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Report on research activities carried out for determination of mercury neuro-toxicological effects on cetacean brain

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Summary

In this study we analyzed Hg and Se concentrations in dolphin brain tissues of fifteen specimens of striped dolphin (*Stenella coeruleoalba*) and eight specimens of bottlenose dolphin (*Tursiops truncatus*) stranded in the Tyrrhenian and Adriatic Seas, in order to assess the toxicological risks associated with Hg exposure. High Hg concentrations were found in brain tissues of both analyzed specie (1.86–243mg/kg dw for striped dolphin and 2.1–98.7 mg/kg dw for bottlenose dolphin), exceeding levels associated with marine mammals neurotoxicity. Although the results clearly suggest that the protective effects of Se against Hg toxicity occur in cetaceans' brain tissues, a molar excess of mercury with respect to selenium was found, particularly in adult specimens of *Stenella coeruleoalba*. On contrary, negligible neuro-toxicological risks were found for *Tursiops truncatus* specimens, due to detoxification processes. Data obtained allowed to prove a more marked neuro-toxicological risk for adult specimens of *Stenella coeruleoalba* in both Tyrrhenian and Adriatic Seas.

Introduction

Among trace metals, mercury is one of the pollutants of most concern because of its toxicity and accumulative behavior in the environment and biota (Borrell et al. 2014). Due to the biomagnification effect and their long life span, the toothed cetaceans, inhabitants of the highest trophic levels of marine ecosystem, bioaccumulate generally a great amount of Hg (Bellante et al. 2012). Mercury pollution in the Mediterranean Sea is particularly relevant because it is a semi enclosed sea with high natural and anthropogenic inflows containing this metal. Particularly, Hg concentrations in cetacean tissues from the North Tyrrhenian Sea and the North Adriatic Sea are generally higher than those found in other Mediterranean regions (Bellante et al. 2012). Most of marine mammals monitoring programs developed in these regions, were focused on investigation of Hg concentrations in cetacean liver and kidney, because of the high capability of these tissues to accumulate this metal (Capelli et al. 2008; Leonzio et al. 1992). However recent studies have shown that the brain is the main target organ, where Hg chronic toxicity is underlined (Krey et al. 2015). Several in vivo and in vitro experimental studies showed that MeHg, the most toxic form of Hg, combines covalently with sulfhydryl (thiol) groups, leading to the sulfhydryl- containing enzymes inhibition (Farina et al. 2011). These observations led to postulate that MeHg-induced neurotoxicity is determined by the direct chemical interaction among MeHg and thiol groups from proteins and non protein molecules, such as glutathione (GSH; γ—glutamyl- cysteinylglycine) (Farina et al. 2011). Typical manifestations of Hg neurotoxicity include structural degeneration of the occipital cortex and cerebellum, which leads to movement disorders consisting mainly of ataxia, paresthesia and loss of balance (Lucena et al. 2007), sensory impairment and memory loss (Basu and Head 2010). Behaviors related to visual (visual contrast sensitivity task), cognitive (passive avoidance task); and emotional (depression-like behavior) functions are also affected by MeHg exposures (Ferraro et al. 2009). Selenium is

recognized as an essential element for metabolic activities of marine mammals. Moreover it is noteworthy that Se can protect marine mammals from the toxic effects of Hg through the formation of complexes species such as bis-methylmercuric selenide and mercuric selenide or tiemmanite (Watanabe 2002). The relation between Hg and Se in marine mammal tissues was previously documented by Capelli et al. (2008) and Bellante et al. (2012). According to these studies the molar ratio Se/Hg in liver of dolphins was approximately 1. This strong relationship may probably occur also in brain tissues (Ostertag et al. 2013). Thus, the Se/Hg molar ratio in brain tissue could be considered to be a critical parameter in determining Hg toxicity (Berry and Ralston 2009). To date, very few studies have been performed on Hg concentrations in dolphin brains, especially in the Mediterranean Sea. Thus, the risk of neuro-toxicological effects due to Hg exposure of dolphins from the Tyrrhenian and Adriatic Seas is almost unknown. In this study we analyzed Hg and Se concentrations in *Stenella coeruleoalba* and *Tursiops truncatus* brain tissues from the Tyrrhenian and Adriatic Seas in order to assess the toxicological risks associated with Hg exposure.

