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Trophic transfer and accumulation of mercury in ray species in coastal waters affected by historic mercury mining (Gulf of Trieste, northern Adriatic Sea)

Milena Horvat · Nina Degenek · Lovrenc Lipej · Janja Snoj Tratnik · Jadran Faganeli

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Abstract Total mercury (Hg) and monomethylmercury (MMHg) were analysed in the gills, liver and muscle of four cartilaginous fish species (top predators), namely, the eagle ray (*Myliobatis aquila*), the bull ray (*Pteromylaeus bovinus*), the pelagic stingray (*Dasyatis violacea*) and the common stingray (*Dasyatis pastinaca*), collected in the Gulf of Trieste, one of the most Hg-polluted areas in the Mediterranean and worldwide due to past mining activity in Idrija (West Slovenia). The highest Hg and MMHg concentrations expressed on a dry weight (d.w.) basis were found in the muscle of the pelagic stingray (mean, 2.529 mg/kg; range, 1.179–4.398 mg/kg, d.w.), followed by the bull ray (mean, 1.582 mg/kg; range, 0.129–3.050 mg/kg d.w.) and the eagle ray (mean, 0.222 mg/kg; range, 0.070–0.467 mg/kg, d.w.). Only one specimen of the common stingray was analysed, with a mean value in the muscle of 1.596 mg/kg, d.w. Hg and MMHg contents in the bull ray were found to be positively correlated with species length and weight. The highest MMHg accumulation was found in muscle tissue. Hg and MMHg were also found in two embryos of a bull ray, indicating Hg transfer from the mother during pregnancy. The number of specimens and the size coverage of the bull rays allowed an assessment of Hg accumulation with age. It was shown that in bigger bull ray specimens, the high uptake of inorganic Hg in the liver and the slower MMHg increase in the muscle were most probably due to the demethylation of MMHg in the liver. The highest Hg and MMHg contents in all organs were found in the pelagic stingray, which first appeared in the northern Adriatic in 1999. High Hg and MMHg concentrations were also found in prey species such as the banded murex (*Hexaplex trunculus*), the principal prey of the eagle rays and bull rays, the anchovy (*Engraulis encrasicholus*) and the red bandfish (*Cepola rubescens*), which are preyed upon by the pelagic stingray, as well as in zooplankton and seawater. Based on previously published data, a tentative estimation of MMHg bioamagnification was established. The average increase in MMHg between seawater, including phytoplankton, and zooplankton in the Gulf was about 10^4, and MMHg in anchovy was about 50-fold higher than in zooplankton. The bioaccumulation of MMHg between seawater and small pelagic fish (anchovy) amounted to 10^6 and between water and the muscle of larger pelagic fish (pelagic stingray) to 10^7. The MMHg increase between surface sediment and benthic invertebrates (murex) and between benthic invertebrates and small benthic fish was 10^2. Ultimately, the trophic transfer resulted in a 10^3 accumulation of MMHg between water and muscle of larger benthic fish (bull ray, eagle ray, common stingray), suggesting lower bioaccumulation by benthic feeding species.

Keywords Mercury · Methylmercury · Fish · Rays · Bioaccumulation · Northern Adriatic

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M. Horvat · J. Snoj Tratnik
Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, Ljubljana, Slovenia

M. Horvat (✉) · J. Snoj Tratnik
International Postgraduate School Jožef Stefan, Jamova 39, Ljubljana, Slovenia
e-mail: milena.horvat@ijs.si

N. Degenek · L. Lipej · J. Faganeli
Marine Biological Station, National Institute of Biology, Fornače 41, Piran, Slovenia

Introduction

Mercury (Hg) is nowadays the subject of intensive environmental research especially because of the neurotoxicity of monomethylmercury (MMHg), which is mainly formed by

microbial Hg methylation. The bioaccumulation and biomagnification of MMHg in marine food webs can lead to high Hg contents in fish that are consumed by wildlife and humans (Wiener et al. 2003; Munthe et al. 2007). Studies of its toxicological effects in fish, including behavioural, developmental and endocrine effects, and effects on proliferation are rare (Wiener et al. 2003). In humans, MMHg neurotoxicity is manifested especially in the foetus (Meyers et al. 2000; National Academy of Science 2000; Karagas et al. 2012). The neurotoxicological effects are mitigated by Se present in fish (Hansen and Gilman 2005) and antioxidants such as *N*-acetyl-L-cysteine (Ornaghi et al. 1993).

Northern Adriatic with the Gulf of Trieste as its northernmost part is a shallow marine basin between Italy, Slovenia and Croatia with heavily populated coasts and an industrialized area with many different anthropogenic pressures. One of the main pollution concerns in the investigated area is eutrophication, but recent analysis pointed towards more oligotrophic condition (Turk et al. 2007) and pollution with toxic metals (Ščančar et al. 2007), with mercury as the main concern (Horvat et al. 1999; Covelli et al. 2001).

The trophic enrichment of Hg in coastal marine environments remains poorly understood (Fitzgerald et al. 2007, Chen et al. 2013), as it is in the Gulf of Trieste (northern Adriatic Sea), one of the most polluted areas in the Mediterranean (Horvat et al. 1999; Covelli et al. 2001), into which the Hg-polluted waters from the River Isonzo/Soča empty (Hines et al. 2000). The Hg pollution of the river water is a consequence of nearly 500 years of mining activity in Idrija in western Slovenia (Horvat et al. 1999, 2003a, b), the second largest Hg mine in the world. Hg levels in the surface sediments of the Gulf of Trieste show a progressive southward decrease from about >20 mg/kg in the Isonzo/Soča estuarine region to <0.2 mg/kg in the southern part of the Gulf (Covelli et al. 2001). Similarly, MMHg, originating mostly from sedimentary production (Hines et al. 2000, 2006; Bratkič et al. 2013), tends to decrease from 4 μg/kg in the estuarine region to <0.5 μg/kg in the south (Horvat et al. 1999; Covelli et al. 2001). Mercury levels found at locations of the ray species catch range between 0.5 and 1.0 mg/kg (Horvat et al. 1999; Covelli et al. 2001).

Knowledge of the contents, transport and fate of MMHg in the marine ecosystem is important in order to assess its impact on edible marine organisms and on humans (Chen et al. 2008a, 2013). In this context, especially long-lived species at the top of the food chain (predators) and living in a wide area seem useful because their Hg content is a consequence of the long-term contamination of the water basin (Chen et al. 2008a).

It is well known that Hg in fish is mostly accumulated by food intake (Hall et al. 1997). The direct contribution from water varies and depends on various factors governing the production of MMHg and its availability in the marine environment, as well as on the fish species and season (Downs et al. 1998). Hg distribution in fish tissues, on the other hand, is governed by the uptake route which is connected with the chemical speciation of Hg, the physical and chemical properties of the environment affecting chemical speciation and the physiological functions of organisms, as well as the physiological and biochemical properties of fish which influence Hg uptake through biological barriers (digest wall and gills), accumulation in cells and tissues, and excretion (Boudou and Ribeyre 1997; Chen et al. 2008a; Choy et al. 2009). Since long-lived cartilaginous fish, which are located at the top of marine food webs, are known to be sensitive to high levels of mercury contamination through their food, they are valuable bioindicators for measuring the bioaccumulation of MMHg in the marine environment (Pethybridge et al. 2010).

