Bull Environ Contam Toxicol (2016) 97:367–373
DOI 10.1007/s00128-016-1845-0

Copper Levels in Tissues of Dolphins *Tursiops truncatus, Stenella coeruleoalba* and *Grampus griseus* from the Croatian Adriatic Coast

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Received: 16 March 2016 / Accepted: 25 May 2016 / Published online: 31 May 2016

Abstract Copper concentrations were determined in muscle, liver, kidney, spleen and lung tissues of three dolphin species. Dolphins of *Tursiops truncatus* (young and adult), *Stenella coeruleoalba* and *Grampus griseus* were stranded along the Croatian coast. Concentrations in tissues of all three dolphin species were highest in the liver (4.92–16.5 µg/g) followed by kidney (2.85–5.29 µg/g). Similar levels were measured in muscle, spleen and lung in range 1.13–3.67 µg/g. Statistics analysis showed significant differences of Cu concentrations for muscle ($p = 0.008$), kidney ($p = 0.04$) and liver ($p = 0.02$) between the three dolphin species. Also, for all three species significant differences between tissue types of the same species were determined ($p < 0.001$, all). However, there were no significant differences in Cu levels of the same tissues between males and females within same species. Also, significant differences of body length and weight between three dolphin were found ($p < 0.001$, both).

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Keywords Copper · Dolphins · Tissues · Croatian Adriatic coast

As top predators, marine mammals such as whales and dolphins accumulate trace elements including copper (Cu), selenium (Se), mercury (Hg) and cadmium (Cd) through water, mainly by passive diffusion of the metal as part of soluble compounds, though the main source of contamination originates naturally from the diet (Cardellicchio et al. 2000; Capelli et al. 2008). Accumulation of elements in tissues of these species is due to their relatively long life spans, and has been used as a tool for the assessment of marine pollution (Das et al. 2003). The variability of trace metal levels in cetacean species has been shown to be due to food sources, the physiological state of the individual and toxicological dynamics of the specific metal, while there is also a correlation of metal accumulation with age and sex (Roditi-Elasar et al. 2003; Shoham-Frider et al. 2009).

As an essential element, Cu is required for normal growth and metabolism in aquatic mammals. Copper concentrations are dependent on the physiology and metabolism of the specimens and they appear to be homeostatically controlled due to fact that Cu participates in key biochemical and physiological processes. A mechanism for regulating intracellular levels of Cu is controlled by ATP-dependent pumps in cellular uptake, distribution and export (Storelli and Marcotrigiano 2002). Copper uptake is mainly via the gastrointestinal system and is regulated by body stores at the time of exposure. A sufficient quantity of Cu in the diet is required due to its role as a cofactor for enzymes involved in glucose metabolism and the synthesis of haemoglobin, and in optimising the production of connective tissue and phospholipids, for reproduction, iron utilization, and oxidation–reduction reactions (McDonald et al. 2010). However, Cu bioaccumulates and many organisms are unable to excrete Cu efficiently. Therefore, if Cu intake is in small amounts above the animal's daily needs, over time symptoms of toxicity may appear, such as vomiting nausea, abdominal and muscle pain, dermatitis, liver cirrhosis and neurological disorders (McDonald et al. 2010).

Most studies regarding Cu levels in mammals have been carried out along the Mediterranean and Atlantic coasts (Cardellicchio et al. 2002; Roditi-Elasar et al. 2003; Capelli et al. 2008; Wafo et al. 2014; Monteiro et al. 2016), and relatively few data exist on trace element levels in dolphins inhabiting the Adriatic Sea (Storelli and Marcotrigiano 2000, 2002). Cetacean species regularly present in the Mediterranean Sea are Risso's dolphin (*Grampus griseus)*, bottlenose dolphin (*Tursiops truncatus*) and striped dolphin (*S. coeruleoalba)*, whereas *T. truncatus* is the only cetacean species that regularly occurs in the Adriatic Sea (Gomerčić et al. 2002). It is believed that in many regions there are at least two types of *T. truncatus* – coastal and pelagic, which differ in morphology, feeding modes and also parasites. In the Croatian part of the Adriatic Sea, there is only one morphological type of *T. truncatus* (Đuras Gomerčić 2006). On the other hand, Risso's dolphin and striped dolphin are thought to be only occasionally present in the Adriatic Sea and along the entire Croatian coast (Gomerčić et al. 2002).

