

# A comparative study of inorganic elements in the blood of male and female Caspian pond turtles (*Mauremys caspica*) from the southern basin of the Caspian Sea

Milad Adel<sup>1</sup> · Adriana A. Cortés-Gómez<sup>2</sup> · Maryam Dadar<sup>3</sup> · Hossein Riyahi<sup>4</sup> · Marc Girondot<sup>2</sup>

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**Abstract** Due to their bioaccumulation and biomagnification pathways, inorganic elements can accumulate in high-level aquatic organisms in the food web. Then, this species can be used to monitor the quality of the environment. Blood concentration of nine inorganic elements, including possible toxic metals (An, Cu, Mn, Se, As, Ni, Cd, Pb, and Hg), in 20 males and 20 females from eight different locations with high industry and agriculture activities in Iran were evaluated in this work. Additionally, size, sex, condition index, and locations were also

included and analyzed. Among the essential elements, Zn and Se presented very high concentrations ( $56.14 \pm 2.66$  and  $8.44 \pm 0.77$   $\mu\text{g/g}$  ww, respectively) in all locations. Regarding possible toxic elements, Pb and Cd presented concerning concentrations as well ( $0.52$  and  $0.58$   $\mu\text{g/g}$  ww); this is especially true for Pb, an element found in very high concentrations in tissues of turtles from the same area in a previous study. The sex and the size of the individuals also had significant differences in concentration of Pb, Cd, As, and Hg.

**Capsule** Ecology and physiology interplay to explain the dynamic of inorganic elements, including possible toxic metals, in the freshwater turtle *Mauremys caspica* from Iran.

**Highlights** • Concentration of nine elements was determined in 40 individuals from eight populations of the Caspian pond turtle.  
• Concentration of the nine elements have been tested against location, sex, length, and condition index using the PERMANOVA, Mantel test, and generalized linear model.  
• Significant differences between sex and size were found for Pb, Cd, Hg, and As.  
• Pb, Zn, and Se had very high concentration compared with other populations and species.

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✉ Milad Adel  
miladadel85@yahoo.com

- <sup>1</sup> Department of Aquatic Animal Health and Diseases, Iranian Fisheries Science Research Institute, Agriculture Research Education and Extension Organization, Tehran, Iran
- <sup>2</sup> Laboratoire Ecologie, Systématique et Evolution, UMR 8079, Université Paris Sud, CNRS et AgroParisTech, 91405 Orsay cedex, France
- <sup>3</sup> Razi Vaccine and Serum Research Institute, Agricultural Research, Karaj, Iran
- <sup>4</sup> DVM Graduated, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran

**Keywords** Inorganic elements · Caspian pond turtle · Blood

## Introduction

In recent decades, the rapid development of industry and agriculture has resulted in the use and release of many chemical elements in the environment, including trace elements (Olowu et al. 2010). Inorganic elements, including possible toxic metals, occur as trace constituents of primary minerals in igneous rocks (which crystallize from molten magma) (Alloway 1995). The concentration of trace elements in a living organism first depends on their presence in the environment from natural (geological) or anthropogenic (pollution) sources. Second, it depends on the intake of this element in the organism, generally through food, thus impacting the ecology of the organism. Lastly, it depends on the assimilation and excretion of the element as well as on the physiology of the organism (Rainbow 2002). Metals accumulate in aquatic animals almost exclusively through their diet (Clemente et al. 2011; da Silva et al. 2016; Langston and Spence 1995; Yu et al. 2011 or maternal transfer to the eggs (Guirlet et al. 2008; Paez-Osuna et al. 2010a).

Possible toxic metals, such as cadmium (Cd), mercury (Hg), and lead (Pb) are important to be monitored due to their toxicity and bioaccumulative behavior in aquatic organisms (Kalantzi

et al. 2013). These elements are released in the environment in different ways, for example, through extraction, diffusion, combustion, and industrial activities. They subsequently enter the aquatic environment through the discharge of domestic, industrial, and agricultural waste water, accidental leakage, ballast water discharges by ships, and soil erosion (Bahnasawy et al. 2009; Javed 2005). These metals can be strongly accumulated along water and aquatic food chains, thus resulting in sublethal effects or death in local fish populations and, eventually, in humans (Grillitsch and Schiesari 2010; Pérez-López et al. 2008).