The ray species other than those of the family Rajidae have received limited scientific attention in terms of their biology and ecology. Whilst the eagle ray (*Myliobatis aquila*) is occasionally caught in the Gulf of Trieste during pelagic trawls as bycatch, the bull ray (*Pteromylaeus bovinus*) is a rare and lesser known species with only sketchy data available on its distribution in the Adriatic Sea. The common stingray (*Dasyatis pastinaca*) is nowadays a rare species in the area, whilst the pelagic stingray (*Dasyatis violacea*) has been found only recently in the northern Adriatic (Mavrič et al. 2004). The pelagic stingray was formerly recorded mainly along the North African shore, and the recent occurrence of the species in the Mediterranean, such as in the Tyrrhenian Sea and especially the northern Adriatic, can be related to tropicalization, i.e. northward spreading of southern species (Capape et al. 2006).

The bull ray and the eagle ray are benthic dwelling species, whereas pelagic stingray is occurring mostly in the water column—pelagic. Since for all of them neonate specimens are known in the Gulf of Trieste, the studied area is considered to be their reproductive ground. The common eagle ray is considered as a common species, the pelagic stingray as a present one and the bull ray as rather rare species.

The ray species were so far studied in the Gulf (Mavrič et al. 2004; Dulčič et al. 2008; Lipej et al. 2013) with the aim to provide samples for population assessment. Rays are predators with no known natural enemies, and like their relatives, sharks represent the top predators in their environment; most species live and feed on the seafloor and only a few species in the open sea. Three species from the same study, two benthic and one pelagic, were used and investigated in the present study as well. Their feeding habits have been described recently (Lipej et al. 2013).

The aim of this study was to investigate Hg and MMHg levels in the gills, liver and muscle of four cartilaginous fish species (top predators), namely, the eagle ray, bull ray, pelagic stingray and common stingray, collected in the Gulf of Trieste.

To better understand the uptake and accumulation of Hg and MMHg, samples of the prey of the rays were analysed, namely, the banded murex (*Hexaplex trunculus*), the principal

prey for the eagle ray and bull ray, and the anchovy (*Engraulis encrasicholus*) and the red bandfish (*Cepola rubescens*), the principal prey of the pelagic stingray. Finally, using the Hg and MMHg contents in sediment, water, plankton and prey species obtained from previous measurements and published data, the bioaccumulation of Hg and MMHg in ray species and their biomagnification along the food web in the Gulf of Trieste were estimated.

This is the first time such data were reported in these species in the region. As these ray fish species are top predators and are consumed by humans, the information is also valuable in the context of their potential human health impact.

Materials and methods

Samples

The ray species were collected in the period from August to October 2005 in the central part of the Gulf of Trieste (Fig. 1). A total of 5 specimens of eagle ray, 17 specimens of bull ray, 8 specimens of the pelagic stingray and a single specimen of the common stingray were used. The collected ray species were identified at the species level and their size measured to the nearest millimetre (Jardas 1996). Gender was determined according to the presence of claspers. The catch of eagle rays consisted of young specimens, whereas the specimens of bull ray and pelagic stingray were mostly adults (Table 1). In addition, a total of ten specimens of murex, five specimens of anchovy and five specimens of red bandfish collected in the Gulf of Trieste were also analysed.

Gill, liver and muscle tissues were dissected from each fish, transferred to plastic bags and stored in a freezer at −23 °C. Samples were successively freeze-dried (Christ Alpha 1-4) over a period of 4 days at −40 °C and a pressure of 0.02 mbar. The weights before and after freeze drying were recorded and the concentrations found could be reconstructed on a wet weight (w.w.) basis. Homogenization of the samples was performed in a planetary micro-mill (Fritsch pulverisette 7) and then the samples kept in a refrigerator until analysis. Samples (each specimen) of murex, anchovy and red bandfish were homogenized using a laboratory mixer before storage and analysis. The concentrations in prey species, except for gastropods where the shell was removed, were measured in the whole organism, as is the common practise (Hammerschmidt and Fitzgerald 2006). Samples were stored in clean containers at −23 °C.

Chemical analysis

Total Hg (THg) in samples was determined with cold vapour atomic absorption spectrophotometry after acid digestion with HNO_3, $HClO_4$ and H_2SO_4 (Horvat et al. 1991). About 500 mg of sample was weighed directly in a 100-mL volumetric flask followed by the addition of 3 mL of HNO_3 (65 %), 1 mL $HClO_4$ (70 %) and, finally, 5 mL of H_2SO_4 (96 %). The vessels were closed and the mixture was left to react at room temperature for an hour. The vessels were then placed for 20 min on a hot plate at 230 °C. When cool, the digest was diluted with Milli-Q water. An aliquot of the digest was added to the reduction cell; after reduction with $SnCl_2$, mercury was swept from the solution by aeration and concentrated on a gold trap. Mercury was then released from the gold trap by heating and measured on an LDC Milton Roy instrument. A detailed description of the method was presented elsewhere (Horvat et al. 1991). The precision of the method was ±2–3 % (RSD). The limit of detection (LOD), expressed as the SD of the blank, was 0.2 ng/g; the limit of quantification (LOQ) was 1 ng/g.

Determination of MMHg was based on the separation of MMHg from the samples by evaporation of MMHg halides and cyanides in a closed microdiffusion cell onto a paper impregnated with cysteine (Horvat et al. 1990). The paper was successively transferred to a glass vial, acidified and extracted with toluene. An aliquot of the extract was injected into a column consisting of 5 % DEGS-PS on Supelcoport 100–120 mesh, 5 % Carbowax R 20 M on Supelcoport 100–120 mesh and 5 % PEGS on Diatomite »C« 100–120 mesh (length, 160 cm; i.d., 2 mm) installed in a gas chromatograph (Hewlett Packard mod. 5890 II) equipped with an electron capture detector (Horvat et al. 1990). In each set of analyses, a recovery test was performed and it was found to vary between 85 and 95 %. The results were corrected for the recovery factor. The reproducibility of the method was between 3 and 5 % (RSD); the LOD was 2 ng/mL and the LOQ 7 ng/g.

The accuracy of the results was checked by regular analysis of certified reference materials (CRMs) certified for total Hg and MMHg. The CRMs DORM-2 (dogfish muscle), DOLT-3 (dogfish liver) and TORT-2 (lobster hepatopancreas), obtained from NRCC and IAEA-350 (tuna fish homogenate), were regularly used in each batch of analysis. The values obtained agreed with the certified values; the certified values and the values obtained are reported in Electronic supplementary material (ESM) Table 1. All ray samples were measured in duplicate. Blanks were constantly measured to verify the purity of reagents and labware.

Statistical analysis

Pearson's correlation coefficients were used to calculate correlations among variables, and *t* test was used for the statistical significance of differences for THg and MMHg in male and female tissues of the bull ray species using SAS/STAT software (SAS Institute Inc., 2001).