Very few studies have been published on trace element concentrations in dolphin species in the Adriatic Sea. The objective of this study was to collect baseline data on Cu concentrations in *T. truncatus, S. coeruleoalba* and *G. griseus* stranded along the east Adriatic coastline in the period 1999–2003. Obtained data were compared with different studies in dolphins worldwide, in particular to the Mediterranean, Adriatic and Atlantic waters.

Materials and Methods

Stranded individuals of three cetacean species were collected along the Croatian Adriatic coast between 1999 and 2003: bottlenose dolphins (*T. truncatus*), striped dolphins (*S. coeruleoalba*) and Risso's dolphins (*G. griseus*). Prior to dissection, gender, body mass and length were recorded for each animal. Teeth sections of bottlenose and striped dolphins were prepared according to Slooten (1991) and the age was estimated by counting growth layer groups (GLG-s) according to Hohn et al. (1998). Dolphins older than 7 years were considered adult specimens (Andre et al. 1991). In a total of 44 dolphins for three species young speciments were 9 *T. truncatus* (3 male, 6 female) and all other were adults: 21 *T. truncatus* (11 male, 10 female), 8 *S. coeruleoalba* (3 male, 5 female) and 6 *G. griseus* (5 male, 1 female). During necropsies muscle, liver, kidney, spleen and lung tissues were collected. Following collection, tissue samples were frozen in polyethylene bags and stored frozen at −18°C until analysis.

All reagents were of analytical grade (Kemika, Zagreb, Croatia). Ultra-pure water (18.2 MΩ/cm resistivity, Milli-Q, Millipore, USA) was used for all dilutions. All plastic and glassware were cleaned by soaking in diluted HNO_3 (1/9, v/v) and rinsed with distilled water prior to use. Calibrations were prepared with element standard solutions of 1000 mg/L supplied by Perkin Elmer (Waltham, Massachusetts, USA). Stock solution was diluted in HNO_3 (0.2 %).

Samples (0.5 g) were digested with 4 mL HNO_3 (65 % v/v), 2 mL H_2O_2 (30 % v/v) with a microwave closed system Multiwave 3000 (Anton Paar, Ostfildern, Germany). The digestion program began at a potency of 500 W then ramped for 1 min, after which samples were held for 4 min at 500 W. The second step began at a potency of 1000 W and held for 5 min. The third step began at a potency of 1400 W and held for 10 min. The fourth step was cooling at a power of 0 W for 15 min. Digested samples were diluted to a final volume of 50 mL with double deionised water. All metal concentrations were determined on a wet weight basis as µg/g.

The analyses of Cu were conducted by graphite furnace-atomic absorption spectroscopy using an AAnalyst 800 spectrometer equipped with an AS 800 autosampler (Perkin Elmer, Waltham, Massachusetts, USA) set at 228.8 nm. For graphite furnace measurements, argon was used as the inert gas (flow rate 250 mL/min). Pyrolytic-coated graphite tubes with a platform were used.

Detection limits (LOD) were determined as the concentration corresponding to three times the standard deviation of ten blanks samples of the results generated by GFAAS analysis. Obtained LOD (µg/g, wet weight, w.w.) were: muscle 0.0005, liver 0.0005; kidney 0.005, spleen 0.0005, lung 0.0005. All specimens were run in batches that included blanks, a standard calibration curve and two spiked specimens.

Precision and accuracy of the analytical methods were determined using certified reference materials DORM-2, dogfish muscle sample (National Research Council, Canada). The reference material was treated and analysed under the same conditions as the samples. Good agreement was obtained between certified (2.34 ± 0.16 µg/g) and analysed (2.31 ± 0.24 µg/g) and 98.7 % recovery was obtained. The coefficient of variation (CV) in samples was less than 10 %.

