Hg transformations and bioaccumulation. Only in the absence of ^{P}P-limitation conditions ($^{NO-P}$P-limitation) is MeHg significantly related to most chemical and microbial parameters, indicating metabolism-dependent Hg transformations. The heterotrophic activity of low nucleic acid bacteria (abundant in oligotrophic regions) seems responsible for most of Hg methylation under $^{NO-P}$P-limitation. Under these conditions, DGM is strongly related to microbial fractions and chlorophyll *a*, indicating biological DGM production, which is probably not metabolically induced, as most of these relations are also observed under ^{P}P-limitation. MMHg biomagnification was observed through an increased

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bioaccumulation factor from microseston to mesozooplankton. Our results indicate that Hg transformations and uptake might be enhanced under $^{NO-P}$P-limitation conditions, emphasizing their impact on the transfer of Hg to higher trophic levels.
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Introduction

Mercury (Hg) is a contaminant of great concern due to its toxicity, persistence, and accumulative behavior in the environment, especially its organic form monomethylmercury (MMHg) (Mason et al., 2012). Natural and anthropogenic Hg sources represent major problems in the Mediterranean and Adriatic Seas where elevated concentrations in seawater can be found (Horvat et al., 2003; Živković et al., 2017b). Natural biological and (photo)chemical processes can convert oxidized Hg to Hg^0, which can travel long distances before deposition and conversion to MMHg. A major MMHg source is biotic Hg methylation by sulfate-reducing bacteria in anoxic sediments (Barkay and Wagner-Döbler, 2005), which can represent a major burden to the shallow Adriatic environments (Petranich et al., 2017). Recent research suggests that oxic ocean waters may also host biotic methylation (Cossa et al., 2017; Heimbürger et al., 2010; Lehnherr et al., 2011). Hg methylation has been linked to organic carbon remineralization (Cossa et al., 2009; Heimbürger et al., 2010), oxygen-deficient zones (Cossa et al., 2017; Heimbürger et al., 2010), and the presence of small-sized phytoplankton that dominate under oligotrophic conditions (Heimbürger et al., 2010). Although MMHg biomagnifies along the food web, initial microbial MMHg bioaccumulation represents the greatest contribution to Hg bioconcentration (Gosnell et al., 2017; Mason et al., 2012).

Microorganisms are of great importance to marine biogeochemical cycles and processes. In oligotrophic seas, such as the Central Adriatic Sea, autotrophic picoplankton (APP) and heterotrophic picoplankton (mostly heterotrophic bacteria) represent major components of the picoplankton community (Grob et al., 2007). In these waters, autotrophic picoplankton make a large contribution to carbon fixation, biomass, and energy transfer. The specific structure of the microbial food web depends on the environmental trophic status, and it causes variability in the microbial role in biogeochemical transformations (Berglund et al., 2007), especially in nutrient-limited environments.

The effects of limited nutrient accessibility on Hg transformations and its bioaccumulation in marine plankton are still not well known (Gosnell et al., 2017). Phosphorus limitation (P-limitation) can induce severe physiological and metabolic changes in autotrophic and heterotrophic microorganisms, including morphology and ecophysiology alterations, phosphorus-containing biomass depletion, polyphosphate and acetyl coenzyme A degradation, changes in the central metabolism and polyhydroxyalkanoate production, and the downregulation of enzymes involved in DNA replication, protein synthesis, and phosphate transformations (Bertilsson et al., 2003; Casey et al., 2016; Miles et al., 2001; Romano et al., 2015; Wen et al., 2010). In the Adriatic estuaries, prokaryotic autotrophs reach their highest biomasses under P-limitation, while heterotrophic bacteria reach higher biomasses under conditions that are not P-limited (Šolić et al., 2015) due to the lower bacterial production under P-limitation (Šolić et al., 2017). The combination of microbial metabolic changes and the abundances of autotrophic and heterotrophic communities might have a significant influence on Hg speciation under P-limitation conditions.

A recent conceptual model suggested that an increase in reactive nitrogen loadings in coastal marine environments alters Hg dynamics by decreasing its bioavailability and trophic transfer (Driscoll et al., 2012). However, eutrophication can also increase phytoplankton MMHg in waters where MMHg is produced under normoxic conditions (Soerensen et al., 2016). Although eutrophication effects were studied, the effects of P-limitation on Hg bioaccumulation are still unknown.

The objective of this study is to determine the effect of the microbial community composition on Hg speciation in the Adriatic Sea under the presence and absence of probable phosphorus limitation conditions (^{P}P-limitation and $^{NO-P}$P-limitation). Our analysis evaluates the correlations between the concentrations of Hg fractions and physico-chemical properties and the composition of marine microbial communities. We examine the effects of ^{P}P-limitation on Hg-microorganism relations and discuss the biological parameters affecting Hg speciation in seawater.

1. Materials and methods

1.1. Study area

The Adriatic Sea is a semi-enclosed basin in the northernmost part of the Mediterranean Sea. It is an oligotrophic and phosphorus-limited sea (Šolić et al., 2015). The main Hg sources in the Adriatic Sea include chlor-alkali plants and the discharge of the Hg-rich Isonzo River, resulting in elevated Hg concentrations in seawaters and sediments (Živković et al., 2017b).

Our research was restricted to transect between Kaštela Bay and the Island of Vis in the Central Adriatic Sea (Fig. 1). Kaštela Bay is a shallow, semi-enclosed bay in the Central Adriatic Sea. The Jadro River is the most important freshwater source, carrying nutrients from surrounding fields. Water circulation in the bay is wind-induced, with a water renewal time of one month. Renewal time is longer during summer months, when wind-induced circulation and freshwater input are relatively low. However, during strong winds, it can be as short as five days (Zore-Armanda, 1980). It was estimated that at least 750 kmol of Hg was released into the bay during the operation of a chlor-alkali plant (1950–1990; Fig. 1), causing contamination of seawater and sediments (Zvonarić, 1991).

Although located away from coastal waters, surface layers of the station near the Island of Vis (CJ009) are partially influenced by the Neretva River, the largest eastern

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Fig. 1 – (a) Sampling area in the Adriatic Sea indicated with a red square. (b) Sampling site locations mapped using Google Earth Pro 7.1. Yellow pins indicate location of the former chlor-alkali plant (CAP).

Adriatic river. Deeper layers are characterized mostly by the presence of Levantine Intermediate Water and North Adriatic Deep Water, whose chemical and physical characteristics are not as susceptible to great oscillations as those of coastal waters (Buljan and Zore-Armanda, 1976). Therefore, this station is convenient for detecting long-term environmental changes in contaminant concentrations in the open Adriatic seawaters.

1.2. *Sampling*

Water samples were collected on board the R/V *Bios Dva* during monthly oceanographic cruises in the Central Adriatic Sea (Fig. 1) from February 2014 to December 2015. The sampling stations' coordinates and sampling dates are presented in Appendix A **Table S1** and **Table S2**, respectively. Cable-mounted Niskin bottles (5 L) were used for seawater sampling. All samples were stored in acid-cleaned borosilicate glass bottles at 5°C until analysis. Only samples for the determination of total mercury (THg) and total methylated mercury (MeHg) were acidified with HCl (0.1%, V/V). Dissolved gaseous mercury (DGM) was analyzed immediately upon arrival at the Institute of Oceanography and Fisheries (Split, Croatia), while THg and MeHg were determined within three months at the Jožef Stefan Institute (Ljubljana, Slovenia). Samples for cell counts were transferred into sterile glass containers, fixed with formaldehyde (final concentration 2%), and counted within 2 days of collection.

Microseston and mesozooplankton samples were collected in March and April 2014 with a 53-μm mesh Nansen net (0.55 m diameter, total length 2.75 m) and a 200-μm mesh Nansen net (0.60 m diameter, total length 3.35 m), respectively by consecutive vertical hauls from near-bottom to the surface. Collected samples were rinsed with ambient seawater and Milli-Q, and were freeze-dried at the Institute of Oceanography and Fisheries.

1.3. *Analytical methods for mercury determinations*

THg in seawater and precipitations was determined using cold vapor atomic fluorescence spectrometry (CVAFS; Tekran 2600, Tekran Inc., Canada) by following instrument manual after bromine monochloride digestion, UV irradiation, and reduction with tin chloride (Horvat et al., 2003). Corresponding field blanks were below the method detection limit (Appendix A **Table S3**). DGM was determined by purging seawater with argon gas onto gold traps (double amalgamation), followed by thermal desorption and CVAFS detection (Brooks Rand III, Brooks Rand Instruments, USA) (Horvat et al., 2003; Kotnik et al., 2007). Measured DGM concentrations represent the sum of the dissolved elemental mercury (DEM) and dimethylmercury (DMHg).

MeHg in samples was determined by a direct method based on derivatization by hydride generation, which is precisely described by Živković et al. (2017a). The limit of detection (LOD) of this method range from 3.94 to 15.9 fmol/L, primarily due to very low reagent and measurement blanks. The only drawback of this method is that in case inorganic mercury was high in seawater, the resulting peaks corresponding to elemental mercury and methylmercury hydride in chromatograms were not well separated. In such cases, samples were processed by a conventional method based on solvent extraction, ethylation and CVAFS detection (MERX, Brooks Rand Instruments, USA) (Horvat et al., 1993). This method provides comparable results, but its' LOD is slightly higher (from 7.73 to 25.3 fmol/L) as shown in Appendix A **Table S3**. The percentage of samples below ethylation LOD was 14.6%. Both methods were validated and the results and corresponding uncertainties are comparable (Živković et al., 2017a). Reported MeHg values represent the sum of MMHg and DMHg.

THg in freeze-dried plankton was determined using atomic absorption spectrometry (Direct Mercury Analyzer DMA-80, Milestone, Italy) after thermal decomposition, catalytic reduction, gold trapping, and desorption (Battuello et al., 2016).

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MMHg in plankton was determined with CVAFS after HNO_3 digestion, aqueous phase ethylation, Tenax trapping, chromatographic separation, and pyrolysis (Tekran 2700, Tekran Inc., Canada) (Gosnell et al., 2017; Hammerschmidt et al., 2013). The detection limits and quality assurance of methods for mercury determinations are presented in **Table S3** and **Table S4**, respectively.

1.4. Determination of microbial community components

Abundances of *Synechococcus* (*Syn*), *Prochlorococcus* (*Proc*), and picoeukaryotes (Pico) were determined using flow cytometry (Marie et al., 1999). Based on the orange emission of phycoerythrin-rich cells, the red emission of cellular chlorophyll, and light scattering, autotrophic organisms were divided into cyanobacteria (*Synechococcus* and *Prochlorococcus*) and picoeukaryotes. Flow cytometry (EPICS XL-MCL, Beckman Coulter, USA) was used for the determination of SYBR® Green-I-stained non-pigmented bacteria (Marie et al., 1997) and heterotrophic nanoflagellates (HNF) (Christaki et al., 2011). The standardization of bacterial cell fluorescence intensity was achieved using fluorescein calibration beads (Coulter). Based on light scattering and relative green fluorescence (proxy for nucleic acid content) (Jochem, 2001), high nucleic acid (HNA) bacteria were distinguished from low nucleic acid (LNA) bacteria.

Bacterial cell production was determined from the bacterial cell number and ^{3}H-thymidine incorporation rate into bacterial DNA during bacterial growth (Fuhrman and Azam, 1982; Riemann et al., 1987). Epifluorescence microscopy was used to determine phototrophic nanoflagellates (PNFs). Seawater samples were filtered through polycarbonate filters and obtained microscope slides were examined under UV light (Olympus BX51, Olympus Corporation, Japan; 1000× magnification) (Porter and Feig, 1980).

The following cell-to-carbon conversion factors were used for picoplankton biomass estimation: 1.665 fmol C/cell for heterotrophic bacteria (Kirchman et al., 1993; Lee and Fuhrman, 1987), 2.998 fmol C/cell for *Prochlorococcus*, 21.23 fmol C/cell for *Synechococcus*, and 215.7 fmol C/cell for picoeukaryotes (Buitenhuis et al., 2012). Determinations of microbial species were not performed at station CJ008.