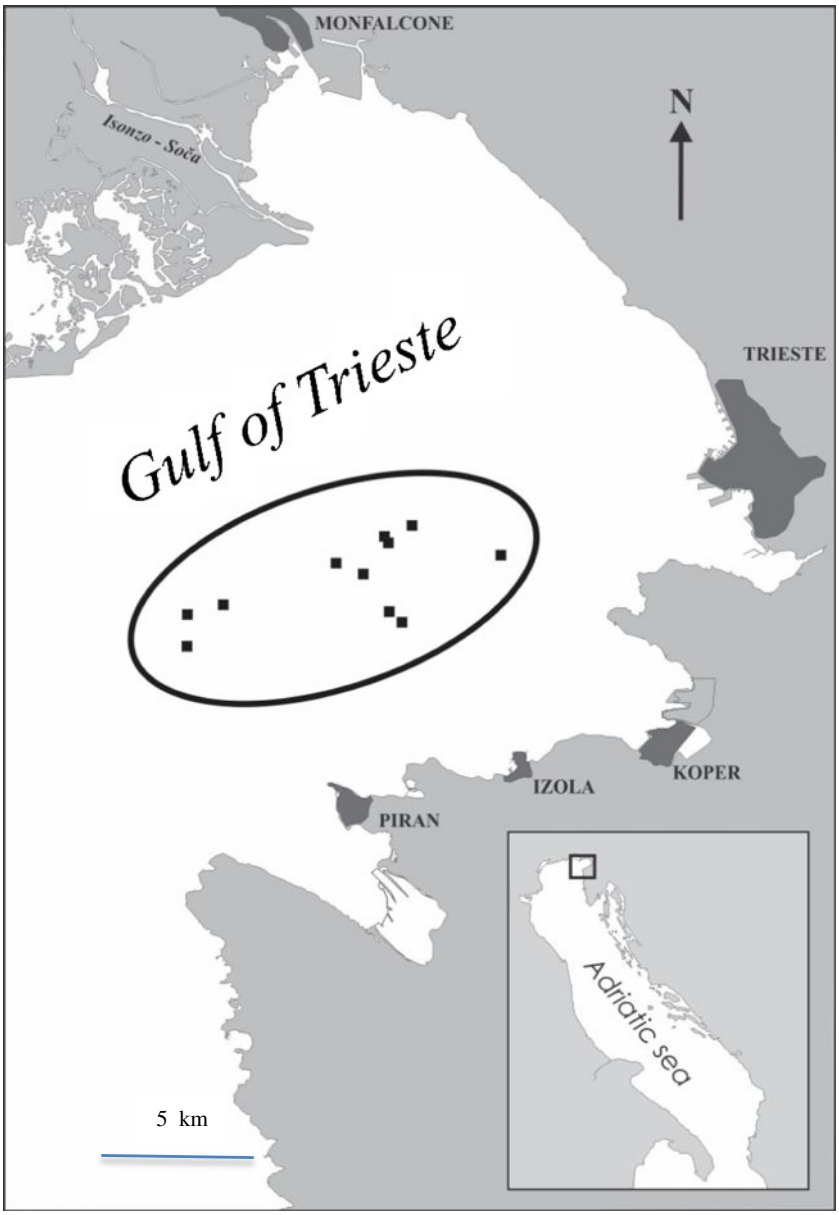

Fig. 1 Map of the study area with sampling locations in the Gulf of Trieste (northern Adriatic Sea) where the four ray species were collected

Results

A summary of the biometric data of the four studied ray species is presented in Table 1; the summary results for total Hg and MMHg based on both wet and dry basis are shown in Table 2. The data for each individual species are provided in ESM Tables 2, 3 and 4. Results expressed on a dry weight basis were used for statistical evaluation.

Table 1 Biometric data of the four studied ray species

Species	*n* (m, f, juv)	Disc width (mm)	Disc length (mm)	Weight (kg)
Eagle ray (*Myliobatis aquila*)	5 (1, 1, 3)	273–380, 310±50	142–225, 176±38.9	0.26–0.98, 0.50±0.31
Bull ray (*Pteromylaeus bovinus*)	17 (6, 9, 2)	450–2,220, 727±422	760–2,940, 1,714±806	1.50–116.0, 47.0±43.0
Pelagic stingray (*Dasyatis violacea*)	8 (5, 3, 0)	437–600, 531±61	1,010–1,392, 1,240±146	2.40–7.56, 4.83±1.78
Common stingray (*Dasyatis pastinaca*)	1 (1, 0, 0)	455	367	4.00

The numbers of males, females and juveniles (or embryos) are given in parentheses

m males, *f* females, *juv* juveniles

Table 2 Summary data for THg and MMHg in tissues (in milligrams per kilogram), expressed on the basis of dry and wet weights, of the eagle ray (*M. aquila*), bull ray (*P. bovinus*) pelagic stingray (*D. Violacea*), and common stingray (*D. pastinaca*), and the percentage of MMHg

		Gills					Liver					Muscle				
		THg (mg/kg d.w.)	THg (mg/kg w.w.)	MMHg (mg/kg d.w.)	MMHg (mg/kg w.w.)	% MMHg	THg (mg/kg d.w.)	THg (mg/kg w.w.)	MMHg (mg/kg d.w.)	MMHg (mg/kg w.w.)	% MMHg	THg (mg/kg d.w.)	THg (mg/kg w.w.)	MMHg (mg/kg d.w.)	MMHg (mg/kg w.w.)	% MMHg
Eagle ray ($N=5$)	Average	0.222	0.083	0.190	0.073	90	0.078	0.045	0.076	0.044	98	0.352	0.086	0.326	0.080	90
	Min	0.070	0.032	0.070	0.032	80	0.050	0.026	0.050	0.026	90	0.213	0.051	0.192	0.046	82
	Max	0.467	0.151	0.374	0.121	100	0.120	0.075	0.120	0.075	100	0.756	0.183	0.734	0.178	97
Bull ray ($N=15$)	Average	1.582	0.343	1.162	0.251	71	1.467	0.951	0.546	0.355	60	3.752	1.028	3.710	1.017	98
	Min	0.120	0.030	0.080	0.020	45	0.020	0.020	0.020	0.020	24	0.300	0.080	0.280	0.080	88
	Max	3.050	0.670	2.320	0.510	89	5.260	3.300	1.320	0.830	100	6.540	1.790	6.370	1.740	100
Pelagic stingray ($N=8$)	Average	2.529	0.686	2.155	0.586	85	1.277	0.752	1.049	0.618	84	4.086	0.871	3.970	0.846	98
	Min	1.179	0.375	1.048	0.252	67	0.581	0.394	0.473	0.321	65	2.364	0.499	2.364	0.499	93
	Max	4.398	1.012	3.907	0.899	96	2.872	1.642	1.877	1.073	97	6.772	1.344	6.305	1.285	100
Common stingray ($N=1$)		0.995	0.270	0.885	0.240	89	0.238	0.140	0.238	0.140	100	1.876	0.400	1.876	0.400	100

Data for MMHg are expressed as milligrams Hg per kilogram

The correlations between total Hg and MMHg and size are given in Table 3. Evidently, in all species analysed, the highest values were found in muscle, followed by liver and gills.

For the eagle ray, the correlations between total Hg and MMHg and the specimen's size seem to be strong, but due to the small number of specimens, these were statistically non-significant (Table 3). Also, total Hg and MMHg are not correlated within and between various tissues due to the unrepresentative and small number of specimens.

For bull ray, the correlations between total Hg and MMHg and size were strong and statistically highly significant ($p < 0.001$; Table 3). Total Hg and MMHg in the gills, muscle and liver are strongly correlated with the weight of the fish specimens (Fig. 2). Interestingly, in the liver, the total Hg content increases nearly exponentially, whilst MMHg showed a slower increase (Fig. 2b). The correlation between the percentage of Hg as MMHg in the liver and weight is strongly negative, probably due to the demethylation process occurring in the liver in older specimens (Fig. 2b). The resulting inorganic mercury from demethylation seems to be retained in the liver of older species, which might be due to the formation of insoluble Hg compounds, such as HgSe. However, in muscle, MMHg approaches almost 100 % in older specimens (Fig. 2c).

In Fig. 3a-c, correlations between total Hg and MMHg, as well as the percentage of MMHg in different organs of the bull rays, are shown. Strong and positive correlations were found in the muscle, liver and gills. Hg in muscle was primarily in MMHg form. In the liver, the correlation between total Hg and MMHg was also strong (log-transformed correlation), but the percentage of MMHg significantly decreased with the total Hg content. In gills, total Hg and MMHg were in linear correlation, but the percentage of MMHg was variable, between 45 and 100 %, and not dependent on total Hg. These observations further support the hypothesis that MMHg is metabolized in the liver and the resulting inorganic Hg retained as a stable insoluble compound.