Materials and methods

Samples of brain tissues (central nervous system) were collected from 15 specimens of striped dolphin (*S. coeruleoalba*) and 8 specimens of bottlenose dolphin (*T. truncatus*) stranded along the Tyrrhenian and Adriatic coasts during the period 2010–2013 (Table 1). The total length of each specimen was measured in a straight line from the tip of the snout to the tip of the tail. Brain samples were collected only from carcass in good condition (carcass condition code II and III, following the carcass classification by Geraci and Lounsbury 1993) with clean stainless steel instruments. After collection, samples were wrapped in trace metals free rinsed glass ware and immediately stored at −20 °C. Brain samples were dried at 35 °C for 48 h and homogenized in an agate mortar before analysis. Total Hg concentrations were measured by atomic absorption spectrophotometry, using a direct mercury analyzer Milestone DMA-80 (Figure 1), according to analytical procedures reported in EPA 7473 (EPA Methods 7473). Approximately 0.1 g of each dried tissue was loaded in nickel boats and transferred to the DMA-80 system. In order to minimize contamination risks, acid-cleaned laboratory materials were used during sample preparation and analytical determination. Selenium was measured by an ICP-AES iCAP-6000 (Figure 2). About 0.25g of each air-dried and homogenized sample was digested in a microwave oven CEM MARS-5 (Figure 2) using 10mL of ultra-grade HNO_3 in Teflon liners, for 4h at 200W and at T=160°C. A reference standard material, supplied by the National Research Council of Canada (TORT-2; HgT certificate value = 0.27 ± 0.06 mg/kg; Se certificate value = 5.63 ± 0.67) was used to assess the accuracy and precision for metal. For Hg analysis, the accuracy was estimated to be approximately 97% and precision expressed as RSD% (n

= 3) resulted routinely lower than 4%. Duplicate samples (about 20% of the total number of samples) were measured to estimate reproducibility, which always resulted in differences less than 7%. In the analytical procedure for Se determination, accuracy was estimated between 85–93% and precision, always expressed as RSD% (n = 10), ranged between ±3–5%. Results of the quality control show an excellent agreement with certified data for both metals. Concentrations in brain tissues were expressed in mg kg^{-1} of dry weight (dw).

Figure 1: Direct Mercury Analyzer (Milestone DMA-80) used for mercury determination.

Figure 2: microwave oven (CEM MARS-5) and ICP-AES iCAP-6000 used for selenium determination.

Results and discussions

Mercury and selenium concentrations measured in cetaceans brain tissues, are shown in Table 1, together with the relative total length of single specimens. Wide ranges of Hg and Se concentrations were found in brain tissue of striped dolphin (from 1.9 to 243 mg/kg dw for Hg; from 1.9 to 42.5 mg/kg dw for Se) and bottlenose dolphin (from 1.1 to 98.7 mg/kg dw for Hg; from 2.2 to 27.5 mg/kg dw for Se) from the two sampling areas. Significant positive correlations were found between Hg concentrations and length for both dolphin species. Spearman's correlation coefficients (R), equal to 0.751 for striped dolphin and 0.857 for bottlenose dolphin respectively, suggested an overtime storage

of Hg in brain tissues. Based on analysis of covariance (using length as a covariate), statistically significant differences in Hg concentrations were found between the two species analyzed (ANCOVA test: $F=5.403$, $p=0.03$). Particularly, higher concentrations of Hg were recorded in brain tissues of striped dolphin. Higher Hg concentrations in liver and kidney of striped dolphins, with respect to the other species, were previously found in the same studied areas (Bellante et al. 2012). Mammals, especially those at high trophic position, can accumulate Hg in the brain tissue at concentration levels associated with the evidence of neuro-toxicological damages. The structural (brain lesions) and functional (clinical outcomes) effects of Hg exposure are similar among mammals because, due to its high affinity for sulfhydryl group, Hg interacts with proteins in a non-discriminate way (Basu et al. 2010). For this reason, interspecific differences of Hg neuro-toxicological mechanisms are not considered in this study. The risk of toxicity in wildlife is generally assessed by comparing concentrations of contaminants in tissues of wild animals with those of suitable laboratory animal toxicity models. Threshold levels of Hg toxicity established in laboratory for animals are important indicators of the neuro-toxicological effects that can be expected in wildlife species (Krey et al. 2015). According to Krey et al. (2015), clinical signs of neurotoxicity for mammals are generally observed at total Hg brain concentrations higher than 27.1 mg/kg dw. Neuropathological signs have been observed at total Hg brain concentration higher than 16.1 mg/kg dw. Neurochemical disruptors have been detected in the brain tissues as Hg concentration exceeds 1.61 mg/kg dw. In general, changes in behavior, genetic or immune response have been found at Hg brain concentration above 0.4 mg/kg dw. We found a strong significant relationship between Hg and Se in brain tissues of striped dolphin and bottlenose dolphin (Spearman R correlation = 0.92 and 0.87 respectively). These results allowed us to make the hypothesis that in the brain tissue Se interacts with Hg probably forming inert compounds of mercury selenide (tiemanite) as occurs in dolphins liver (Bellante et al. 2012; Cardellicchio et al. 2002). Values of Se/Hg molar ratio higher than 1 are assumed to counteract the adverse effects of Hg (Kehrig et al. 2013). However, as shown in Table 2, only 5 individuals among the 15 total species of striped dolphin and 5 individuals among the 8 species of bottlenose dolphin, showed a Se/Hg molar ratio higher than 1. Thus, 10 specimens of striped dolphin and 3 specimens of bottlenose dolphin show a variable amount of free Hg in their brain tissues (from 0.4 to 99.6 mg/kg dw for striped dolphin and from 0.33 to 28.8 mg/kg dw for bottlenose dolphin; Table 3). Comparing Hg striped dolphins concentrations with the threshold limits proposed by Krey et al. (2015) for Hg neurotoxicity in mammals, our results suggest that three specimens could be affected by clinical signs of Hg intoxication, three specimens by neuropathological disease, two specimens by neurochemical disease and two specimens by neurobehavioral disease. As regard bottlenose dolphin, only one specimen could show clinical signs of Hg intoxication and other two specimens could experience