Statistical analyses were performed using the Stata 6.0 statistical package (Stata Corp. USA). Concentrations of Cu in tissues, body lengths and body weights are presented as mean and range (min–max). The significance of differences of Cu concentrations in the same tissues, between different tissues, between body length and weight between three dolphin species was assessed by the Mann–Whitney test and the Kruskal–Wallis test. Concentrations of Cu in two age groups of *T. truncates* species were compared using the Mann–Whitney test. Relationships between Cu concentrations and length and weight of dolphins were assessed using linear regression. Results were considered significant at $p \leq 0.05$.

Results and Discussion

Most previous studies have been conducted on trace element concentrations in the tissues of stranded *S. coeruleoalba* and *T. truncatus* dolphins. Few studies have provided trace element concentrations in *G. griseus* in the Mediterranean and Adriatic Seas (Capelli et al. 2008; Shoham-Frider et al. 2009). This is the first study of Cu levels in muscle, liver, kidney, lung and spleen tissues of three dolphin species stranded in the Croatian part of the Adriatic Sea.

Copper concentrations in muscle, kidney, liver, spleen and lung tissues and also length and weight of *S. coeruleoalba*, *G. griseus* and young and adult *T. truncatus* are presented in Table 1. Statistical analysis showed significant differences of Cu concentrations for muscle ($p = 0.008$), kidney ($p = 0.04$) and liver ($p = 0.02$) between the three dolphin species (adult). There were no significant differences in Cu concentrations between the species for spleen and lung tissues. Analysis between young and adult specimens of *T. truncatus* showed significant differences only for Cu levels in muscle tissues ($p = 0.02$). Also, significant differences of body length and weight between three dolphin were found ($p < 0.001$, both). However, there were no linear relationship between Cu concentrations and length, and Cu levels and weight of dolphins ($F(1,42) = 4.44$; $p = 0.04$).

Significant differences in Cu concentrations between tissue types of the same species were determined for all three dolphin species ($p < 0.001$, all). Copper concentrations in tissues of all three dolphin species tested, were highest in liver followed by kidney, while levels measured in muscle, spleen and lung were very similar and ranged from 1.13 to 3.67 µg/g. Liver Cu levels were 4.4 to 6.4-times and 1.7- to 3.1-times higher than in muscle and kidney tissues in all three dolphin species tested. The lowest Cu concentration of 0.63 µg/g was determined in the lung tissue of *G. griseus,* while the highest level of 47.9 µg/g was found in the liver tissue of a young female specimen of *T. truncatus* found in 2000 near the island Cres. Further, statistical significance of Cu levels between sex in the same species was investigated. There were no statistically significant differences in Cu concentrations of the same tissue types between males and females within same species. However recently was reported sex-related differences for *T. truncatus* (Monteiro et al. 2016).

In marine mammals, homeostatic control of Cu may be mediated by metallothionein and the induction of metallothionein synthesis is very important in metal detoxification. Different metallothioneins able to bind Cu, Zn and Cd have been identified in marine mammals and reduces metal mobility in the organism and protect from intoxication (Cardellicchio et al. 2002). High hepatic levels of Cu are related to the liver's role in detoxifying and excreting harmful substances, metabolizing nutrients and essential elements, and removing certain non-essential elements and toxins from the bloodstream (Frodello et al. 2002). The essential element concentrations of Cu and Zn are homeostatically controlled, and possible variations may be affected by physiology and metabolism. It has been suggested that the range of Cu in liver within which this regulation is active, is approximately 3–30 µg/g (Law et al. 1992). When concentrations are outside this range, it may be assumed that the regulating mechanism is impaired. In the present study, concentrations higher than the upper limit of 30 µg/g were found in the liver of two *T. truncatus* specimens less than 1 year of age and stranded on the islands of Cres and Murter (47.9 and 36.9 µg/g) and an adult specimens (12 years old) stranded in the Novigrad Sea (43.3 µg/g). Liver Cu levels were below the lower limit of 3 µg/g (1.38 µg/g) in a 3-year old *T. truncatus* found in the Maloston Canal (south Adriatic Sea). However, for the remaining 40 specimens, liver Cu levels were within the physiological safe range.