In pelagic stingray, the highest Hg and MMHg contents as well as the highest percentage of MMHg were found in the muscle and the lowest in the liver (Table 2). The concentrations of Hg and MMHg in the gills and muscle seem to be correlated with the size of the specimens, but not significantly (Table 3). The percentage of MMHg was not well correlated within the organs of individual animals, nor between them. This is probably due to the relatively small number of fish with a small size range of the specimens (Table 1).

For the common stingray, the measurements of total Hg and MeHg in one specimen only are provided (Table 2) for information.

Interspecies comparison

The biometric data indicate that the ray species cannot be directly compared (Table 1). For example, the largest

Table 3 Linear regression correlation coefficients for associations between Hg in different tissues of ray species

		Gills		Liver		Muscle	
		THg	MMHg	THg	MMHg	THg	MMHg
Eagle ray ($N=5$)	Weight (kg)	$r=0.639$	$r=0.626$	$r=0.790$	$r=0.790$	$r=0.945$	$r=0.952$
		$p=0.246$	$p=0.258$	$p=0.112$	$p=0.112$	$p=0.015$	$p=0.013$
	Disk width (mm)	$r=0.509$	$r=0.490$	$r=0.775$	$r=0.849$	$r=0.879$	$r=0.887$
		$p=0.382$	$p=0.402$	$p=0.124$	$p=0.069$	$p=0.049$	$p=0.045$
	Disk length (mm)	$r=0.382$	$r=0.375$	$r=0.698$	$r=0.777$	$r=0.803$	$r=0.810$
		$p=0.525$	$p=0.535$	$p=0.190$	$p=0.122$	$p=0.102$	$p=0.097$
	Total length (mm)	$r=0.380$	$r=0.369$	$r=0.655$	$r=0.745$	$r=0.807$	$r=0.820$
		$p=0.528$	$p=0.542$	$p=0.230$	$p=0.149$	$p=0.099$	$p=0.089$
Bull ray ($N=15$)	Weight (kg)	**0.897**	**0.864**	**0.839**	**0.869**	**0.969**	**0.971**
		$p<0.001$	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**
	Disk width (mm)	**0.905**	**0.862**	**0.897**	**0.879**	**0.938**	**0.942**
		$p<0.001$	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**
	Disk length (mm)	**0.902**	**0.855**	**0.924**	**0.908**	**0.965**	**0.967**
		$p<0.001$	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**
	Total length (mm)	**0.847**	**0.847**	**0.883**	**0.865**	**0.903**	**0.900**
		$p<0.001$	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**	**$p<0.001$**
Pelagic stingray ($N=8$)	Weight (kg)	$r=0.384$	$r=0.339$	$r=-0.045$	$r=0.166$	$r=0.508$	$r=0.496$
		$p=0.348$	$p=0.411$	$p=0.915$	$p=0.694$	$p=0.198$	$p=0.211$
	Disk width (mm)	$r=0.445$	$r=0.406$	$r=0.159$	$r=0.366$	$r=0.482$	$r=0.472$
		$p=0.269$	$p=0.318$	$p=0.707$	$p=0.372$	$p=0.226$	$p=0.238$
	Disk length (mm)	$r=0.563$	$r=0.560$	$r=0.134$	$r=0.320$	$r=0.685$	$r=0.657$
		$p=0.146$	$p=0.149$	$p=0.752$	$p=0.440$	$p=0.061$	$p=0.077$
	Total length (mm)	$r=0.682$	$r=0.661$	$r=0.502$	$r=0.561$	$r=0.609$	$r=0.602$
		$p=0.092$	$p=0.106$	$p=0.251$	$p=0.191$	$p=0.147$	$p=0.153$

Significance level was set at $\alpha=0.002$ as the number of comparisons (correlations) calculated was $n=24$ for each species. According to Bonferroni correction, significance level was calculated as α/n. Significant correlations are in bold

specimens of bull ray ranged from 1.5 to 116 kg, whilst in the case of the eagle ray, all specimens were <1 kg. Also, the number of specimens was relatively small, especially for the eagle ray and pelagic stingray. Comparison of the values for male, female and juvenile specimens was not possible due to the small sample size. Although female and male bull ray specimens numbered 6 and 9, respectively, comparisons were not possible due to the different size classes of the two genders.

To facilitate comparisons of the data for total Hg and MMHg in different ray species and tissues, the results are summarized in Fig. 4a–g, showing the highest values in the pelagic string ray and the lowest in the eagle ray. Interestingly, the proportion of Hg as MMHg in muscle (Fig. 4g) was significantly lower in the eagle ray than in the bull ray ($p=0.012$ and $p=0.026$, respectively). The proportion of MMHg does not differ significantly between the bull ray and the pelagic stingray ($p=0.500$). The proportion of Hg as MMHg in the liver (Fig. 4f), however, is significantly higher in the eagle ray and pelagic stingray than in the bull ray ($p=0.032$ and $p=0.013$, respectively). The proportion of Hg as MMHg did not differ significantly between the bull ray and the pelagic stingray ($p=0.197$). The proportion of Hg as MMHg in gills (Fig. 4e) was significantly higher in the eagle ray and pelagic stingray than in the bull ray ($p=0.016$ and $p=0.008$, respectively). The proportion of Hg as MMHg did not differ significantly between the eagle ray and pelagic stingray ($p=0.661$).

As the size range of the various species was different, a comparison between specimens only of similar size classes was also performed between the species. Therefore, large bull ray specimens were excluded; the results are shown in Fig. 5a–d. Very clear differences were observed for total Hg and MMHg in the liver and muscle of benthic (eagle ray and bull ray) compared to pelagic stingray.

The pelagic stingray displayed a higher Hg concentration in gills (Fig. 5b) than the bull ray and eagle ray species ($p=0.002$ and 0.003, respectively). The concentrations in gills did not differ significantly between the bull ray and eagle ray ($p>0.05$). The pelagic stingray had higher Hg concentration in the liver compared to the bull ray and eagle ray ($p=0.002$ and 0.003, respectively; Fig. 5c). The concentrations in the liver do not differ significantly between the bull ray and eagle ray