1.5. Determination of seawater physico-chemical parameters

Seawater temperature and salinity were measured with the Seabird-25 CTD probe (Sea-Bird Scientific, USA). Following the manufacturer's procedure, obtained temperatures and salinities were averaged for every meter of vertical profile. Dissolved oxygen concentrations were determined using Winkler titration (Grasshoff, 1976). Apparent oxygen utilization (AOU) was calculated from dissolved oxygen concentrations and oxygen saturation data. Seawater pH was measured potentiometrically (Strickland and Parsons, 1972) using a Metrohm glass electrode (Metrohm, Switzerland) calibrated in NIST buffer solutions prior to every set of measurements. Concentrations of dissolved inorganic nutrients (nitrate, nitrite, ammonia, phosphate, and silicate) were determined colorimetrically (AutoAnalyzer, Seal Analytical, UK) following modified methods originally proposed by Grasshoff (1976). Total dissolved nitrogen and total dissolved phosphorous were determined as nitrate and phosphate after wet digestion (Grasshoff, 1976). Chlorophyll *a* (Chl-*a*) was determined fluorometrically (Turner 112, Sequoia-Turner Corporation, USA) after acetone extraction (Strickland and Parsons, 1972).

1.6. Data analysis

Results were statistically analyzed using the Stata/SE program (StataCorp, USA; version 12.0). As a normal distribution was not achieved (Shapiro–Wilk test), data were transformed using common logarithm. For the same reason, results are presented using geometric means, which are adequate statistical descriptors (Cossa et al., 2009). Significant differences between sample groups were determined using non-parametric tests (Mann–Whitney, signed-rank test) when data significantly deviated from normal distribution. The p values of <0.05 were considered statistically significant. Correlations between variables are presented using Pearson's product–moment correlation coefficients (r), and they refer to log-transformed data. The Fisher r-to-z test was used to determine significant differences between two correlation coefficients (Soper, 2018).

The significance of the differences among concentrations of Hg fractions to the spatial scale was tested *via* a two-way ANOVA using Kruskal–Wallis statistics, followed by the post-hoc Dunn's Multiple Comparison test.

A principal component analysis (PCA) was performed to summarize the main patterns of variations between the microbial food web components as active variables and environmental variables as supplementary variables across the samples. The use of a linear method is appropriate, as the response data are compositional and have a gradient of 0.4 standard deviation units long. The input data consisted of two sets of variables representing the spatial and seasonal variability of microbial food web parameters (high and low nucleic acid bacteria, *Prochlorococcus*, *Synechococcus*, picoeukaryotes, heterotrophic and phototrophic nanoflagellates, bacterial production, and chlorophyll *a*) and abiotic variables, including Hg fractions. The values of the squared cosines of the variables were used to estimate the best link between a particular variable and the extracted principal component. The Varimax rotation of extracted principal components produced better insight into the behavior of observation variables. Kruskal–Wallis statistics and PCA were performed using the XLSTAT statistical package (Addinsoft, USA; version 2015.4.01.21575).

Multi-dimensional scaling (MDS) was used to describe the (dis)similarity patterns between Hg fractions in the Central Adriatic Sea (Clarke and Warwick, 2006). The matrix consisted of log $(x + 1)$-transformed concentrations of Hg species converted to a lower triangular similarity matrix using Euclidean distance coefficients. A grouping variable (sampling station) was used to extract distribution patterns from the data matrix, and this analysis was performed using the Primer 6 statistical package (PRIMER-E, New Zealand; version 6.1.13) (Clarke and Gorley, 2006).

2. Results and discussion

2.1. Probable phosphorus limitation

Phosphate concentrations (Fig. 2) show significant differences among stations (Kruskal–Wallis, $p < 0.001$). ^{P}P-limitation can

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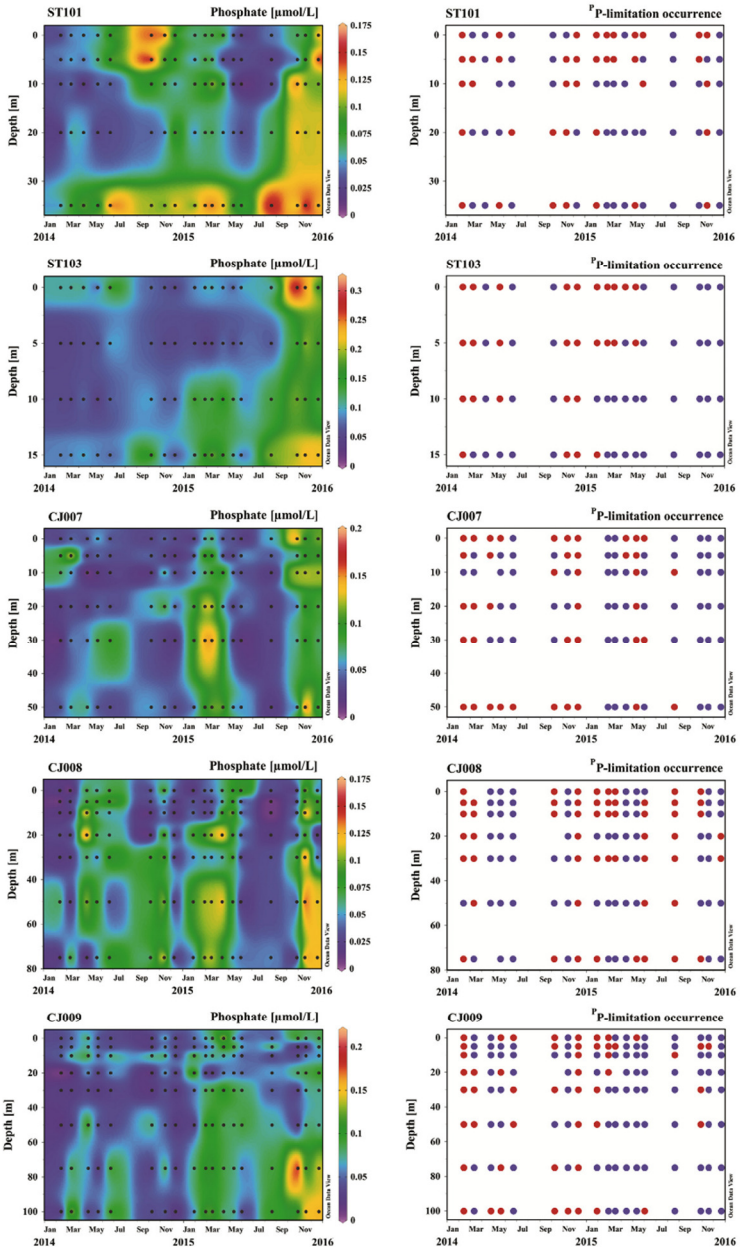


Fig. 2 – Vertical profiles of phosphate concentrations (left) and the occurrence of probable phosphorus limitation conditions (^{P}P-limitation) in the Central Adriatic Sea during 2014–2015 sampling campaign (right; blue dot – absence of ^{P}P-limitation, red dot – presence of ^{P}P-limitation).

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be assessed using the criteria for stoichiometric P-limitation (Dortch and Whitledge, 1992; Justić et al., 1995). By applying these criteria, ^{P}P-limitation was defined using literature stoichiometric ratios previously determined in the Adriatic Sea (total dissolved inorganic nitrogen–TIN:PO_4 > 22:1 and SiO_4:PO_4 > 22:1) (Šolić et al., 2015, 2017).

^{P}P-limitation conditions occur in 40.5% of samples, and they are not significantly related to any specific station (χ^2 test, $p = 0.508$; Fig. 2) or any depth (χ^2 tests, $p > 0.05$). However, ^{P}P-limitation conditions occur more frequently during winter than during other seasons (χ^2 test, $p < 0.001$), when an excess of dissolved nitrogen species can shift the stoichiometric TIN:PO_4 ratio to more than 100. Relations between mercury fractions and biological/chemical parameters show differences under the presence of ^{P}P-limitation and the absence of ^{P}P-limitation ($^{NO-P}$P-limitation); specific correlations are discussed below, while summaries are presented in Table 1 and Table 2 for MeHg and DGM, respectively.

2.2. *Mercury abundance and distribution in the Central Adriatic Sea*

THg concentrations (Fig. 3) range from 0.54 to 27.8 pmol/L, with an overall geometric mean of 2.55 pmol/L. THg concentrations show a decreasing gradient from the coastal stations to the open sea, with a statistically significant difference among stations (Kruskal–Wallis, $p < 0.001$; Fig. 4). (Dis)similarities among the sampling stations were illustrated in the MDS ordination diagram (Appendix A **Fig. S1**). The separation of the neritic station (ST103) in comparison to the open water stations, particularly station CJ009, was evident in the distributions of all target Hg fractions. These differences were statistically significant (Kruskal–Wallis, $p < 0.0001$). The lowest THg concentrations at the open sea station (CJ009, geomean 1.72 pmol/L) are similar to the average values (1.5 pmol/L) previously found in the whole Mediterranean Sea (Horvat et al., 2003). However, this value is lower than the concentration (2.99 pmol/L) recently measured at the near-by station during summer 2005 (Kotnik et al., 2015), indicating high seasonal variability. The highest THg values in Kaštela Bay (ST103, geomean 8.89 pmol/L) are influenced by a former chlor-alkali plant. Horvat et al. (2003) measured a similar value (7.70 pmol/L) near the plant and observed a similar decreasing gradient toward the open sea. Even though the seawater in Kaštela Bay has elevated THg values that can present a significant Hg source to the region, their influence seems to be localized, as THg values outside Kaštela Bay are similar (Fig. 4).

THg concentrations do not show significant differences between colder (December–April) and warmer times (May–

Table 1 – Pearson's product–moment correlation coefficient (*r*) between MeHg and various chemical/biological parameters, and the difference between correlation coefficients (*r*-to-z test) under the presence and absence of probable phosphorus limitation conditions (^{P}P-limitation and $^{NO-P}$P-limitation).

MeHg vs.	*r* (all dataset)	*r* ($^{NO-P}$P-limitation)	*r* (^{P}P-limitation)	*r*-to-z test
Total mercury	0.214 ****	0.206 ***	0.211 **	ns
Dissolved gaseous mercury	0.287 ****	0.310 ****	0.262 ***	ns
Bacterial biomass	0.108 *	0.176 **	0.025 ns	ns
High nucleic acid bacteria	0.042 ns	0.071 ns	0.060 ns	ns
Low nucleic acid bacteria	0.137 **	0.229 ***	−0.024 ns	*
Bacterial cell production	−0.218 ***	−0.265 ***	−0.191 *	ns
Specific bacterial production	−0.258 ****	−0.345 ****	−0.184 *	ns
Chlorophyll *a*	0.163 ***	0.289 ****	0.010 ns	**
Autotrophic picoplankton	0.148 **	0.123 ns	0.183 *	ns
Picoeukaryotes	0.194 ***	0.192 **	0.221 **	ns
Prochlorococcus	0.131 *	0.287 ***	−0.070 ns	***
Synechococcus	0.030 ns	−0.005 ns	0.073 ns	ns
Heterotrophic nanoflagellates	0.052 ns	0.186 **	−0.088 ns	**
Nanoflagellates:bacteria ratio	−0.015 ns	0.099 ns	−0.138 ns	*
Phototrophic nanoflagellates	−0.084 ns	−0.045 ns	−0.095 ns	ns
Sea temperature	−0.179 ***	−0.302 ****	−0.048 ns	**
Salinity	0.027 ns	−0.063 ns	0.082 ns	ns
Dissolved oxygen	0.030 ns	0.136 *	−0.106 ns	**
Apparent oxygen utilization	0.099 *	0.078 ns	0.202 **	ns
pH	−0.048 ns	−0.041 ns	0.019 ns	ns
Nitrate	0.165 ***	0.270 ****	0.040 ns	**
Nitrite	0.143 ***	0.295 ****	−0.036 ns	***
Ammonium	−0.073 ns	−0.195 ***	0.116 ns	***
Phosphate	0.085 *	0.205 ***	0.052 ns	ns
Silicate	0.169 ***	0.286 ****	0.067 ns	*

Correlations refer to log-transformed data, except for pH (original data), and phototrophic nanoflagellates and apparent oxygen utilization where log(x + 1) and log(x + 100) transformations were used respectively, due to existence of non-positive values. ns — not significant.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.
**** $p < 0.00001$.

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Table 2 – Pearson's product-moment correlation coefficient (r) for entire dataset and difference among correlation coefficients (r-to-z test) between DGM and various chemical/biological parameters under the presence and absence of probable phosphorus limitation conditions (^{P}P-limitation and $^{NO-P}$P-limitation).