Previous studies have suggested that high Cu concentrations in the liver of foetal and new-born mammals, including marine species, demonstrate the essential nature of Cu in mammalian development (Law et al. 1992; Storelli and Marcotrigiano 2000). Accordingly, in the present study, liver Cu concentrations measured in young *T. truncatus* were higher (16.5 µg/g) than in adult animals (14.5 µg/g). Age-dependent higher Cu level in the liver of juveniles were also found previously (Kunito et al. 2004; Wafo et al. 2014). These higher Cu concentrations in juveniles might be related to a biochemical requirement in newborns for growth and development (Wagemann et al. 1988). Furthermore, decrease of Cu in liver with the age of dolphins could be a result of dilution of Cu levels as consequence of increasing tissue mass with age or body length or related to metabolic regulation with age and reduced requirements Cu tissue level (Woshner et al. 2001). Regarding feeding preferences, *T. truncatus* in the Adriatic Sea mostly feeds on fish, but often consumes

Table 1 Copper concentrations (mean ± SD, range) in tissues of young and adult *T. truncatus, S. coeruleoalba* and *G. griseus* from the Croatian Adriatic coast

Species	Muscle (μg/g)	Kidney (μg/g)	Liver (μg/g)	Spleen (μg/g)	Lung (μg/g)	Length (cm)	Weight (kg)
T. truncatus	6.12 ± 4.02	5.78 ± 2.32	11.6 ± 9.17	4.52 ± 6.04	0.92 ± 0.33	202 ± 14.0	109 ± 16.5
M – young	1.56–9.18	3.17–7.60	3.53–21.6	0.81–11.5	0.69–1.16	186–212	98.0–128
	3	3	3	3	2	3	3
T. truncatus	2.44 ± 1.21	5.04 ± 1.96	18.9 ± 19.0	2.56 ± 1.41	3.75 ± 3.54	164 ± 31.0	48.0 ± 19.2
F – young	1.32–4.19	2.96–8.49	1.38–47.9	0.99–4.31	1.04–9.32	122–200	23.0–66.0
	6	6	6	6	6	6	5
T. truncatus	3.67 ± 2.89[a,d]	5.29 ± 1.97[b]	16.5 ± 16.1[c]	3.21 ± 3.36	3.04 ± 3.27	177 ± 31.8	177 ± 31.8
All – young	1.32–9.18	2.96–8.49	1.38–47.9	0.81–11.5	0.69–9.32	122–212	23.0–128
	9	9	9	9	8	9	8
T. truncatus	3.04 ± 3.73	5.88 ± 3.96	16.3 ± 12.0	2.39 ± 1.87	2.55 ± 2.15	275 ± 22.0	215 ± 67.2
M – adult	0.75–14.0	2.49–16.1	3.99–43.3	0.74–6.63	0.79–6.68	235–312	135–324
	11	11	11	11	9	11	11
T. truncatus	1.68 ± 1.38	4.79 ± 2.18	12.7 ± 7.70	1.79 ± 1.16	1.31 ± 0.37	269 ± 11.8	200 ± 30.4
F – adult	0.87–5.52	2.86–9.62	4.52–31.6	0.77–4.63	0.76–1.87	246–283	163–246
	10	10	10	10	8	10	8
T. truncatus	2.27 ± 2.77[a,d]	5.23 ± 3.09[b]	14.5 ± 10.1[c]	2.02 ± 1.52	1.89 ± 1.65	272 ± 17.7[e]	209 ± 54.1[f]
All – adult	0.75–14.0	2.48–16.1	3.87–43.3	0.74–6.63	0.65–6.68	235–312	135–324
	21	21	21	21	17	21	19
S. coeruleoalba	2.05 ± 0.85	3.99 ± 0.82	12.7 ± 9.35	1.35 ± 0.38	1.04 ± 0.89	202 ± 11.8	88.0 ± 18.2
M	1.15–2.82	3.21–4.84	6.04–23.4	1.07–1.78	0.41–2.07	188–209	67.0–99.0
	3	3	3	3	3	3	3
S. coeruleoalba	1.99 ± 0.47	5.63 ± 2.55	9.18 ± 2.44	1.67 ± 0.42	1.81 ± 1.86	201 ± 4.04	92.4 ± 5.59
F	1.42–2.66	3.08–8.83	7.67–13.4	1.25–2.34	0.39–4.84	197–207	86.0–100
	5	5	5	5	5	5	5
S. coeruleoalba	2.02 ± 0.57[a]	5.01 ± 2.15[b]	10.5 ± 5.63[c]	1.55 ± 0.41	1.53 ± 1.54	201 ± 7.05[e]	90.8 ± 10.8[f]
All	1.15–2.82	3.08–8.83	6.04–23.4	1.07–2.34	0.39–4.84	188–209	67.0–100
	8	8	8	8	8	8	8
G.griseus	1.18 ± 0.37	2.77 ± 0.79	4.59 ± 1.62	1.39 ± 0.16	1.24 ± 1.05	299.6 ± 11.8	261.5 ± 53.2
M	0.83–1.58	1.48–3.64	2.99–6.98	1.19–1.54	0.63–2.81	286–318	185–305
	5	5	5	4	4	5	4
G.griseus	0.79	3.29	6.58	1.09	0.71	303	248
F	–	–	–	–	–	–	–
	1	1	1	1	1	1	1
G.griseus	1.11 ± 0.36[a]	2.85 ± 0.74[b]	4.92 ± 1.66[c]	1.34 ± 0.19	1.13 ± 0.94	300 ± 10.6[e]	259 ± 46.5[f]
All	0.79–1.58	1.48–3.64	2.99–6.98	1.09–1.54	0.63–2.81	286–318	185–305
	6	6	6	5	5	6	5