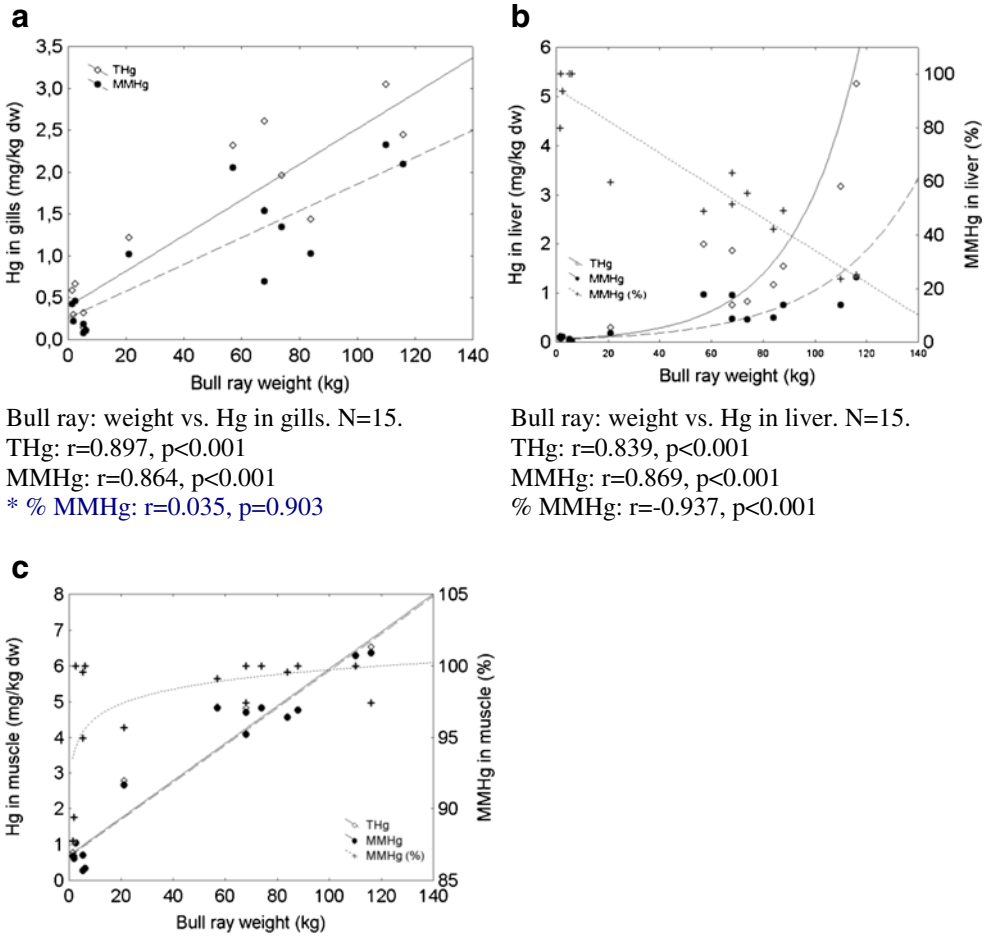

Fig. 2 Correlation between weight and the total Hg and MMHg concentrations in the bull ray

Bull ray: weight vs. Hg in gills. N=15.
THg: r=0.897, p<0.001
MMHg: r=0.864, p<0.001
* % MMHg: r=0.035, p=0.903

Bull ray: weight vs. Hg in liver. N=15.
THg: r=0.839, p<0.001
MMHg: r=0.869, p<0.001
% MMHg: r=-0.937, p<0.001

Bull ray: weight vs. Hg in muscle. N=15.
THg: r=0.969, p<0.001
MMHg: r=0.971, p<0.001
% MMHg: r=0.612, p=0.072

($p > 0.05$). The pelagic stingray had higher Hg concentration in the muscle than the bull ray and eagle ray ($p = 0.002$ and 0.003, respectively; Fig. 5d). Concentrations in the muscle did not differ significantly between the bull ray and eagle ray ($p > 0.05$).

In gills, a significantly lower proportion of Hg as MMHg was found in the bull ray than in the eagle ray ($p = 0.028$) and pelagic stingray ($p = 0.020$). In the liver, a significantly lower proportion was found in the pelagic stingray than in the bull ray ($p = 0.039$) and eagle ray ($p = 0.013$). In the muscle, a significantly higher proportion was found in the pelagic stingray compared to the eagle ray ($p = 0.028$).

Hg in the prey of the rays

The levels of Hg in the prey of the rays, namely, the banded murex, red bandfish and anchovy, averaged 0.28±0.03, 0.04±0.01 and 0.16±0.06 mg/kg w.w., respectively. The MMHg contents averaged 0.14±0.03, 0.02±0.01 and 0.11±0.02 mg/kg w.w., respectively. The percentage of Hg as MMHg varied between 38 % in red bandfish, 52 % in banded murex and 71 % in anchovy. All values in prey are given on a fresh weight basis in order to be able to calculate the accumulation factors in the web structures.

Discussion

Comparison between species

Since the absorption of Hg in aquatic organisms proceeds directly by uptake from the surrounding contaminated water and through the contaminated food web, we focused our study on gills, a potential indicator of direct uptake from water; the liver, an indicator of accumulation and detoxification; and muscle tissue, an indicator of bioaccumulation through the food web, i.e. indirect contamination (Boening 2000). Because the eagle ray specimens were rather young, the

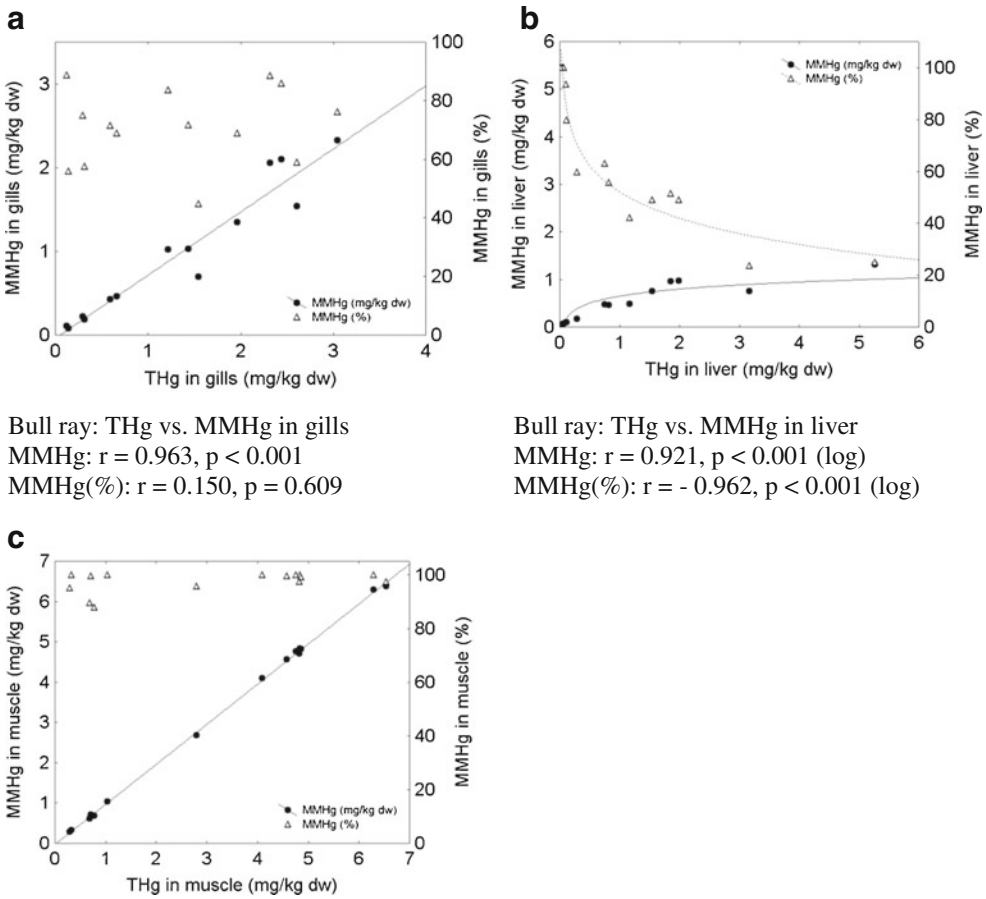

Fig. 3 Correlation between the total Hg and MMHg concentrations in the muscle, liver and gills in the bull ray

Bull ray: THg vs. MMHg in gills
MMHg: r = 0.963, p < 0.001
MMHg(%): r = 0.150, p = 0.609

Bull ray: THg vs. MMHg in liver
MMHg: r = 0.921, p < 0.001 (log)
MMHg(%): r = - 0.962, p < 0.001 (log)

Bull ray: THg vs. MMHg in muscle
MMHg: r = 0.9997, p < 0.001
MMHg(%): r = 0.451, p = 0.092

correlations between Hg contents in the gills and the biometric data were rather low. Hg contents in all tissues of the bull ray were lower compared to the other species studied. Only a comparison with data for Hg concentrations in the muscle of eagle rays from the southern Adriatic Sea reported by Storelli et al. (2002) was possible. They found up to tenfold higher contents in these fish from the southern Adriatic, considered less anthropogenically Hg-polluted compared to the northern Adriatic (Horvat et al. 2003a; Kotnik et al. 2013), but the differences found could arise from their analysis of larger and older specimens.