DGM vs.	r (all dataset)	r ($^{NO-P}$P-limitation)	r (^{P}P-limitation)	r-to-z test
Total mercury	0.192 ***	0.202 ***	0.181 *	ns
Methylmercury	0.287 ****	0.310 ****	0.262 ***	ns
Bacterial biomass	0.427 ****	0.466 ****	0.359 ***	ns
High nucleic acid bacteria	0.369 ****	0.363 ****	0.391 ****	ns
Low nucleic acid bacteria	0.357 ****	0.457 ****	0.206 *	**
Bacterial cell production	−0.247 ****	−0.247 ***	−0.266 **	ns
Specific bacterial production	−0.487 ****	−0.528 ****	−0.451 ****	ns
Chlorophyll *a*	0.369 ****	0.468 ****	0.207 **	***
Autotrophic picoplankton	0.465 ****	0.423 ****	0.514 ****	ns
Picoeukaryotes	0.393 ****	0.390 ****	0.397 ****	ns
Prochlorococcus	0.348 ****	0.473 ****	0.158 ns	**
Synechococcus	0.260 ****	0.204 **	0.318 ***	ns
Heterotrophic nanoflagellates	0.410 ****	0.536 ****	0.215 *	***
Nanoflagellates:bacteria ratio	0.214 ***	0.319 ****	0.035 ns	**
Phototrophic nanoflagellates	0.008 ns	0.134 *	−0.230 **	***
Sea temperature	−0.079 ns	−0.091 ns	−0.017 ns	ns
Salinity	−0.183 ***	−0.301 ****	−0.099 ns	*
Dissolved oxygen	0.027 ns	0.088 ns	−0.065 ns	ns
Apparent oxygen utilization	−0.001 ns	−0.075 ns	0.135 ns	*
pH	0.259 ****	0.121 ns	0.409 ****	**
Nitrate	0.161 ***	0.088 ns	0.233 **	ns
Nitrite	0.091 *	0.137 *	0.001 ns	ns
Ammonium	0.020 ns	−0.098 ns	0.172 *	**
Phosphate	−0.017 ns	0.102 ns	−0.039 ns	ns
Silicate	0.207 ****	0.138 *	0.266 ***	ns

Correlations refer to log-transformed data, except for pH (original data), and phototrophic nanoflagellates and apparent oxygen utilization where log(x + 1) and log(x + 100) transformations were used respectively, due to existence of non-positive values. ns – not significant.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.
**** $p < 0.00001$.

November) of the year (Mann–Whitney, $p = 0.503$). The surface and bottom THg concentrations are significantly different at neritic station ST103 (signed-rank test; $p < 0.01$), where THg is correlated with depth ($r = 0.488$, $p < 0.0001$). These results show that sediment resuspension at the shallow coastal station presents an important source of mercury to the water column. However, the distribution of THg in the water column can be occasionally completely inversed, with the highest values found in the surface layer, even at the coastal station. In June 2015, the surface THg at ST103 reached 22.9 pmol/L, and it was 3-fold higher than the bottom THg. Several days prior to sampling, the considerable amount of precipitations (94.6 L/m^2) containing 77.8 pmol THg/L alongside the freshwater input from the Jadro River were the most probable reasons for the observed THg distribution. In these multiple-input coastal waters, allochthonous Hg requires time before natural environmental concentrations are restored; therefore, allochthonous THg might not correlate with autochthonous microbial communities.

The results of the PCA suggest that microbial parameters were always better related with THg and DGM than with MeHg, especially in neritic and $^{NO-P}$P-limited ecosystems (ST103) (**Fig. S2a, b**). THg was considerably linked with bacterial production during the spring, and these relations were better pronounced at the coastal station ST103 (PC1 32.61%) than at the open sea stations (PC2 19.72%).

DGM concentrations (Fig. 3) range from 0.10 to 3.39 pmol/L (geomean 0.47 pmol/L). Similar to THg, DGM shows a decreasing gradient from the coastal area toward the open sea, with a significant difference between stations (Kruskal–Wallis, $p < 0.001$), although the decrease is less sharp than for THg (Fig. 4). The geometric mean of the DGM concentrations at open sea station CJ009 (0.34 pmol/L) is higher than the value obtained at the near-by station during June 2005 (0.21 pmol/L) (Kotnik et al., 2015) or in the surface layer of the Eastern Mediterranean Sea (0.22 pmol/L) (Horvat et al., 2003). However, lower values in June 2014 (geomean 0.14 pmol/L) emphasize DGM variability and the importance of specific photochemical and/or microbial reactions in DGM transformations (Horvat et al., 2003). The highest DGM concentrations were measured at the neritic station during December 2014. The night before sampling, a strong northern wind (16.3 m/sec) was blowing from the former chlor-alkali plant (Fig. 1) toward the sea. Consequently, waves probably disrupted Hg^0-rich sediments (Kwokal et al., 2002) that were enriched due to historical Hg^0 release from the chlor-alkali plant into the sea (Zvonarić, 1991). The wave-induced water transfer might have caused the southward dispersal of elemental mercury to the shallow neritic station.

Contrary to THg, DGM concentrations are significantly higher during colder (geomean 0.60 pmol/L) than during warmer times of the year (0.38 pmol/L) (Mann–Whitney,

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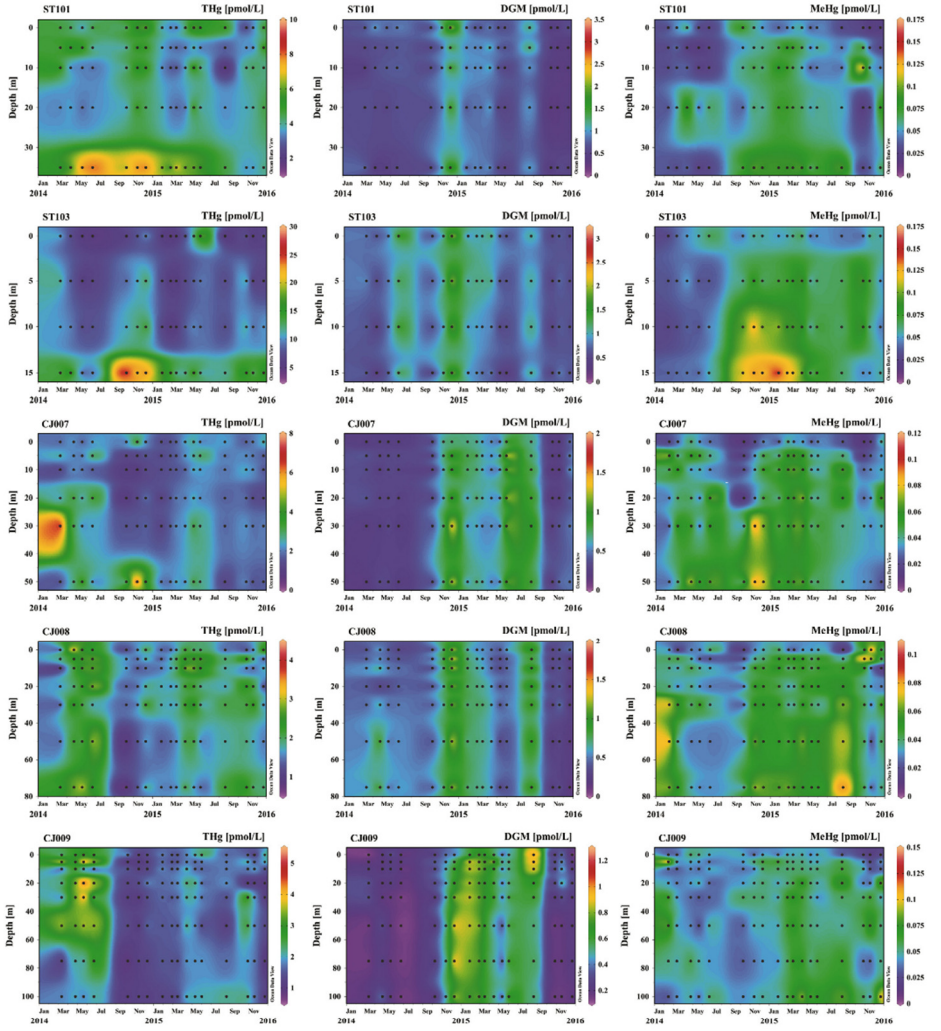


Fig. 3 – Vertical profiles of mercury fractions (total mercury, THg; dissolved gaseous mercury, DGM; and total methylated mercury, MeHg) in the Central Adriatic Sea during 2014–2015 sampling campaign.

$p < 0.001$), indicating the importance of photo- and biologically mediated reactions, and DGM solubility in colder waters. In $^{NO-P}$P-limited marine environments, DGM was related positively and strongly with numerous microbial parameters, such as the abundances of HNA bacteria, LNA bacteria, *Proc*, *Syn*, HNF, Chl-*a*, and seawater temperature. Such relations were recorded at neritic and open sea stations with an almost equal intensity. The only relation in ^{P}P-limited marine environments was found within PC1 (28.36%), which consists of DGM and THg, seawater temperature, and *Syn* (not shown).

Contrary to THg, DGM does not show a significant correlation with depth ($p > 0.25$) or a significant difference between the surface and bottom layers at any station (signed-rank test; $p > 0.05$). DGM volatility probably causes its vertical transfer and subsequent release into the atmosphere, as most surface waters are supersaturated relative to the atmosphere (Kotnik

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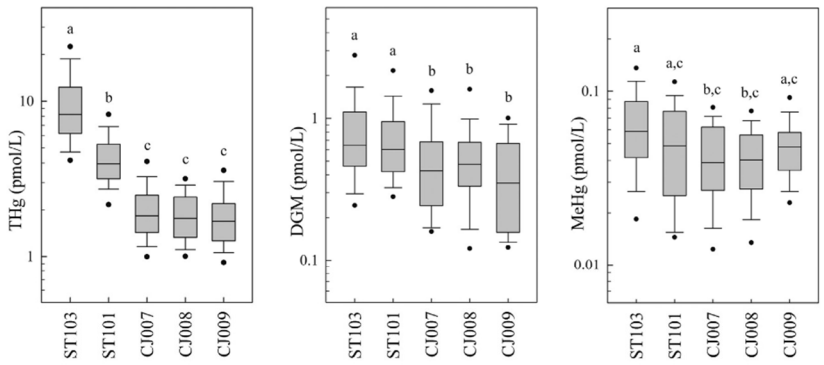


Fig. 4 – Box-and-whiskers plots (5th, 10th, 25th, median, 75th, 90th, and 95th percentile) of THg (left), DGM (center), and MeHg concentrations (right), sorted by station distance from the chlor-alkali plant. All mercury fractions show significantly different concentrations among stations (Kruskal–Wallis, $p < 0.001$). Different lowercase letters correspond to pairwise stations with significant difference in concentrations (post-hoc Dunn's test, $p < 0.05$). Vertical axes are different among graphs and represented on a logarithmic scale.

et al., 2017; Mason et al., 2017). On average, DGM accounts for 18.3% of THg, which is similar to the values previously found in the Adriatic (12.7%–35.1%) and Mediterranean Seas (20%) (Kotnik et al., 2017). As DMHg concentrations are low in the surface waters of the Central Adriatic (geomean 0.003 pmol/L) (Kotnik et al., 2015), the DGM measured in our study probably consists mostly of Hg^0.

MeHg concentrations (Fig. 3) are low and range from the detection limit to 0.17 pmol/L (geomean 0.043 pmol/L). Contrary to THg and DGM gradients, MeHg shows a somewhat different spatial distribution (Fig. 4). MeHg concentrations first decrease from the neritic station (geomean 0.056 pmol/L at ST103) and then reach minimal values at CJ007 (0.037 pmol/L). Toward the open sea, the average MeHg concentration rises to 0.046 pmol/L at CJ009, showing a significant difference between stations (Kruskal–Wallis, $p < 0.001$; Fig. 4). This might be due to elevated DMHg concentrations (0.011 pmol/L) previously found in the Levantine Intermediate Water at open sea stations (Kotnik et al., 2015). Although the evasion of DMHg and its decomposition into MMHg in surface layers could explain this observation, sediment input is also possible. The bottom MeHg concentrations are significantly higher than the surface values at any station (signed-rank test; $p < 0.05$). As THg does not correlate with depth at open sea stations, it seems that either MeHg has much higher sediment diffusion rates than THg or MeHg is produced in the bottom water layer at open sea stations. At the coastal stations, sediment resuspension is most likely to occur due to low depth. Occasional MeHg hotspots in the surface and sub-surface layers at ST101 and CJ009 might originate from wet deposition. The precipitation samples collected at the Institute of Oceanography and Fisheries (near ST101) have elevated MeHg concentrations (0.24–0.70 pmol/L) compared to seawater. This might also explain the observations at CJ009, but precipitation samples were not collected at the Island of Vis (near CJ009).