M Male, *F* Female

Statistically significant differences between species for tissues: [a] muscle ($p = 0.008$), [b] kidney ($p = 0.04$), [c] liver ($p = 0.02$)

Statistically significant differences between young and adult specimens of *T. truncatus* for muscle: [d] ($p = 0.02$)

Statistically significant differences between three species: length [e] ($p = 0.0004$), weight [f] ($p = 0.0006$)

cephalopods such as squid, shortfin squid, musky octopus and cuttlefish (Poldan 2004).

Table 2 shows the Cu concentrations reported in three dolphin species from different marine areas in the Mediterranean, Adriatic and Atlantic Seas. Element levels found in *S. coeruleoalba* were similar to previously reported concentrations in muscle, liver and kidney in *S. coeruleoalba* from the Mediterranean (Monaci et al. 1998; Cardellicchio et al. 2000, 2002; Roditi-Elasar et al. 2003; Capelli et al. 2008; Wafo et al. 2014), Atlantic (Das et al.

Table 2 Copper concentrations (µg/g) in tissues of young and adult *T. truncatus*, *S. coeruleoalba* and *G. griseus* from different locations in Adriatic, Mediterranean and European Atlantic

Location	Muscle	Liver	Kidney	Spleen	Lung	Reference
Tursiops truncatus						
French Atlantic coast	0.73–1.25[a]	1.1–7.5[a]	1.88–6.0[a]			Holsbeek et al. (1998)
South Adriatic Sea		8.29	2.78		1.12	Storelli and Marcotrigiano (2000)
Mediterranean Corsican coast	1.4[a]	10.25[a]	3.58[a]		1.23[a]	Frodello et al. (2002)
Mediterranean coast of Israeli	1.2	8.9	3.2			Roditi-Elasar et al. (2003)
Mediterranean north-west Italy (Ligurian Sea)	1.0–1.35[a]	5.45–23.8[a]	3.38–9.73[a]	1.23[a]	0.85–1.98[a]	Capelli et al. (2008)
Mediterranean coast of Israeli	1.1	11.4	3.0			Shoham-Frider et al. (2009)
Atlantic coast of Portugal	1.505–1.827	11.358–12.919	4.567–4.910			Monteiro et al. (2016)
Stenella coeruleoalba						
Mediterranean coast of Italy	1.6[a]	5.5[a]	3.6[a]			Monaci et al. (1998)
Mediterranean coast of Italy (Adriatic and Ionian coasts)	0.85	7.73	1.45		0.64	Cardellicchio et al. (2000)
Mediterranean coast of Italy (Ligurian Sea)	1.7[a]	7.95[a]	3.3[a]	0.8[a]	1.4[a]	Capelli et al. (2000)
South Adriatic Sea		4.05–10.71	2.50–36.3			Storelli and Marcotrigiano (2002)
Mediterranean coast of Italy (Apulian coasts)	1.78	9.99	4.06		1.20	Cardellicchio et al. (2002)
Mediterranean coast of Israeli	1.4	9.7	2.8			Roditi-Elasar et al. (2003)
Mediterranean north-west Italy (Ligurian Sea)	0.73–1.6[a]	4.9–10.3[a]	2.65–3.88[a]	1.03[a]	0.63–0.85[a]	Capelli et al. (2008)
French Mediterranean coast	1.35[a]	8.43[a]	2.83[a]		0.95[a]	Wafo et al. (2014)
Grampus griseus						
Mediterranean coast of Israeli	2.86	6.11	2.93			Shoham-Frider et al. (2002)
Mediterranean north-west Italy (Ligurian Sea)	0.73–0.93[a]	2.38–2.68[a]	1.67–1.85[a]	0.53–0.85[a]	0.55–0.9[a]	Capelli et al. (2008)