Because the studied bull ray specimens encompassed ages from embryos to adults, they were more suitable for studying correlations between Hg and MMHg, as well as between Hg species and biometric data, and differences between genders. A parallel increase in Hg and MMHg contents in bull ray liver with increasing disc width was evident up to about 1,600 mm. Beyond this, an abrupt nearly exponential increase of Hg and a slow increase of MMHg appeared (Fig. 2b), probably due to the demethylation process in the presence of high Hg contents, as observed in dolphins (Palmisano et al. 1995), when the liver starts to accumulate Se which is involved in detoxification mechanisms (Parizek and Ostadalova 1967). The high Hg and MMHg contents in bull ray embryos may originate from food intake through mother liquor rich in mucous, proteins and lipids, and their birth may reduce the Hg levels in mature females, as suggested for sharks (Walker 1976). In our case, comparison between genders was unfortunately not possible due to the different size classes of the two genders, although female and male bull ray specimens numbered 6 and 9, respectively.

The highest contents of Hg and MMHg in all three tissues analysed from all the studied species were found in the pelagic stingray. The pelagic stingray, compared to the eagle ray and the bull ray, is a typical pelagic species feeding on pelagic fish containing higher Hg levels compared to benthic invertebrates, the principal food of the eagle ray and bull ray. The reason could also lie in a not yet adapted mechanism for Hg excretion and/or demethylation of MMHg in this species which only recently (after 1999) appeared in the northern Adriatic, probably as a result of increased seawater temperatures and a vacant niche for shark-like predators (Mavrič et al. 2004).