Contrary to THg, MeHg concentrations are significantly higher during colder (geomean 0.049 pmol/L) than during warmer times of the year (0.038 pmol/L; Mann–Whitney, $p < 0.001$), indicating the importance of photo- and biodemethylation of MeHg. PCA analysis revealed that under $^{NO-P}$P-limitation conditions, MeHg was related with the abundance of cyanobacteria (*Proc* and *Syn*) at the neritic station (PC3 14.05%), while at open sea stations, this Hg fraction was linked with Pico and *Proc* (PC2 19.72%). The average MeHg values are lower than the corresponding values found during previous measurements in Kaštela Bay in 1998 (0.14 pmol/L) (Horvat et al., 2003) and near Vis in 2005 (0.45 pmol/L) (Kotnik et al., 2015). However, the average MeHg values are similar to concentrations found at the Rhône River pro-delta in the Gulf of Lions (0.041 pmol/L) (Cossa et al., 2017). On average, MeHg accounts for 1.69% of THg in the Central Adriatic Sea. This value is similar to the average concentration at the Gulf of Lions shelf (1.9%) (Cossa et al., 2017), but it is much lower than values previously calculated for the open Adriatic (22.2%) (Kotnik et al., 2015) and Mediterranean Seas (14%–20%) (Cossa et al., 2009; Horvat et al., 2003). As DMHg represents at most 10.8% of the MeHg in the deeper layers at the station near the Island of Vis (Kotnik et al., 2015), the MeHg measured in our study probably consists mostly of MMHg.

2.3. Mercury speciation and physico-chemical parameters

Recent studies found correlations between MeHg and AOU in the Mediterranean Sea (Cossa et al., 2009, 2017; Heimbürger et al., 2010) and Pacific Ocean (Sunderland et al., 2009), where the remineralization of organic matter is a driving force for bacterial Hg methylation. In this study, MeHg is correlated with AOU ($r = 0.285$, $p < 0.05$) only at the neritic station, which is characterized by higher MeHg and mostly positive AOU

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values in the bottom layer (Appendix A **Fig. S3**). Generally, there are no significant differences between MeHg–AOU correlations under ^{P}P-limitation and $^{NO-P}$P-limitation (Table 1). As primary production prevails over organic matter degradation, the activity of heterotrophic bacteria might represent a source of MeHg in the water column of the Central Adriatic Sea, but it cannot be distinguished from possible autotrophic MeHg production. In addition, photochemical MeHg demethylation in surface waters and probable MeHg input from the sediments are possible explanations for low correlation coefficients between MeHg and AOU (and other chemical and biological parameters as well).

MeHg is significantly correlated with all major nutrients (Table 1). However, this might mask differences in mercury and nutrient sources, such as concomitant release from the sediments or coastal input. Correlations under $^{NO-P}$P-limitation are statistically different from correlations under ^{P}P-limitation (Table 1). Because ^{P}P-limitation conditions in the water column are not associated with any station and do not show specific vertical distributions, localized allochthonous input does not represent a factor when considering ^{P}P-limitation effects.

Relation between MeHg and phosphate during the winter in the open sea is especially important (Appendix A **Fig. S2c**), as MeHg formation was linked to organic carbon remineralization and the microbial food web (Cossa et al., 2009, 2017; Heimbürger et al., 2010). Better correlations under $^{NO-P}$P-limitation, when microbial metabolism is not nutrient-limited, indicate that MeHg production is nutrient co-dependent, and it might be produced by marine microorganisms.

However, MeHg shows a significant negative correlation with ammonium and the seawater temperature under $^{NO-P}$P-limitation (Table 1), whose higher values are related to organic matter degradation. When the metabolism of microbes is $^{NO-P}$P-limited, MeHg demethylation during warmer times of the year seems to be the major MeHg transformation, as the lowest MeHg concentrations (geomean 0.036 pmol/L) are found during autumn under $^{NO-P}$P-limitation. Even though photodemethylation during warmer times of the year might explain these correlations, MeHg–temperature correlations are not significant under ^{P}P-limitation conditions ($p > 0.05$) indicating the existence of fundamental differences in mercury biogeochemistry under the presence and absence of ^{P}P-limitation. As bacterial production in the Adriatic Sea is higher during $^{NO-P}$P-limitation conditions and it increases with temperature (Šolić et al., 2017), climate change might actually decrease MeHg concentrations in this area. The predicted rise in the seawater temperature by 3°C in the near future could increase bacterial growth during winter and in deeper waters. This effect could be two-fold greater under $^{NO-P}$P-limitation (Šolić et al., 2017), which would increase the importance of the microbial food web and consequently influence Hg transformations.

The effects of ^{P}P-limitation are best observed at CJ007, where DGM positively correlates with temperature under ^{P}P-limitation (Fig. 5) and negatively under $^{NO-P}$P-limitation ($r = -0.600$, $p < 0.001$). The same trends can be observed through significant correlations between percentage of DGM and temperature (not shown, $p < 0.05$). Under ^{P}P-limitation, higher concentrations of free inorganic Hg in seawater might be available for abiotic (photo)reduction at higher temperatures (solar radiation) resulting in higher DGM concentrations. Consequently, DGM has significantly higher concentrations under ^{P}P-limitation (0.51 pmol/L) compared to $^{NO-P}$P-limitation conditions (0.44 pmol/L) (Mann–Whitney, $p < 0.05$). The same positive and negative correlations with temperature can be calculated in the Southern Atlantic Ocean when considering a probable nitrogen limitation and its absence, respectively (Bratkič et al., 2016; personal communication).

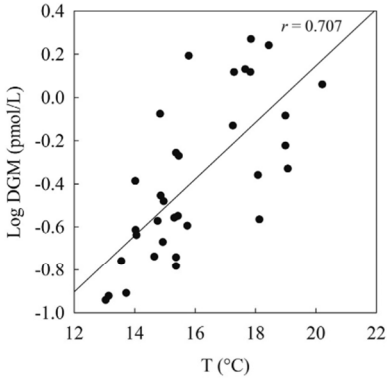


Fig. 5 – Correlations between DGM and temperature at station CJ007 under ^{P}P-limitation conditions ($p < 0.001$).

DGM photoreduction in surface waters is a probable explanation for the observed low correlation coefficients between DGM and chemical and biological parameters. DGM has relatively higher correlation coefficients with these parameters when compared to MeHg, probably because DGM does not show a considerable sedimentary input as MeHg does. Therefore, correlations between mercury fractions and microbial community components can only explain a part of variance of mercury data.

2.4. *MeHg and heterotrophic bacteria*

The activity of heterotrophic microorganisms plays an important role in MMHg and DGM formation (Cossa et al., 2009; Heimbürger et al., 2010; Jonsson et al., 2017; Mason et al., 1998; Sunderland et al., 2009). The correlation between MMHg and heterotrophic activity has been previously observed in the microbial food web and the microbial loop (Heimbürger et al., 2010), which designates the almost closed system of heterotrophic bacteria and their grazers (Rassoulzadegan, 1993).

In our study, the correlation between heterotrophic bacteria and MeHg is significant with a small correlation coefficient (Table 1), as bacterial biomass (BB, Fig. 6) accounts for only 24.8% of picoplankton biomass, and primary production prevails over organic matter decomposition in photic layers. This percentage is the highest in the open sea (CJ009, 29.5%), characterized by a slightly higher correlation coefficient between BB and MeHg ($r = 0.165$, $p < 0.05$). Therefore, the increasing percentage of BB in picoplankton biomass might be

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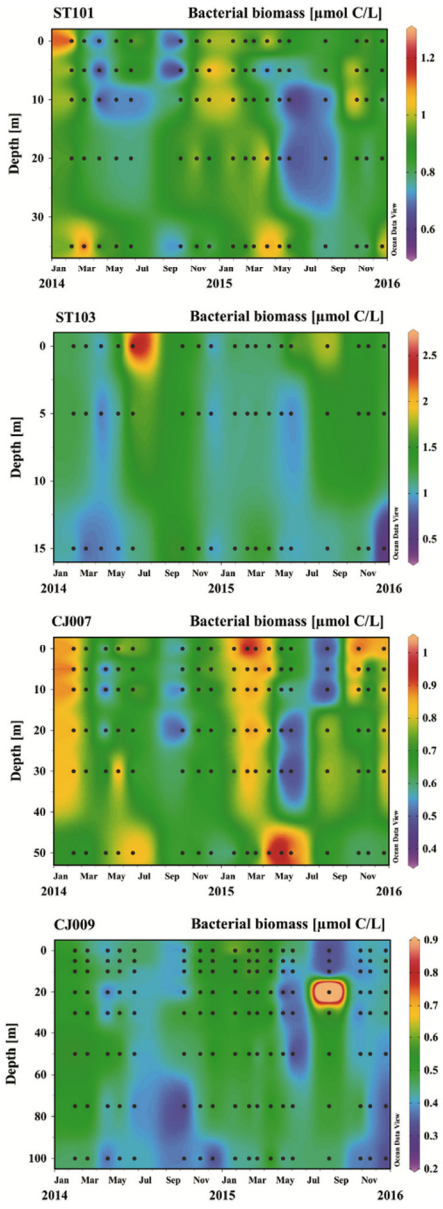


Fig. 6 – Vertical profiles of bacterial biomass in the Central Adriatic Sea during 2014–2015 sampling campaign.

used as an indicator of microbial loop and heterotrophic activity. This might be a reason for the observed increase in MeHg concentrations from the stations CJ007/CJ008 to the station CJ009 (Fig. 4).

MeHg is significantly correlated with BB under $^{NO-P}$P-limitation and not under ^{P}P-limitation (Table 1), as ^{P}P-limitation possibly decreases Hg uptake in relation to polyphosphate complexation (Pan-Hou et al., 2002) and limits Hg methylation. BB has significantly higher values during colder (0.70 µmol C/L) than during warmer times of the year (0.64 µmol C/L) (Mann–Whitney, $p < 0.01$). Similar to MeHg, BB shows a significant negative correlation with temperature under $^{NO-P}$P-limitation at all stations ($0.001 < p < 0.05$; except at neritic station) and no significant correlation under ^{P}P-limitation. It seems that bacterial demethylation under nutrient-replete conditions might be the main biotic transformation in the open waters during warmer times of the year. Alongside photodemethylation, metabolically dependent demethylation is possible at CJ007, as the correlation coefficient between MeHg and specific bacterial production (Appendix A **Fig. S4**) under $^{NO-P}$P-limitation ($r = -0.426$, $p < 0.001$) is significantly different from that under ^{P}P-limitation ($r = -0.003$, $p = 0.98$).

Small correlation coefficient between BB and MeHg could be explained by different behaviors of two groups of heterotrophic bacteria. Heterotrophic bacteria can be divided into two groups based on nucleic acid content: high nucleic acid (HNA) and low nucleic acid groups (LNA) (Gasol et al., 1999). HNA predominates in eutrophic and mesotrophic environments and LNA in oligotrophic environments (Moran et al., 2007). MeHg correlations with HNA or LNA under ^{P}P-limitation are not significant. However, under $^{NO-P}$P-limitation, MeHg shows a significant correlation only with LNA (Table 1), especially at the neritic station. HNA cells were previously considered an active part of the bacterial community, while LNA cells were considered dead cell fragments (Gasol et al., 1999), which would connect MeHg to the degradation of dead bacterial cells (Ramamoorthy et al., 1982). However, recent research showed that LNA bacteria might represent the most active and predominant bacterial group in oligotrophic environments (Jochem et al., 2004; Longnecker et al., 2005). Our results indicate that biotic MeHg transformations can be attributed only to LNA activity under $^{NO-P}$P-limitation and not to the overall heterotrophic bacterial community in oligotrophic waters. Therefore, high MeHg concentrations found in water column of various world oceans could be due to the activity of this bacterial group because oligotrophic ocean waters are usually not limited by phosphorus, but rather by nitrogen (Moore et al., 2008).