[a] Value expressed as µg/g wet weight: dry weight were converted to wet weight using the factor (ww/dw) of 0.25 established for dolphins (Becker et al. 1995)

2003) and southern Adriatic Sea (Storelli and Marcotrigiano 2002). Copper concentrations in the muscle of all three dolphin species in Mediterranean were found in the range from 0.73 µg/g in *S. coeruleoalba* (Capelli et al. 2008) to the highest level of 2.86 µg/g reported in *G. griseus* (Shoham-Frider et al. (2002). Regarding kidney, Cu concentrations three times higher than those in this study were measured in *S. coeruleoalba* from the southern Adriatic Sea (36.3 µg/g; Storelli and Marcotrigiano 2002) and Irish coast (37.5 µg/g; Das et al. 2003). Copper levels detected in tissues of *G. griseus* were similar to previous reports from the Mediterranean and Italian coasts (Cardellicchio et al. 2000, 2002; Capelli et al. 2008).

In the present study, higher Cu levels were measured in the muscle of young and adult *T. truncatus* than those found on the Mediterranean coasts (0.73–1.4 µg/g; (Storelli and Marcotrigiano 2000; Frodello et al. 2002; Roditi-Elasar et al. 2003; Shoham-Frider et al. 2009). Copper concentrations in liver and kidney found in this study are comparable to those measured in the southern Adriatic and Mediterranean coast and also to Portugal Atlantic coast measured in the ranges (µg/g): 5.45–23.8; 2.78–9.73 and 4.564–12.913 (Storelli and Marcotrigiano 2000; Capelli et al. 2008; Monteiro et al. 2016).

There are very few data regarding Cu concentrations in cetacean spleen and lung tissues (Cardellicchio et al. 2000, 2002; Storelli and Marcotrigiano 2000, 2002; Frodello et al. 2002; Capelli et al. 2008). In these studies, Cu levels in three dolphin species were measured in the spleen in the range from 0.53 to 1.23 µg/g and in lung tissues from

0.55 to 1.98 μg/g. In this study, comparable Cu levels were measured in spleen and lung of the three examined dolphin species.

It was concluded that after the lactation period, the metal intake of marine mammals is primarily through the diet, and such introduction can lead to element accumulation in tissues (Zhou et al. 2001). In addition to the long life span of dolphins, which is responsible for the bioaccumulation of elements and also Cu and variations in concentrations in different tissues of dolphins, there are other physiological factors that should be taken into consideration, such as age, gender and dietary preferences, and the condition of stranded dolphins (Monaci et al. 1998).

In conclusion, relatively comparable concentrations of Cu were found in the liver, muscle and kidney tissue of three dolphin species when compared with other reports from the Mediterranean basin. Copper is an essential element highly regulated in tissues and suggested range within this regulation in liver is active is approximately 3–30 μg/g. In this study Cu concentrations in the liver of dolphins were measured within this range and therefore it may be confirmed that liver functions were not impaired. However, in four specimens of *T. truncates* the concentrations outside this range were found which could be an indication of health damage.

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