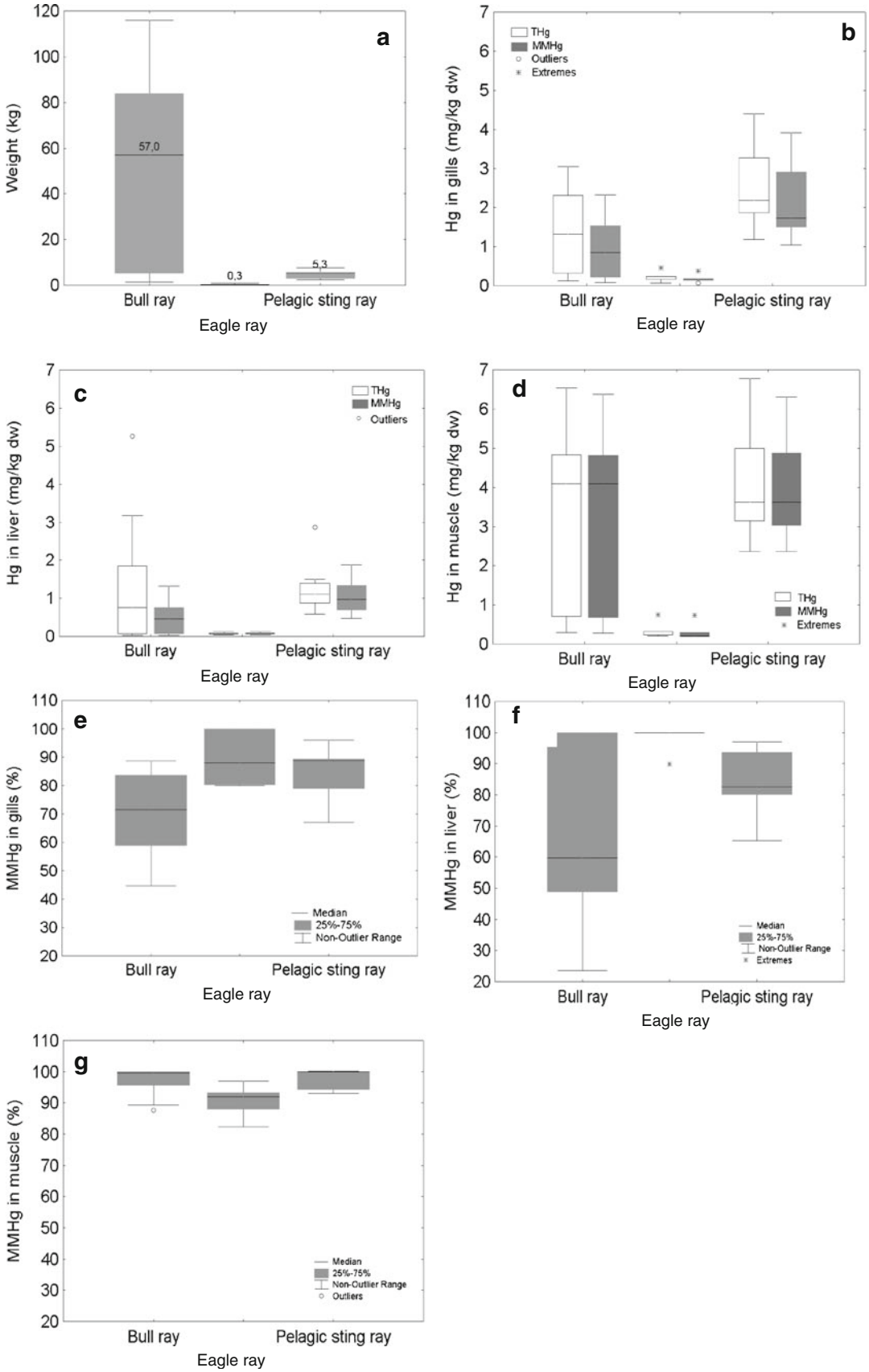

Fig. 4 Comparison of the total Hg and MMHg concentrations in ray species

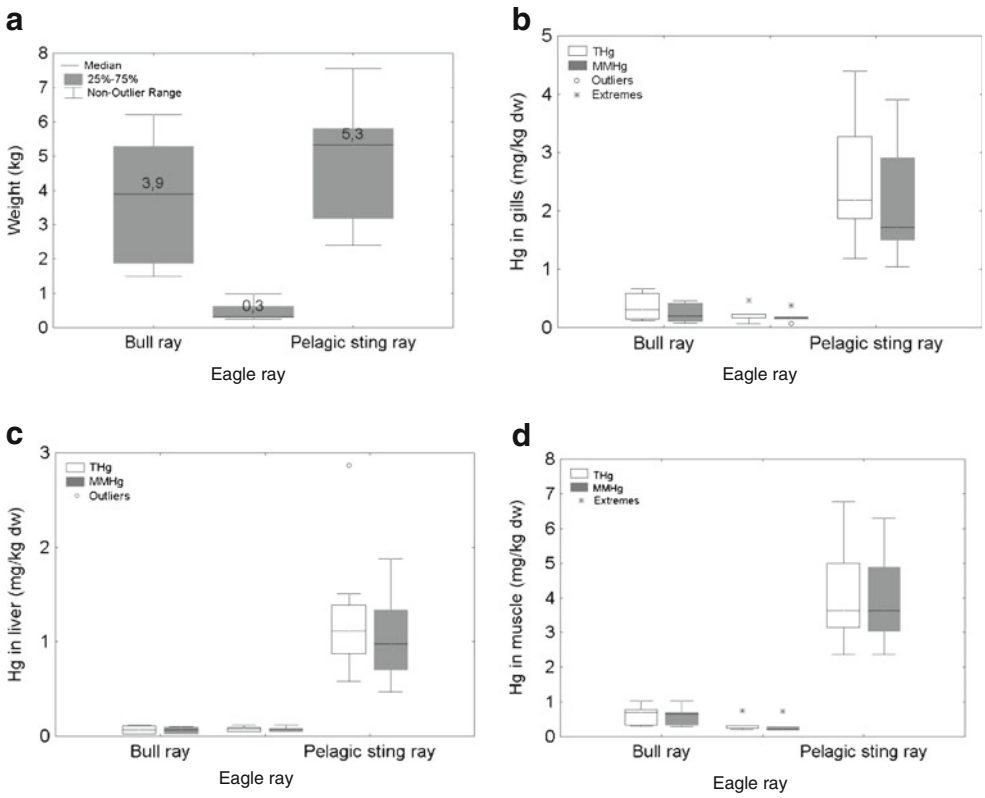

Fig. 5 Comparison of the total Hg and MMHg concentrations in ray species, excluding larger specimens

Bioaccumulation in various tissues differs among species due to their feeding behaviour (Régine et al. 2006). Among the species analysed, we found the highest Hg contents in muscle tissue followed by the gills and liver. The relatively high Hg and MMHg contents in the gills of all the studied species suggest the capability of gills to accumulate a rather high quantity of Hg species from water (Malatt 1985), passively or actively (Andres et al. 2002), especially in the inorganic form (Oliviera Ribeiro et al. 2002). Up to about a tenfold higher Hg content was found in the pelagic stingray compared to the bull ray of similar size. Due to the rather similar surface and bottom water concentrations in the Gulf (Horvat et al. 1999; Faganeli et al. 2003; Bratkič et al. 2013), except near the Soča/Isonzo River mouth, we would expect that the benthic bull ray would exhibit similar (or higher) Hg and MMHg contents. However, it seems possible that metal transfer from the gills to the internal organs of the organism is lower than that through the gut (Andres et al. 2002). The liver is an important accumulation site in aquatic vertebrates (Olsvik et al. 2001), generally containing high Hg^{2+} levels depending on the species properties (Riisgard and Hansen 1990). In the bull ray, the level of Hg in the liver is highly correlated with size, whilst in muscle this increase is far less pronounced, especially after the total Hg value in muscle reaches 1 mg/kg. It appears that at certain levels of Hg accumulated, the demethylation mechanism is induced and the supply of MMHg to other target organs such as the muscle is reduced. The reported deleterious effects of Hg^{2+} include the inhibition of glucose release and biosynthesis of cAMP in the eel (*Anguilla Anguilla*; Trombini et al. 2003) and an impact on Ca^{2+} deficiency in skate hepatocytes (Nathanson et al. 1995). MMHg detoxification proceeds through Se in the liver (Peterson et al. 2009) leading to HgSe (Kojadinović et al. 2007), which is accumulated in liver cells in lysosomes (Cardellicchio et al. 2002). Other detoxifying processes can be operative in the liver, including a linkage to metallothioneins (Tušek-Žnidarič et al. 2006) or thiols like glutathione (GSH; Zalups and Lash 1996), as observed in dolphins (André et al. 1990), as well as secretion into the bile, most probably as a GSH complex. As a general rule, endogenous GSH represents the first line of cellular defence against Hg, induction of metallothioneins the second and lysosomal formation of insoluble Se/Hg/S complexes the third (Cuvin-Aralar and Furness 1991).

Hg and MMHg measured in murex, the prey of the eagle and bull ray, showed high concentrations; 52 % of Hg was present as MMHg. In pelagic stingray gut, anchovy remains were found with lower Hg and MMHg contents, but a higher percentage of Hg (71 %) as MMHg. The pelagic stingray also feeds on red bandfish, where the lowest Hg content and percentage of Hg as MMHg (38 %) were found. In muscle, the majority of Hg is accumulated in methylated form, ranging between 63 and 86 % in various fish species (Andersen and Depledge 1997), >85 % in pilchard (*Sardina pilchardus*;

Joiris et al. 1999) and 75–100 % in tuna (Storelli et al. 2002, 2005). Among the cartilaginous fish, 66 % of Hg as MMHg was generally found in shark muscle (Walker 1976), but higher percentages (>90 %) were detected in spiny dogfish (*Squalus acanthias*; Pethybridge et al. 2010) and blue shark (*Prionace glauca*; Davenport 1995). The published percentages of Hg as MMHg in the marbled electric ray *(Torpedo marmorata*, 81 %) and eagle ray (72 %) are in the range reported for other cartilaginous fish (Storelli et al. 2002). The values reported in the present study are between 81 and 100 %, thus a lot higher than those reported by Storelli et al. (2002).

The high correlation between Hg and MMHg contents and specimen size, i.e. disc width, disc length, total length and weight, observed in the bull ray, encompassing a wider size/age spectrum, confirms the higher contents in older specimens (Storelli et al. 2005) due to longer exposure to pollutant impact (Pellegrini and Barghigiani 1989). Similar conclusions about Hg contents and size (age) were already reported for sharks and other fish species (Walker 1976; Pethybridge et al. 2010, 2013; Chen et al. 2008b; Choy et al. 2009).

Bioamagnification of MMHg in food webs

We tentatively elucidated MMHg biomagnification in the local food webs (Fig. 6) using our data from ray species and their prey as well as published data on mean Hg and MMHg contents based on multi-season and multi-year analyses of seawater and zooplankton (Horvat et al. 1999; Faganeli et al. 2003; Bratkič et al. 2013) from the Gulf of Trieste. It should be noted that Hg concentrations in these figures are expressed on a fresh weight basis. It is well documented that the percentage of Hg as MMHg increases along the aquatic food web, averaging about 10 % in particulate matter, 15 % in phytoplankton and 30 % in zooplankton, and about 95 % in fish muscle (Watras and Bloom 1992), due to trophic variability and differences in ecology and metabolism (Back and Watras 1995). However, little is known about Hg and MMHg accumulation in marine as well as Mediterranean food webs (Chen et al. 2008a). For example, it was reported that in a Mediterranean food web composed of seawater→plankton→pilchard→tuna, the percentage of Hg as MMHg increases from 2 % in seawater through 60–90 % in pilchard to 100 % in tuna (Bernhard 1988).