2.5. *DGM and heterotrophic bacteria*

A significant correlation between DGM and BB (Table 2) conforms with the hypothesis that heterotrophic bacteria are one of the most important biotic producers of DGM (Ahn et al., 2010; Mason et al., 1995). The threshold THg concentration for the expression of mercuric reductase (Barkay and Wagner-Döbler, 2005) was not observed, indicating that an enzymatic reduction is unlikely and occurs photochemically or through other pathways (Grégoire and Poulain, 2014). The absence of a significant difference between $^{NO-P}$P-limitation

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and ^{P}P-limitation conditions (Table 2) indicates the presence of a metabolically independent Hg reduction or the dominance of photoreduction. This can be further corroborated by the fact that DGM is significantly correlated with both HNA and LNA bacteria under both $^{NO-P}$P-limitation and ^{P}P-limitation (Table 2).

Active and inactive anaerobic bacterial cells can oxidize DEM passively into inorganic Hg through the physicochemical sorption of DEM to thiol functional groups followed by oxidation (Colombo et al., 2014). At the neritic station (ST103), a negative correlation between DGM and BB under ^{P}P-limitation (Fig. 7) indicates DGM oxidation by heterotrophic bacteria. Even though this correlation might be due to light penetration, significant correlation between DGM and depth was not observed under these conditions ($p = 0.762$). DGM oxidation in freshwater has been linked to the activity of Hg oxidase/catalase in the presence of H_2O_2 (Siciliano et al., 2002). The strong solar radiation, shallow depth, and low salinity at ST103 might create conditions for H_2O_2 production (O'Sullivan et al., 2005). ^{P}P-limitation conditions were found to increase the catalase gene expression in heterotrophic bacteria by at least five-fold (Yuan et al., 2005). Under ^{P}P-limitation, an altered cell membrane composition leads to greater cell sensitivity against oxidative stress (Romano et al., 2015; Yuan et al., 2005). The activity of protective enzymes could subsequently oxidize DGM to Hg^{II} (Siciliano et al., 2002). Therefore, DGM might be oxidized by heterotrophic bacteria, but additional research is required to delineate between biological oxidation and photooxidation under ^{P}P-limitation conditions.

2.6. Relations between mercury fractions and heterotrophic nanoflagellates in seawater

Heterotrophic nanoflagellates (HNFs) feed on marine bacteria and therefore control their abundance in seawater (Šolić et al., 2016). The highest HNF content found during summer (Kruskal–Wallis, $p < 0.001$) indicates that HNF grazing controls bacterial abundance during warmer times of the year (Ordulj et al., 2014) and enhances the bacterial carbon transfer to higher trophic levels (Šolić et al., 2017). The increase in the HNF carbon biomass is an indicator of a prolonged food web length (Jonsson et al., 2017; Šestanović et al., 2004; Šolić et al., 2015), which can lead to an elevated MeHg concentration in higher organisms due to MeHg biomagnification. The MeHg transfer from bacteria to HNF under $^{NO-P}$P-limitation conditions is probable, although the results show a weak correlation. This is probably because HNF correlate much better with high nucleic acid bacteria (possibly through predation) compared to low nucleic acid bacteria, which is the only bacterial group strongly related to MeHg. Results indicate that MeHg might not be readily transferred along microbial food web under ^{P}P-limitation conditions which might have strong implications for mercury content in fish and consequently to human consumers. However, this hypothesis should be further tested using direct measurements in marine organisms.

Even though photoreduction is probably the main reduction mechanism, microorganisms smaller than 3 μm are important biotic reducers in natural waters (Mason et al., 1995). In the Central Adriatic Sea, the median maximal cell diameter for the whole heterotrophic flagellate community was 2.27 μm (Šolić et al., 2015), indicating that small HNF might play an important role in Hg reduction.

DGM correlates with HNF, with significant differences between ^{P}P-limitation and $^{NO-P}$P-limitation conditions (Table 2), especially at the neritic station. In addition, DGM is positively correlated with the HNF-to-heterotrophic bacteria ratio (HNF:HB; Table 2), showing that greater HNF grazing on bacteria corresponds to higher DGM concentrations. Either HNF or bacterial dissolved organic matter released upon grazing can reduce Hg. A significant correlation between DGM and the HNF:HB ratio under $^{NO-P}$P-limitation but not under ^{P}P-limitation indicates that small HNF might reduce actively inorganic Hg under $^{NO-P}$P-limitation, although to a lesser extent than heterotrophic bacteria.

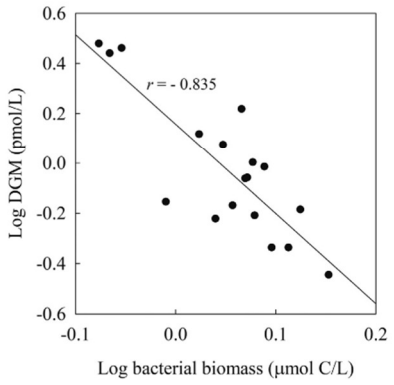


Fig. 7 – Correlation between DGM and bacterial biomass under ^{P}P-limitation at ST103 ($p < 0.001$).

2.7. Autotrophic picoplankton

The Chl-*a* maximum, a proxy for primary biological production, is related to DGM (Poulain et al., 2004) and MeHg peaks (Monperrus et al., 2007) and high Hg methylation rates (Lehnherr et al., 2011). Our results show that Chl-*a* coincides with higher DGM and MeHg concentrations, and $^{NO-P}$P-limitation promotes their production (Tables 1 and 2). Heimbürger et al. (2010) showed that MeHg peaks coincide with the highest abundance of autotrophic picoplankton (APP) and not with the most productive periods. APP has a great importance in mercury transformations in oligotrophic seawaters due to its higher contribution to carbon production (>50%) and energy transfer under a nutrient limitation compared to large phytoplankton (Campbell et al., 1994; Magazzù and Decembrini, 1995; Šolić et al., 2015). Our analysis agrees with previous findings, but shows that ^{P}P-limitation plays a considerable role in mercury transformations.

2.7.1. MeHg and autotrophic picoplankton communities

The most important APP components (*Proc*, *Syn*, and Pico; Fig. 8) show abundances that differ significantly between stations and seasons (Kruskal–Wallis, $p < 0.001$). Different

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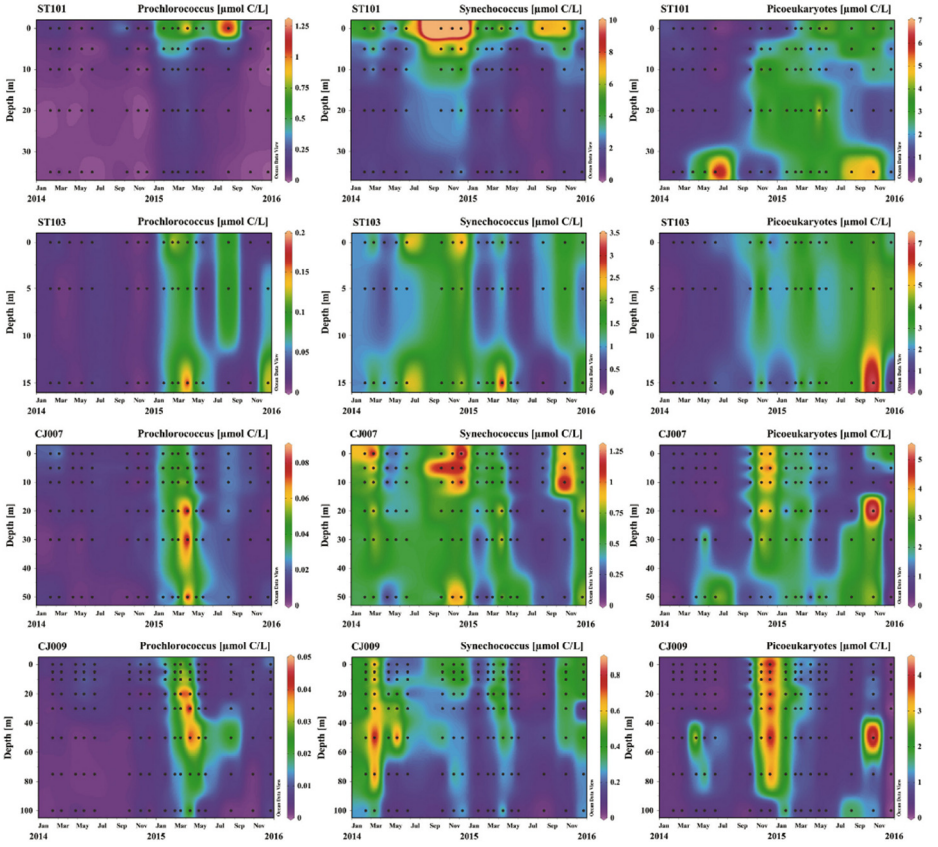


Fig. 8 – Vertical profiles of *Prochlorococcus*, *Synechococcus* and picoeukaryotes in the Central Adriatic Sea during 2014–2015 sampling campaign.

spatial and seasonal variations in APP composition, alongside the biological differences between APP fractions (physiological, genetic), likely cause the observed differences in the correlations between MeHg and individual APP fractions under both ^{P}P-limitation and $^{NO-P}$P-limitation (Table 1).

Phototrophic organisms can directly methylate Hg under laboratory conditions (Deng et al., 2013) or indirectly through HgS solubilization via iodomethane production (Minganti et al., 2007). A significant correlation between MeHg and *Proc* under $^{NO-P}$P-limitation could be due to Hg methylation in the water column. However, low nucleic acid bacteria also show a similar pattern under these conditions. Due to their low mass, *Proc* have a slow sinking velocity and a long residence time in the water column. Therefore, *Proc* can present a source of organic matter that stimulates heterotrophic methylation (Heimbürger et al., 2010) under $^{NO-P}$P-limitation, while ^{P}P-limitation seems to impede microbiological methylation. On the contrary, *Syn* and Pico have much greater biomass than *Proc* and corresponding sinking velocities are much higher. Therefore, they might not represent a good organic substrate for the heterotrophic activity of low nucleic acid bacteria.

Proc have the greatest surface-to-volume ratio and a possibly higher MeHg bioaccumulation (Kim et al., 2014) in comparison to *Syn* or Pico. The inhibition of phosphorylation can lower MeHg uptake in cells (Moye et al., 2002), as P-limitation can alter cell ecophysiology and impact MeHg partitioning between water and cells (Miles et al., 2001), indicating active MeHg uptake under $^{NO-P}$P-limitation. ^{P}P-limitation might therefore have a positive effect by lowering the MeHg transfer to higher trophic levels.

Pico show behaviors that differ from the other APP fractions. MeHg is significantly correlated with Pico under

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both ^{P}P-limitation and $^{NO-P}$P-limitation (Table 1), especially in open seawaters, which might be attributed to the different structure of eukaryotic cells compared to bacterial cells.

2.7.2. DGM and autotrophic picoplankton communities

DEM production by autotrophic plankton is a possible detoxification mechanism linked with their photosynthetic apparatus or released photoreactive compounds (Grégoire and Poulain, 2014). Cyanobacteria *Synechocystis* can reduce inorganic Hg through the *mer*A-like enzymatic pathway (Marteyn et al., 2013). Previous research showed that *Proc*, *Syn*, and eukaryotic microorganisms are able to reduce inorganic Hg (Mason et al., 1995). Eukaryotic Hg reductions probably involve cell surface reductions, and not a gene-coded Hg resistance mechanism (Mason et al., 1995). Our results seem to agree with this hypothesis, as DGM–Pico correlation coefficients are the same under $^{NO-P}$P-limitation and ^{P}P-limitation (Table 2), indicating metabolically independent mechanism. On the contrary, a possible *Proc*-mediated reduction seems influenced by metabolism (whether or not genetically based), as DGM is significantly correlated with *Proc* under $^{NO-P}$P-limitation, but not under ^{P}P-limitation (Table 2).

The effects of ^{P}P-limitation on a possible *Syn*-mediated reduction are hard to assess due to the existence of a positive DGM–*Syn* correlation at the coastal stations under ^{P}P-limitation ($r = 0.300$, $p < 0.05$) and a negative correlation at open sea stations under $^{NO-P}$P-limitation ($r = -0.200$, $p < 0.05$). Observed differences could be explained by the fact that the genus *Syn* includes ecologically distinct ecotypes showing a highly specific affinity to different phosphorus and nitrogen compounds (Ernst et al., 2005; Rajaneesh and Mitbavkar, 2013). In addition, the open sea *Syn* strain has a higher affinity for phosphate than the coastal *Syn* strain, because genetically distinctive strains have different physiological responses to P-limitation (Kretz et al., 2015). Further research is therefore required to assess the effects of P-limitation on *Syn*-mediated mercury reduction.