The increasing concerns about global declines in reptilian populations have derived investigations to evaluate the effects of contaminants on this group of species (Martínez-López et al. 2017). Additionally, their high trophic-level position of carnivorous and omnivorous species (such as *Mauremys caspica*) and their long life spans make them of particular risk to environmental contaminants. In turtles, metals have been shown to correlate with health biomarkers (Day et al. 2010; Day et al. 2007) and hatching success (Perrault et al. 2011). Pb, for instance, influences survival and righting (ability to turn over from their back) of hatchling slider turtles (Burger et al. 1998). Cd can disrupt reproduction in turtles by decreasing germ cell numbers and increasing oocyte apoptosis (Kitana and Callard 2008). However, few studies have investigated the population-concentration impact of environmental contamination in turtles. Albers et al. (1986a) observed a lower abundance of common snapping turtles (*Chelydra serpentina*) at sites with high concentrations of metals (Hg, Cd, Cr, Cu, Ni, Pb, and Zn) compared to sites with lower concentrations, but a similar effect was not detected in a more recent study (Yu et al. 2013). Inorganic elements, defined as those not present among the 10 most frequent elements in the earth's crust (Alloway 1995), have been measured in the seven marine turtle species from several populations, but only in five freshwater turtle species worldwide.

Caspian pond turtle (*M. caspica*) is a freshwater turtle from the family *Geoemydidae* (Guillon et al. 2012), from the Eastern Mediterranean region, located in the Northwest Saudi Arabia, Iraq, Bahrain, Turkey, Caucasus, and Tbilisi to the northern, central, and southwestern parts of Iran (Vamberger et al. 2013). This species is widely dispersed in different provinces of Iran, such as Mazandaran, Golestan, Guilan, Ardabil, Azerbaijan, Kurdistan, Fars, and Khuzestan (Honda et al. 2002). There are two previous works done in *M. caspica* from Iran, where Pb, Cd, and Hg were determined in the liver, muscle, and shell (Adel et al. 2015; Yadollahvand et al. 2014). Pb in the liver and muscle of these turtles was found to be extremely high compared with other turtle species, such as *Aspideretes gangeticus* previously reported by Malik et al. (2013) or *Trachemys scripta elegans* (Yu et al. 2011) from other contaminated areas. However, the use of blood to assess the level of exposure to elements is an attractive option for studies on wildlife health because blood can be non-

lethally collected and allows the determination of many environmental pollutants with a small blood sample.

The aims of this work were (1) to assess the concentrations of nine inorganic elements (Zn, Cu, Mn, Se, As, Ni, Cd, Pb, and Hg) in the blood of free-ranging male and female turtles from 8 different locations of Caspian pond turtles from the Southern basin of Caspian Sea and (2) to evaluate the influence of location, sex, size, and corporal index on metal concentrations.

## Material and methods

### Sampling

Permission to collect of Caspian pond turtle was authorized by the Iran Department of Environment (Permission Number: 1184-N/14/2; 2014; 22th September). The sample sites are located in Northern Iran within the Kopet Dagh and Alborz mountain ranges in Mazandaran and Golestan provinces (Fig. 1a). These mountains form a barrier between the South Caspian Sea and the Iranian plateau (Ghorbani 2013). A total of 40 Caspian pond turtles were collected from the southern basin of the Caspian Sea during September and October 2014. Individuals (males and females) were caught in eight different continental aquatic systems (Fig. 1b); three males and three females originated from each Noor, Babolsar, Sari, and Miankaleh locations and two males and two females originated from each Alagol, Aliabad, Gorgan, and Gonbad-e Qabus locations.