neurochemical and neurobehavioral effect (Table 2). The reported data set clearly documents that the populations of striped dolphin from the Tyrrhenian and Adriatic Seas are affected by very high Hg concentrations in their brain tissues, reflecting the highly contaminated status of these areas. These concentrations exceed the threshold limits previously reported for mammalian Hg neurotoxicity and pose a serious health risk for this specie. On contrary, negligible neuro-toxicological risks were found for bottlenose dolphin specimens. Neuro-toxicological risk assessment for this specie requires further investigations based on a larger number of adult specimens.

Table 1 Total length (cm) and Hg–Se concentrations in brain tissues of *S. coeruleoalba* and *T. truncatus* from Tyrrhenian and Adriatic Sea

Species	Area	Length	Hg	Se
S. coeruleoalba	Tyrrhenian	91	7.4	2.7
S. coeruleoalba	Tyrrhenian	153	1.9	2.7
S. coeruleoalba	Tyrrhenian	201	69.8	20.8
S. coeruleoalba	Tyrrhenian	198	2.4	1.9
S. coeruleoalba	Tyrrhenian	167	7.5	3.1
S. coeruleoalba	Tyrrhenian	192	40.1	9.4
S. coeruleoalba	Tyrrhenian	200	23.1	8.9
S. coeruleoalba	Tyrrhenian	230	243.0	56.4
S. coeruleoalba	Adriatic	199	65.2	22.0
S. coeruleoalba	Adriatic	199	170.0	42.3
S. coeruleoalba	Adriatic	198	17.7	5.9
S. coeruleoalba	Adriatic	220	19.5	10.1
S. coeruleoalba	Tyrrhenian	204	79.3	15.2
S. coeruleoalba	Adriatic	137	1.9	5.6
S. coeruleoalba	Adriatic	205	65.2	19.1
T. truncatus	Adriatic	119	2.2	2.9
T. truncatus	Tyrrhenian	297	50.9	18.5
T. truncatus	Tyrrhenian	220	2.1	3.12
T. truncatus	Tyrrhenian	172	1.1	3.0
T. truncatus	Adriatic	310	98.7	27.5
T. truncatus	Adriatic	240	2.6	2.2
T. truncatus	Adriatic	284	5.4	4.7
T. truncatus	Adriatic	230	8.5	3.2

Note: Concentrations in tissues are expressed in mg kg^{-1} dw

Table 2 Se/Hg molar ratio and concentration of unbound Hg (mg/kg dw) in brain tissues of *S coeruleoalba* and *T. truncatus*

Species	Length	Se/Hg (mM)	Hg unbound (mg/kg dw)	Neurotoxic effects
S. coeruleoalba	91	0.92	0.58	Behavioral signs
S. coeruleoalba	153	3.58	0	Protected
S. coeruleoalba	201	0.76	16.93	Neuropathological signs
S. coeruleoalba	198	2.03	0	Protected
S. coeruleoalba	167	1.05	0	Protected
S. coeruleoalba	192	0.59	16.32	Neuropathological signs
S. coeruleoalba	200	0.98	0.52	Behavioral signs
S. coeruleoalba	230	0.59	99.62	Clinical signs
S. coeruleoalba	199	0.86	9.39	Neurochemical signs
S. coeruleoalba	199	0.63	62.64	Clinical signs
S. coeruleoalba	198	0.84	2.81	Neurochemical signs
S. coeruleoalba	220	1.32	0	Protected
S. coeruleoalba	204	0.49	40.61	Clinical signs
S. coeruleoalba	137	7.70	0	Protected
S. coeruleoalba	205	0.75	16.60	Neuropathological signs
T. truncatus	119	3.37	0	Protected
T. truncatus	297	0.92	4.85	Neurochemical signs
T. truncatus	220	3.77	0	Protected
T. truncatus	172	7.19	0	Protected
T. truncatus	310	0.71	28.86	Clinical signs
T. truncatus	240	2.14	0	Protected
T. truncatus	284	2.23	0	Protected
T. truncatus	230	0.96	0.33	Behavioral signs

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