The low abundance of autotrophic picoplankton compared to the heterotrophic bacteria abundance indicates that heterotrophic activity is probably the main biotic Hg reduction pathway (Mason et al., 1995).

2.8. Mercury in microseston and mesozooplankton

THg concentrations in both plankton fractions show a decreasing gradient from the coast to the open sea following the THg distribution in seawater. On the contrary, MMHg concentrations and the percentage of THg present as MMHg increase toward the open sea (Fig. 9). The plankton MMHg concentrations are generally higher in oligotrophic open waters due to higher surface area-to-volume ratios and growth dilution (Gosnell et al., 2017; Gosnell and Mason, 2015; Hammerschmidt et al., 2013). Approximate bioaccumulation factors (BAFs, L/kg) are calculated under the assumptions that MeHg is entirely composed of dissolved MMHg and that the water contents in mesozooplankton and microseston are 90% and 95%, respectively (Hammerschmidt et al., 2013; Knauer and Martin, 1972). Average MMHg log BAFs are significantly different (paired *t*-test, $p < 0.001$) in microseston (4.08 ± 0.60) and mesozooplankton (5.05 ± 0.34), indicating MMHg biomagnification. These values are similar to MMHg log BAFs in the Northwest Atlantic Ocean (Gosnell et al., 2017; Hammerschmidt et al., 2013). On the contrary, THg is not biomagnified, as its log BAFs are not significantly different (paired *t*-test, $p = 0.17$) in microseston (3.79 ± 0.13) and mesozooplankton (3.99 ± 0.41).

April samples had a higher occurrence of $^{NO-P}$P-limitation conditions within the water column compared to March (Fisher's exact, $p = 0.003$), and mesozooplankton THg values in April were significantly higher than in March (paired *t*-test, $p = 0.003$), even though the THg values in the seawater did not differ (paired *t*-test, $p = 0.344$). Even though mesozooplankton THg values at coastal stations were higher than at open sea stations, overall dataset was tested because two-way repeated measures ANOVA showed no statistically significant interactions between types of seawaters (coastal or open) and sampling months ($p = 0.157$). Although the sample size is small and plankton was sampled only during two months, $^{NO-P}$P-limitation might be responsible for increased planktonic Hg uptake in relation to polyphosphate complexation (Pan-Hou et al., 2002); therefore, it may have strong implications for Hg biomagnification in oligotrophic waters.

3. Conclusions

In the Central Adriatic Sea, THg distribution profiles characterized with enhanced deep concentrations indicate sediment resuspension of previous Hg releases. The variability of DGM concentrations usually follows picoplankton variability emphasizing the importance of mercury reduction by microbial food web components. MeHg concentrations decrease from the neritic station to CJ007 and then again increase toward the open sea, which might be due to MeHg production in the open waters. MeHg concentrations generally follow the distribution of heterotrophic bacteria supporting the hypothesis of *in situ* MeHg production.

Only under the absence of probable phosphorus limitation conditions, MeHg is related to physico-chemical parameters as well as microbial food web components in seawater. As both biological methylation and demethylation are influenced by the microbial food web under the absence of probable phosphorus limitation, the overall net methylation seems to be a nutrient- and/or metabolism-dependent process. Mercury methylation in the water column is promoted only under the absence of probable phosphorus limitation through the heterotrophic activity of low nucleic acid bacteria. On the other hand, biological DGM reduction is not metabolically dependent since DGM relations with microbial community components exist under both phosphorous conditions. Mercury biomagnification from microseston to mesozooplankton is observed through the increase of estimated MMHg bioaccumulation factors in the same direction.

Since the correlation coefficients between mercury fractions and microbial community components are significant but relatively low, the majority of variance in mercury concentrations could be related to abiotic factors, such as photochemistry and allochthonous mercury sources. Accordingly, mercury speciation in the Adriatic Sea at least partially depends on the composition of microbial community.

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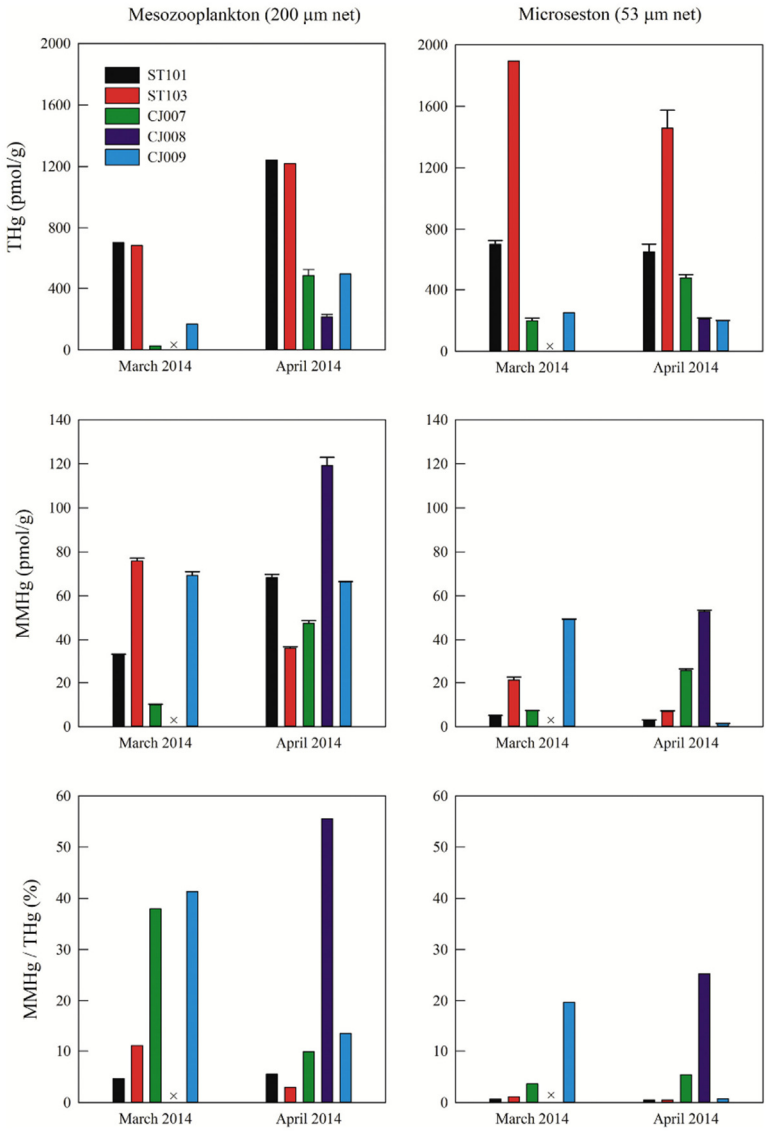


Fig. 9 – THg and MMHg concentrations (on dry weight basis) and percent of THg present as MMHg in mesozooplankton and microseston fraction. March samples at station CJ008 were not collected. Error bars represent one standard deviation of parallel measurements when sample biomass was quantitatively enough to allow duplicate measurements.

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Further, marine chemistry and phosphorus limitation condition influence microbial community structure and consequently mercury speciation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2018.03.012.

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Appendix A. Supplementary data

Relations between mercury fractions and microbial community components in seawater under the presence and absence of probable phosphorus limitation conditions

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Table S1 Sampling sites GPS coordinates and sampling depths.

Station name	Latitude	Longitude	Sampling depths (m)
ST101	43°31'06"N	16°22'54"E	0, 5, 10, 20, 35
ST103	43°31'48"N	16°27'12"E	0, 5, 10, 15
CJ007	43°25'36"N	16°23'54"E	0, 5, 10, 20, 30, 50
CJ008	43°12'00"N	16°19'00"E	0, 5, 10, 20, 30, 50, 75
CJ009	43°00'00"N	16°20'00"E	0, 5, 10, 20, 30, 50, 75, 100

Table S2 Sampling campaign details.

ST101	ST103	CJ007	CJ008	CJ009
02/13/2014	02/13/2014	02/13/2014	02/13/2014	02/14/2014
09:00	11:00	15:55	18:25	08:05
03/12/2014	03/12/2014	03/12/2014	03/12/2014	03/13/2014
08:55	12:00	16:10	18:40	08:15
04/14/2014	04/14/2014	04/24/2014	04/25/2014	04/26/2014
07:50	10:00	08:10	11:45	07:15
05/21/2014	05/21/2014	05/21/2014	05/21/2014	05/22/2014
07:50	09:35	14:10	17:25	07:30
06/23/2014	06/23/2014	06/23/2014	06/23/2014	06/24/2014
07:45	10:05	14:50	17:50	07:00
10/09/2014	10/09/2014	10/09/2014	10/09/2014	10/10/2014
08:05	10:00	15:00	17:45	07:50
11/13/2014	11/13/2014	11/13/2014	11/14/2014	11/14/2014
09:25	10:50	16:30	11:15	08:40
12/10/2014	12/10/2014	12/10/2014	12/10/2014	12/11/2014
09:00	10:25	15:50	17:40	08:15
01/31/2015	01/31/2015	01/29/2015	01/29/2015	01/29/2015
08:50	10:05	18:35	10:15	08:00

02/27/2015 09:15	02/27/2015 10:55	02/27/2015 16:00	02/28/2015 11:40	02/28/2015 09:10
03/17/2015 09:15	03/17/2015 10:55	03/17/2015 16:00	03/18/2015 11:40	03/18/2015 09:10
04/15/2015 09:20	04/15/2015 09:50	04/15/2015 14:45	04/15/2015 16:45	04/16/2015 08:15
05/12/2015 08:15	05/12/2015 09:40	05/12/2015 15:00	05/13/2015 10:45	05/13/2015 08:20
06/02/2015 07:55	06/02/2015 09:30	06/02/2015 14:55	06/03/2015 07:00	06/03/2015 08:30
08/20/2015 08:00	08/20/2015 09:50	08/20/2015 15:05	08/21/2015 06:40	08/21/2015 08:15
10/27/2015 09:00	10/27/2015 10:40	10/27/2015 15:40	10/28/2015 11:30	10/28/2015 09:00
11/17/2015 09:05	11/17/2015 10:45	11/17/2015 16:05	11/18/2015 11:45	11/18/2015 08:55
12/21/2015 09:10	12/21/2015 11:00	12/21/2015 16:05	12/21/2015 18:00	12/22/2015 08:00

Date format: month/day/year hr:min, local time

Table S3 Detection limit, repeatability, reproducibility and spike recovery of mercury determinations.

	Detection limit	Repeatability	Reproducibility	Spike recovery
THg (seawater)	0.30–0.62 pmol/L	4%	10%	92.3–106% (99.8±4.66%, n = 39)
THg (plankton, dw)	0.50–0.86 pmol/g	7%	9%	N/A
DGM in seawater	0.02–0.05 pmol/L	5%	9%	N/A
MeHg (seawater, HG)	3.94–15.9 fmol/L	4%	8%	98.5±3.95% (n = 36)
MeHg (seawater, Eth)	7.73–25.3 fmol/L	5%	10%	79.1±15.7% (n = 21)
MMHg (plankton, dw)	0.18–0.52 pmol/g	5%	11%	105±6.69% (n = 16)

HG – hydride generation; Eth – ethylation; N/A – not available; dw – dry weight; k – coverage factor; n – number of measurements; detection limits are calculated as three times the standard deviation of 5–10 blanks, depending on the method

Table S4 Comparison between obtained and certified values of reference materials.