In the Gulf of Trieste (Fig. 6), the percentage of MMHg increases along the benthic and pelagic food webs, from 0.1 % in seawater, including particulate matter, and 0.02 % in surface sediments through 6–17 % in plankton (Horvat et al. 1999; Faganeli et al. 2003; Bratkič et al. 2013), 52 % in benthic invertebrates, 57–77 % in small benthic fishes, 38–71 % in small pelagic fishes to 90–100 % in larger benthic fishes and 100 % in larger pelagic fishes (results of this study). The average increase in MMHg between seawater, including particulate matter, and zooplankton (>200 μm) in the Gulf is 10^4, but this also includes bioconcentration in phytoplankton, which is supposed to be the greatest (Hammerschmidt and Fitzgerald 2006). MMHg in anchovy was about 50-fold higher than that in zooplankton. Finally, the biomagnification of MMHg from seawater to organisms at higher trophic levels (Fitzgerald et al. 2007) in the Gulf of Trieste amounted to 10^6 for small pelagic fish (anchovy) and 10^7 for the muscle of larger pelagic fishes (pelagic stingray). The bioconcentration between mean sediment and benthic invertebrate MMHg

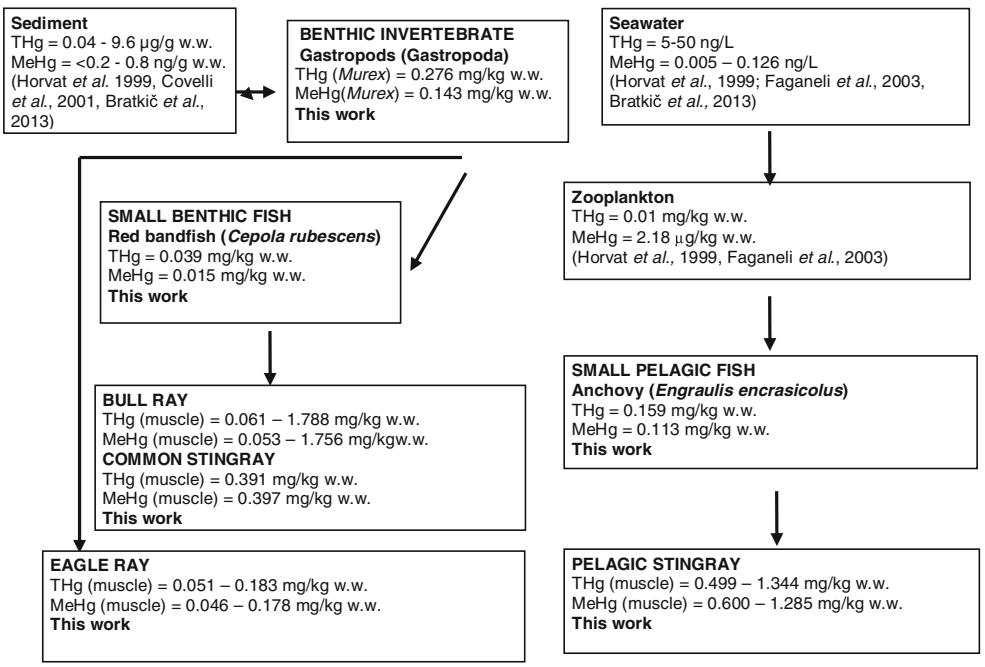

Fig. 6 Schematic representation of the total Hg and MMHg transfer from sediment and seawater to the final trophic level consisting of ray species in the food webs of the Gulf of Trieste (northern Adriatic Sea)

(murex) was 10^2 and that between higher benthic trophic levels ranged between 0.1 and 60. The ultimate trophic transfer resulted in biomagnification of 10^3 between water and muscle of larger benthic fish (bull ray, eagle ray, common stingray). The relative importance of these two pathways suggests greater accumulation of MMHg by pelagic feeding species (Chen et al. 2008b).

Since the Gulf of Trieste is one of the areas most severely polluted by Hg in the Mediterranean and worldwide (Horvat et al. 1999; Fitzgerald et al. 2007), it was expected that mercury in fish would be higher than in other areas of the Adriatic and the Mediterranean. Apart from the present study on ray species, the only systematic study on Hg accumulation in fish of the Gulf included three fish species: grey mullet (*Mugil cephalus*), a herbivorous fish, and common Pandora (*Pagellus erythrinus*) and conger eel (*Conger conger*), which are carnivorous fish (Horvat et al. 1999). The Hg and MeHg concentrations reported in this study were comparable to those obtained in the wider Adriatic (Buzina et al. 1995; Bernhard 1988; Storelli et al. 2005; EFSA 2012), which indicated that high Hg levels in the sediment were not proportionally reflected in fish living in the area, probably due to high demethylation rates (Hines et al. 2000, 2006) and reduction of mercury (Bratkič et al. 2013). However, it has to be mentioned that Hg biomagnification in the Gulf is at the highest limit published between seawater and muscle of predatory fish (Fitzgerald et al. 2007), obviously due to local Hg historical pollution.

The limit for Hg content in seafood in the EU is 0.5 μg/g w.w., whilst that for the fast accumulating species listed in the Directive, encompassing those located in the highest trophic levels (predators) and in benthos, is 1 μg/g w.w. (Commission of EU 2001). Direct comparisons of the Hg levels found in ray species in the Gulf with other areas are rather difficult due to the difference in species studied and, most of all, the size ranges of specimens. The concentrations of total Hg in the muscles of various skates reported by Storelli et al. (2003) ranged from 0.18 to 1.85 mg/kg (wet weight; average, 1.00 mg/kg). For 66.7 % of long nose skate (61.4 % of thornback ray samples, 42.8 % of winter skate samples and 38 % of starry ray samples), the total mercury concentrations exceeded the prescribed legal limit (1.0 mg/kg w.w.). In our study, none of the eagle ray exceeded the limit value, whilst 66 % of the bull ray and 37 % of the pelagic stingray exceeded the limit value of 1.0 mg/kg. It has to be noted that the size range of the specimens in the Gulf of Trieste were much larger than those measured by Storelli et al. (2003). Based on these observations, it can be concluded that the Hg present in the Gulf of Trieste seems to be less available for the uptake in food webs as compared to the Southern Adriatic and Ionian Seas. The reasons for these observations are difficult to describe as information on Hg pollution in the areas investigated by Storelli et al. (2003) are not provided.

In terms of health risks, the recommended Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2012) tolerable weekly intake is 1.6 μg MMHg per kilogram body weight per week. According to the recent European Food and Safety Administration (EFSA) opinion, this value should be reduced to 1.3 μg/kg b.w. (EFSA 2012). Considering these guideline values, consumption of 100 g per week for many specimens of bull ray and pelagic stingray would result in these limits being exceeded.

Conclusions

Hg and MMHg contents were positively correlated with the size/age of bull ray tissues. The highest percentage of mercury as MMHg in all ray species was found in muscle tissue, accounting to nearly 100 % of Hg present as MMHg. The highest Hg and MMHg contents (in all organs) were found in the pelagic stingray, originating from the southern Mediterranean Sea, which first appeared in the northern Adriatic in 1999, suggesting that this species might not be adapted to high Hg levels in the environment. Lower Hg and MMHg contents were found in the eagle ray, where the specimens analysed were rather young. High Hg and MMHg concentrations were also found in two embryos of the bull ray, indicating Hg transfer from mother to foetus during pregnancy. In bull ray liver, the slower MMHg increase was found to be independent of the higher Hg content, probably due to demethylation in the liver.

In parallel, Hg and MMHg contents were determined in the banded murex, the principal prey of the eagle and bull ray, and in anchovy and red bandfish, which are preyed upon by the pelagic stingray, as well as in zooplankton and seawater including particulate matter. Tentative estimation of MMHg bioaccumulation was assessed. It amounted to 10^4 for zooplankton, 10^6 for small pelagic fish (anchovy) and 10^7 for the muscle of the larger pelagic fish (pelagic stingray). MMHg bioaccumulation between sediment and benthic invertebrates (murex) and the muscle of small (red bandfish) and of larger benthic fish (bull ray, eagle ray, common stingray) ranged between 10^2 and 10^3, suggesting greater accumulation by pelagic feeding species. MMHg, originating mostly from sedimentary production and encountered in higher trophic levels in this area including fish, demonstrates dietary bioaccumulation. The outcome of this study can be important from the standpoint of conservation biology and human diet in the North Adriatic area with regard to MMHg toxicity. Since the Se level can greatly affect the bioaccessible and metabolically active fraction of fish Hg for other animals and humans, its potential toxicity cannot be evaluated independently of the Se content and its speciation analyses, a task which remains for future research.

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