	Certified reference material		
	Name	Certified value	Obtained value
THg (seawater)	BCR 579	9.47±2.49 pmol/L (k = 2)	10.2±1.45 pmol/L (k = 2; n = 107)
THg (plankton, dw)	TORT 2	1.35±0.30 nmol/g (k = 2)	1.35±0.10 nmol/g (k = 2; n = 8)
DGM in seawater	N/A	N/A	N/A
MeHg (seawater, HG)	N/A	N/A	N/A
MeHg (seawater, Eth)	N/A	N/A	N/A
MMHg (plankton, dw)	TORT 2	0.76±0.06 nmol/g (k = 2)	0.73±0.06 nmol/g (k = 2; n = 16)

HG – hydride generation; Eth – ethylation; N/A – not available; dw – dry weight; k – coverage factor; n – number of measurements

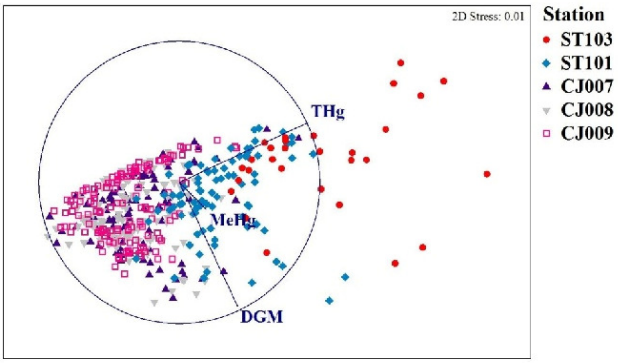


Fig. S1 Non-metric multi-dimensional scaling (MDS) ordination of five stations in the Central Adriatic Sea based on the Euclidean distance from log(x+1)-transformed concentrations of total mercury (THg), total methylated mercury (MeHg), and dissolved gaseous mercury (DGM). Vectors designate Spearman's rank correlations between Hg species and MDS axes.

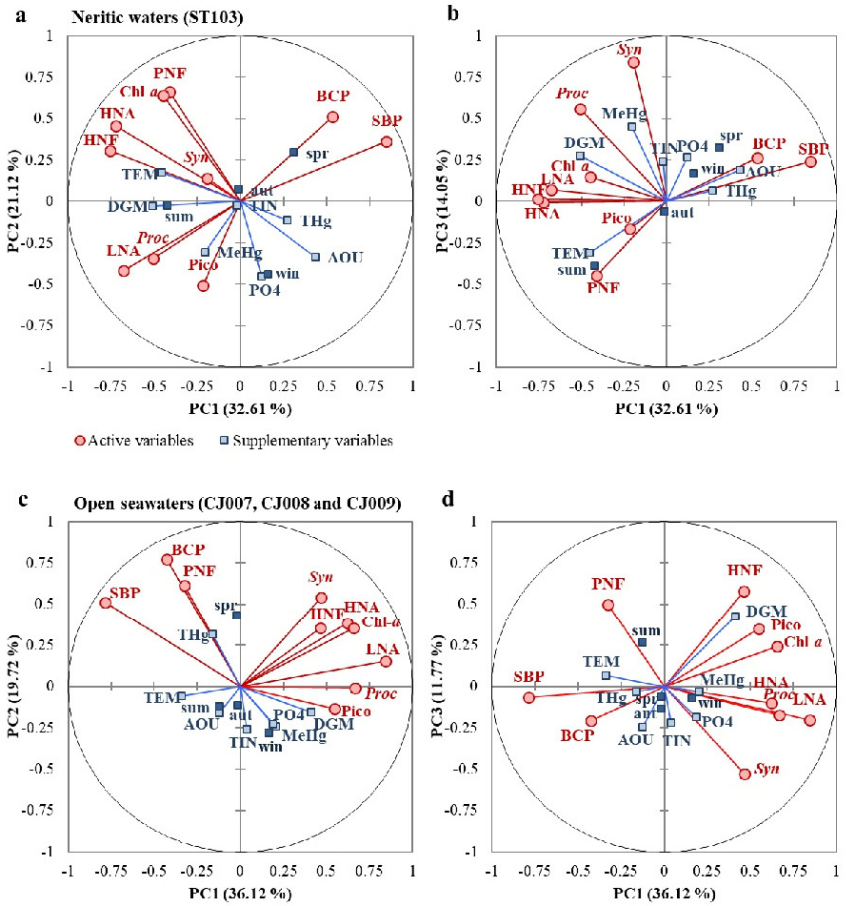


Fig. S2 The ordering of microbial food web parameters as active variables obtained by the Principal Component Analysis (PCA) in $^{NO-P}$P-limited marine ecosystems at the neritic station (a,b) and open sea stations (c,d). Abiotic parameters are superimposed as quantitative or qualitative supplementary variables and plotted as squares. (Active variables: number of HNA, high nucleic acid bacteria; LNA, low nucleic acid bacteria; *Proc*, *Prochlorococcus*; *Syn*, *Synechococcus*; Pico, picoeukaryotes; HNF, heterotrophic nanoflagellates; PNF, phototrophic nanoflagellates; SBP, specific bacterial production; BCP, bacterial cell

production; Chl-*a*, concentration of chlorophyll *a*. Quantitative supplementary variables: concentrations of THg, total mercury; DGM, dissolved gaseous mercury; MeHg, total methylated mercury; TIN, total dissolved inorganic nitrogen; PO_4, phosphates; TEM, sea temperature; AOU, apparent oxygen utilization. Qualitative supplementary variables: win, winter; spr, spring; sum, summer; aut, autumn.)

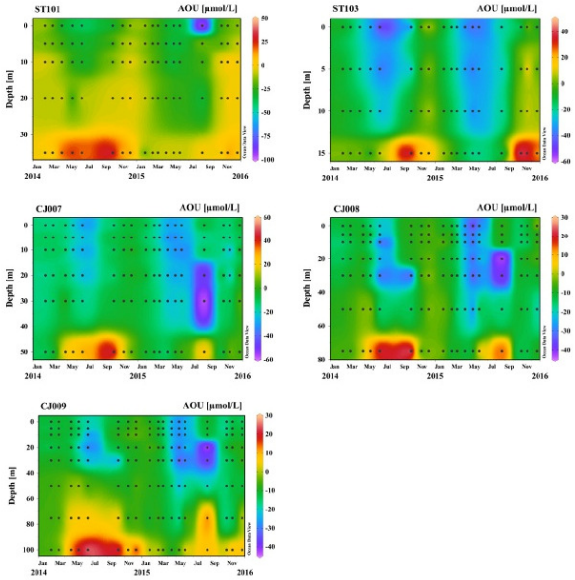


Fig. S3 Vertical profiles of apparent oxygen utilization (AOU) in the Central Adriatic Sea during 2014–2015 sampling campaign.

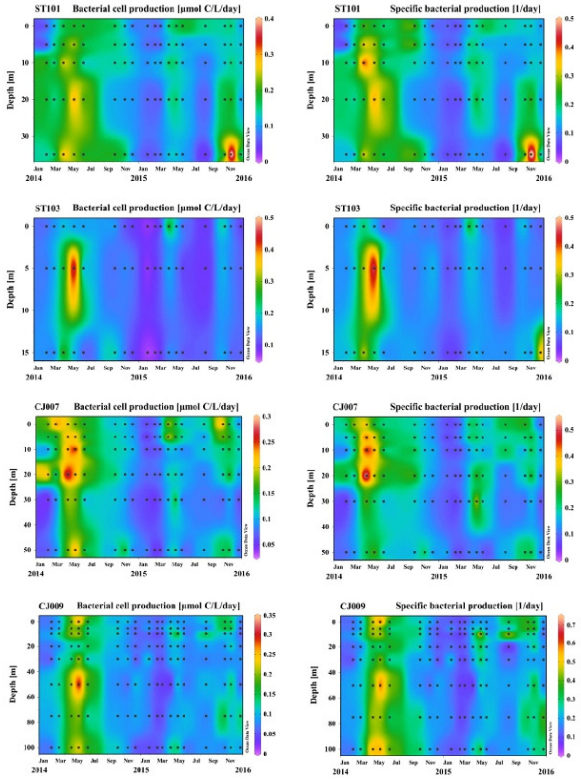


Fig. S4 Vertical profiles of bacterial cell production and specific bacterial production in the Central Adriatic Sea during 2014–2015 sampling campaign.

Chapter 4

Summary and Conclusions

This dissertation contributes to the scientific knowledge about mercury biogeochemistry in seawater and the role of microorganisms in mercury transformations. In this chapter, summaries of two review articles are presented, alongside the main conclusions from two research papers. In this dissertation, concentrations of various mercury fractions in the Adriatic Sea were presented in detail, along with corresponding transport and transformation processes. The existing knowledge about mercury bioaccumulation and biomagnification is described through the comparison of mercury concentrations in biota occupying different trophic levels. In addition, this dissertation introduces the first attempts at quantification and estimation of measurements uncertainty for the determination of methylmercury in seawater. At the end, possible relations between mercury fractions and microorganisms in the central Adriatic Sea were demonstrated.

The importance of the anthropogenic and natural mercury sources in the coastal Adriatic Sea can be observed through elevated mercury concentrations in waters and sediments. The highest THg concentrations in the whole Mediterranean Sea can be found in the northern Adriatic Sea due to the past emissions from the industrial spot sources (mainly chlor-alkali plants) and mercury releases through the Soča/Idrijca Rivers system, which has been draining mercury for centuries from the Idrija mercury mine. Therefore, concentration gradients in both waters and sediments can be observed from the northern to the southern Adriatic. Generally, mercury enrichment is usually found in the upper sediment with a subsequent decrease toward the depth. The termination of the Idrija mine operations in 1994 and the closure of industrial facilities that released mercury are accompanied with the decrease in THg concentrations in environmental compartments. However, the observed decrease in concentrations could also be due to possible previous sample contamination, which cannot be excluded, because trace-metal clean-sampling techniques were not as common as today. Additional problem in comprehensive assessment of Hg biogeochemistry arises from the lack of data on mercury speciation in water and sediments from earlier studies.

DGM is usually low in the Adriatic surface waters and increases towards the bottom, emphasizing the importance of evasion and photochemical reactions at the surface. DGM distribution in deep waters depends on redox processes, microbial production, and diffusion from sediments. Geographical distribution of reactive mercury usually follows that of THg. The highest concentrations of reactive mercury are found in the northern Adriatic, indicating considerable potential for transformation reactions, especially reduction and methylation.

Coastal environments are areas of great importance, but they also present the most affected locations due to anthropogenic mercury releases. Special attention in this dissertation was given to coastal lagoons, where specific conditions exist that enable

mercury methylation. In these coastal areas, mercury methylation by sulfate-reducing and iron-reducing bacteria in the sediments plays an important role in mercury transformations. Mercury methylation rates in the sediments of the Gulf of Trieste and the Venice lagoon are higher than those at the Strait of Otranto and in the Mediterranean Sea (Table 1.1). Therefore, water transfer of MeHg released from the sediments can disperse it to the open sea. A large amount of anthropogenic mercury present in the coastal sediments and high methylation rates can therefore present a considerable source of MeHg to the Adriatic.

Mercury mass balance in the sea shows that the Adriatic Sea is a net source of both inorganic and organic mercury to the open Mediterranean Sea. However, the amount of MeHg produced in sediments is not great enough to account for all MeHg present in the water column. In addition, the spatial distribution of MeHg does not follow that of THg, as the highest MeHg concentrations in the Adriatic seawaters are found in the southern part. Microbial MeHg production in the water column seems to be the major MeHg source in the Adriatic waters, especially in the waters of the southern Adriatic Pit. The important role of heterotrophic activity of microorganisms in mercury methylation processes in the water column was one of the driving forces for the research included in this dissertation. Produced MeHg in water column can be readily bioaccumulated and biomagnified along the trophic levels, and therefore represent a considerable burden to the Adriatic biota.

Differences in mercury bioaccumulation between marine organisms indicate a complex biogeochemical mercury cycle in the Adriatic biota. In this dissertation, variability of mercury concentrations in marine organisms occupying different trophic levels has been demonstrated through their comparison in regard to a different sampling area and periods.

Mercury in the Adriatic phytoplankton has been measured only occasionally. Therefore, the comprehensive estimation of mercury behavior in these organisms was not assessed. In addition, differentiation of biotic from abiotic particles in phytoplankton/microseston mixture and specific attribution of these particles to mercury bioaccumulation is rather challenging.

THg has significantly lower concentrations in zooplankton from the Adriatic Sea than in zooplankton from the Mediterranean Sea, even though the highest THg concentrations in the Mediterranean seawater are found in the Adriatic Sea. Because MeHg concentrations in zooplankton are similar in all studied areas, Adriatic zooplankton consequently has the highest percentage of THg present as MeHg, indicating high methylation potential in seawater accompanied with high MeHg bioaccumulation. Similarly as in seawater, THg in the Adriatic zooplankton shows a significant decrease with time, probably due to decreased anthropogenic mercury releases. Absence of this observation in the Mediterranean plankton from the open sea indicates localized effects of anthropogenic mercury contamination in the marine environment.

Mussels and other bivalves have been used for years as valuable bioindicators of mercury contamination. Mussels reflect localized influence of anthropogenic mercury releases due to their sedentary lifestyle in coastal areas. THg concentrations in mussels from the Adriatic are not significantly higher than in those from the Mediterranean Sea. Mussels from both seas are characterized by approximately a 10-fold decrease in THg during the last 30 years period. Other bivalves are also capable of effective mercury accumulation, but its extent depends on both environmental and biological parameters. Percentage of mercury as MeHg is similar in mussels from the Mediterranean and the pristine areas of the Adriatic Sea. However, previously contaminated locations have lower MeHg percentages indicating low methylation potential, MeHg demethylation or possible detoxification mechanisms. One possible detoxification pathway is *via* metallothioneins, which have a major role in mercury storage and regulation processes in mussels due to their ability to bind mercury efficiently.

The most studied fish in the Adriatic and the Mediterranean Sea, European hake and red mullet, have similar THg concentrations in both seas. Contrary to plankton and mussels, there are no significant differences in THg concentrations during different sampling periods in these fish species. THg and MeHg increase with trophic level of the Adriatic fish; the lowest values are usually found in herbivorous fish and the highest in the top predator species. Mercury is biomagnified through trophic levels, and a subsequent trophic level 'inherits' mercury from the previous level. The percentage of mercury present as MeHg also grows with increasing trophic level, and 100% can be found in top predator species. Mercury concentrations in fish are usually correlated with length (age) and weight, resulting in the highest concentrations found in big older fish. Similar to metallothioneins in mussels, selenium can represent a possible Hg detoxification pathway in fish. Equimolar Se:Hg ratios represent a threshold associated with Hg toxicity and is usually found in fish with elevated THg content in muscle.

Biological, chemical and geological factors influence mercury transformations and lead to MeHg production in marine sediments and the water column. Following its transformations and sequestration by marine microorganisms, mercury biomagnifies along the trophic levels, thus making mercury an element of concern in marine biota. This is the main reason why special attention in this dissertation was attributed to the beginning of this chain, i.e. to the relations between mercury and the lowest marine trophic levels, and to the determination of low MeHg concentrations in seawater.

Hydride generation–cryogenic trapping–gas chromatography–cold vapor atomic fluorescence spectrometry was shown to be a good analytical tool for the determination of MeHg in seawater samples. Hydride generation method was optimized and its performance was compared to the ethylation method; hydride generation had lower LOD, almost quantitative spike recovery, and lower peak asymmetry. Even though hydride generation had lower external calibration slope and relative sensitivity in Milli-Q water, relative sensitivity in seawater samples was similar in both methods due to lower recoveries during the extraction procedure.

Method optimization yielded better performance parameters when compared to previously published research. The most important were 3–12 fold decrease in LOD and 80% decrease in peak asymmetry. Almost perfect peak symmetry enabled better separation of inorganic mercury and MeHg peaks. However, in samples with very high THg concentrations, Hg^0 peak entirely concealed the MeHg peak. Therefore, the best results for hydride generation method were obtained for seawater samples in which THg concentration was less than 5.0 pmol L^{-1} and MeHg represented more than 0.5% of THg concentration.

The expanded relative combined standard uncertainties were 11.1%, 15.0% and 21.3% for hydride generation, and 15.8%, 18.2% and 19.3% for ethylation, at the highest (> 400 fmol L^{-1}), middle (100–150 fmol L^{-1}) and the lowest MeHg concentration level (< 50 fmol L^{-1}), respectively. Hydride generation had lower $U_{ex,r}$ values at higher MeHg concentrations than ethylation, but ethylation had a narrower range of $U_{ex,r}$ values.

For hydride generation, the highest relative contribution to $U_{ex,r}$ values was from sample repeatability, at all concentration levels. Near LOD, sample repeatability accounted for 46.6% of $U_{ex,r}$. In addition, the sample area had a big contribution to $U_{ex,r}$ at the lowest MeHg concentration level due to lower sensitivity of hydride generation compared to ethylation.

Alongside sample repeatability, the recovery had the greatest contribution index to $U_{ex,r}$ in the ethylation method due to relatively low recoveries. Several procedure steps, probable incomplete solvent extraction, and back-extraction into Milli-Q were the main reasons for low and unreproducible recoveries. As hydride generation does not have pre-concentration steps, spike recovery was almost quantitative.

Based on very low LOD, quantitative spike recoveries, and low expanded relative combined standard uncertainties we confirmed the hypothesis that hydride generation can be readily used for the accurate determination of low MeHg concentrations in seawater samples. Using two cryogenic traps/GC columns simultaneously, four measurements can be performed within an hour. Ideally, this corresponds to the determination of MeHg in eight seawater samples *per* day (in duplicates) alongside the measured calibration blanks, sample blanks, standard solutions, and spiked samples. In addition, hydride generation avoids tedious sample pre-treatment and lengthy labware cleaning procedures. The system is simple enough to be easily transported and used directly on-board the research vessel. The only limitation for on-board use represents the availability of liquid nitrogen.

In the central Adriatic Sea, decreasing THg concentration gradient was observed from the Kaštela Bay to the Island of Vis, where the average THg concentration was similar to those previously found in the whole Mediterranean Sea. Elevated THg concentrations in the Kaštela Bay were probably a consequence of previous industrial releases from the chlor-alkali plant. The effects of sediment resuspension at the neritic coastal station in the Kaštela Bay were observed through significantly different THg values at the surface and bottom, and through positive THg correlation with depth. Therefore, past anthropogenic mercury releases could be re-introduced into the marine environment and might be accumulated by the Adriatic biota. THg concentrations did not show any significant variability throughout the year.

Similar to THg, DGM showed a decreasing gradient from the coastal area toward the open sea. DGM concentrations were higher during colder than during warmer times of the year, indicating the importance of possible photo- and biologically mediated reactions on mercury oxidation and reduction. The variability of DGM concentrations usually followed that of heterotrophic bacteria and picoplankton, which might be due to biological mercury reduction. Contrary to THg, DGM did not show correlation with depth or difference between the surface and bottom layers. Facilitated vertical transfer and release into the atmosphere are due to volatility of DGM and its supersaturation in surface waters. On average, DGM accounted for 18.3% of THg, which was similar to values from previous studies.

MeHg concentrations in the central Adriatic Sea were usually around LOD, which emphasized the importance of accurate methods for their determination in seawater. Contrary to THg and DGM gradients, MeHg concentrations showed an interesting spatial distribution. MeHg concentrations decreased from the neritic station, reached minimal values at station CJ007, and then again increased toward the open sea, which might be attributed to MeHg production in open waters. Input of MeHg from the sediments or its production in the deeper water layers could also be possible due to higher bottom MeHg concentrations compared to surface values. Contrary to THg, MeHg concentrations were higher during colder than during warmer times of the year, and generally followed the distribution of heterotrophic bacteria, which might support the hypothesis of *in situ* MeHg production within the water column. On average, MeHg accounted for 1.69% of THg in the central Adriatic Sea, which was similar to values recently measured in the coastal Mediterranean.

Results of mercury speciation in seawater suggest that our hypothesis that chemical processes and seasonal changes drive mercury speciation in the coastal and open marine environments could be partially confirmed. The correlations between the concentrations of nutrients and mercury fractions might not reflect chemical processes, but rather the susceptibility of biologically mediated reactions to the availability of nutrients. The effect of seasonal changes can be observed through higher concentrations of DGM and MeHg in seawater during colder times of the year compared to warmer times of the year.

The distribution of mercury fractions in seawater could confirm the hypothesis that speciation of mercury in the Adriatic Sea depends on sea depth, season, location, marine biology and hydrometeorological parameters. This is demonstrated through correlations of Hg fractions with depth and abundances of microorganisms, differences in concentrations during different times of the year and at sampling stations, and through the effect of precipitations on mercury speciation in surface waters.

The presence and absence of probable phosphorus limitation conditions indicated possible contrasting effects on relations between mercury fractions and microbial community components. Only under $^{NO-P}$P-limitation conditions was MeHg related to most physico-chemical and microbial parameters in seawater. Therefore, mercury methylation within water column might be a nutrient- and/or metabolism-dependent transformation, as phosphorus limitation might induced severe physiological and metabolic changes in both autotrophic and heterotrophic microorganisms. Mercury methylation in the water column could be promoted under $^{NO-P}$P-limitation conditions through the heterotrophic activity of low nucleic acid bacteria. Under these conditions, MeHg concentration could be related with the abundance of small autotrophic picoplankton, whose decay was previously found to promote heterotrophic activity.

Under $^{NO-P}$P-limitation conditions, DGM was correlated with the abundance of most microbial community components, temperature and chlorophyll *a* concentration, which could indicate biological DGM production. However, the mechanism of this reduction might be metabolically independent, as most of these relations are also observed under ^{P}P-limitation conditions.

These relations between MeHg/DGM and microbial community components under ^{P}P-limitation and $^{NO-P}$P-limitation conditions could confirm our hypothesis that Hg speciation in the photic zone depends on physico-chemical properties of seawater and characteristic structure of the marine food web. This is of significant importance for MeHg because its correlations with microbial community components are significant only under $^{NO-P}$P-limitation conditions, which could indicate that Hg methylation might be a nutrient- and metabolism-dependent transformation and thus confirming our hypothesis.

Mercury biomagnification from microseston to mesozooplankton was observed through the increase of estimated MeHg bioaccumulation factors from former to latter fraction. In addition, results showed that mercury uptake by plankton might be enhanced under $^{NO-P}$P-limitation conditions. These results could indicate that our hypothesis about Hg bioaccumulation in the marine food web and its dependence on plankton dynamics and composition might be confirmed. However, due to a low number of microseston and mesozooplankton samples, further research is required to strengthen these findings.

Our results also indicated that physico-chemical properties of seawater and phosphorus availability and limitation might influence the microbial community structure and consequently mercury speciation. Additional research is required to verify these results and to confirm the effects of probable phosphorus (nutrient) limitation on mercury transformations in seawater and transfer to biota occupying higher trophic levels.

Research presented in this dissertation provides a new approach in the assessment of mercury transformations in marine environments. Even though the effects of phosphorus limitation on mercury microbial transformation were statistically significant, more research is required in order to directly observe and assess these relations. In addition, methodological improvements of existing analytical methods were required for more precise and more accurate determinations of investigated mercury species, especially low environmental MeHg concentrations. Further research is also needed to verify the possible effects of nutrient limitations on mercury bioaccumulation and biomagnification in marine food webs. As MeHg in the open ocean waters is mostly produced by the activity of heterotrophic bacteria, the possible effects of climate changes on bacterial abundance and

microbial community structure might have a strong impact on mercury biogeochemistry in the marine environment.

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Biography

The author of this dissertation Igor Živković was born on June 28, 1983 in Split, Croatia. He finished his primary school in Kaštel Gomilica, Croatia, and secondary education in Split, Croatia. In 2006, he started his undergraduate studies in Chemistry at the Faculty of Chemistry and Technology, Split, Croatia. During his undergraduate studies, he was a member of the Committee of Quality Improvement. For his excellent grade point average during undergraduate studies, he received two Rector's awards from the University of Split. He was awarded a univ. bacc. chem. degree for the thesis entitled "Influence of picolinic acid on Cu20Ni alloy corrosion" from the Faculty of Chemistry and Technology in 2009. In the same year, he enrolled graduate studies in Organic Chemistry and Biochemistry at the same faculty. For the defense of the master's thesis entitled "Synthesis of 1*H*- and 2*H*-imidazole derivatives and Beckmann rearrangement of ketoprofen oxime" he was awarded a mag. chem. degree in 2011. Until 2012, he was an assistant at the Department of Organic Chemistry at the Faculty of Chemistry and Technology.

In 2013, he was selected as a PhD candidate at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, under the supervision of Prof. Dr. Milena Horvat. The majority of experimental work related to his PhD was conducted at the Department of Environmental Sciences, Jožef Stefan Institute. Sampling related to his PhD was performed aboard the research vessel *Bios Dva* from the Institute of Oceanography and Fisheries, Split, Croatia. His research focuses on mercury biogeochemistry in the marine environment with relations to marine microorganisms. For his work, he used different methods for mercury determination and speciation in seawater and biota. In addition, he optimized the method for the determination of low concentrations of monomethylmercury in seawater. During PhD studies, he attended workshops on the topic of mercury biogeochemistry in the environment and measurement uncertainty. In addition, he presented his work at several international conferences related to geoscience, environmental biology, toxicology and chemistry, and to mercury as a global contaminant.