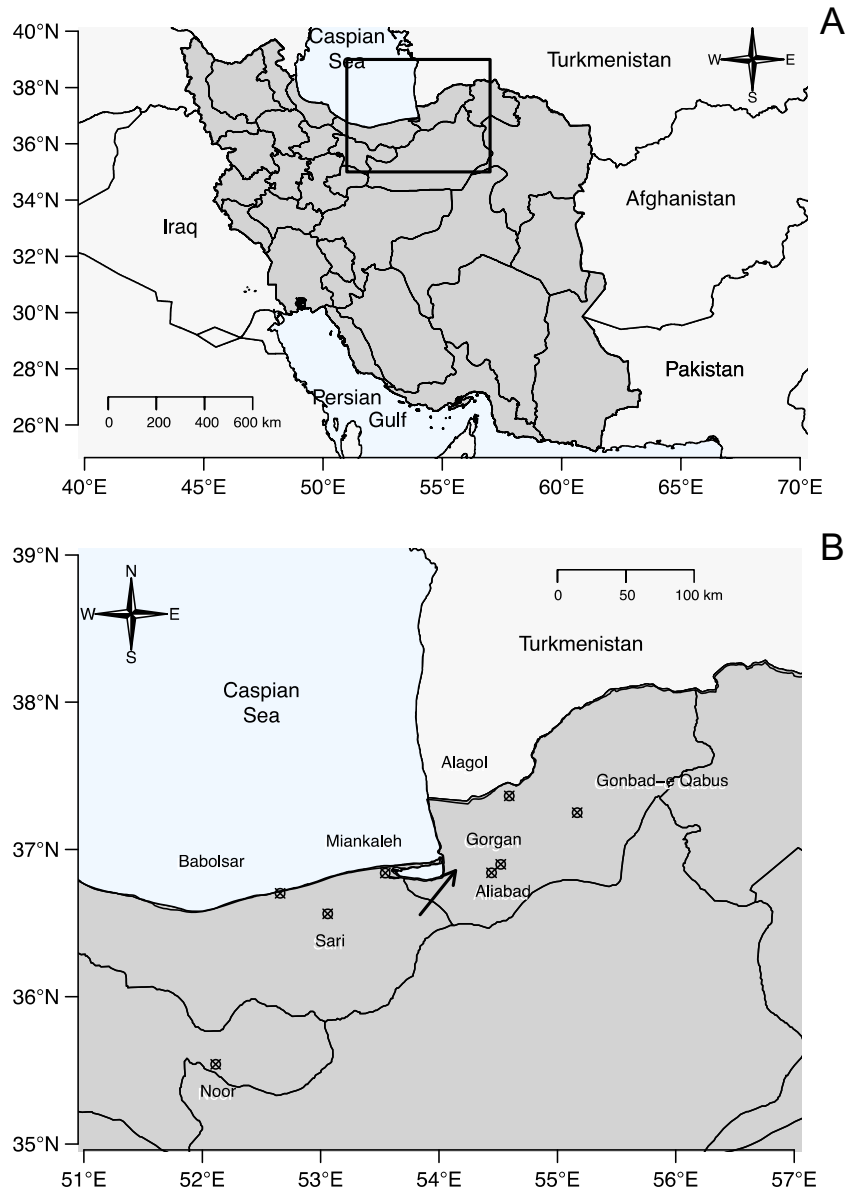
Turtles were transported alive to the central laboratory of the Caspian Sea Ecology Research Centre, and their carapace length (cm) and total mass (g) were measured. A 3-ml blood sample was then collected from the dorsal coccygeal veins and transferred into tubes containing heparin according to the protocol described in Ley-Quiñónez et al. (2011). At the end of the study, turtles were set free. Samples were kept at 4 °C and sent immediately to be analyzed.

For each individual, sex, carapace length, and mass were recorded. Carapace length is used as age indicator in turtles (Suzuki et al. 2012) or age at which individual has reached maturity (Tucek et al. 2014). Mass relates to both the age and health of individuals. To eliminate the strong relationship between length and mass, a condition index (CI) was estimated.

### Condition index analysis

**From mass and length to CI** The model linking mass and length is an exponential model with two fitted parameters,  $a$  and  $b$ :  $\text{mass} = a \times \text{length}^b$ . Values  $a$  and  $b$  were estimated using a generalized linear model (GLM). Likelihood (L) was estimated with a Gaussian distribution of mass and an identity link. The CI for each individual was the residual of the fitted

**Fig. 1** Map of Iran with outlined provinces. **a** Iran is shown in dark gray and the sampled locations included within the rectangle. **b** The detailed map of the northern region shows the eight sampled localities with a symbols



relationship between mass and length (Stevenson and Woods 2006).

Differences between males and females for the relationship between mass and length were tested by the fit of males (m), females (f), and then both sexes together (mf) along with the estimation for each model using the Akaike information criteria (AIC). The AIC (Akaike 1974) for each model ( $AIC_m$ ,  $AIC_f$ , and  $AIC_{mf}$ , respectively) was calculated as  $AIC = -2 \ln L + 2p$ , where  $p$  is the number of parameters in the model and  $L$  the likelihood of the data for the model. The AIC is a measure of the quality of the fit that penalizes the number of parameters. It reduced the risk of over-parameterization. The Akaike weight (Burnham and Anderson 2002) between  $AIC_m + AIC_f$  and  $AIC_{mf}$  estimated the probability of a single model for males and females being sufficient to model the data.

**Metal analysis**

Trace element concentrations in blood samples were determined using the procedure described previously by Ley-Quiñónez et al. (2013). Briefly, all laboratory materials were acid and deionized water washed to prevent samples contamination. Approximately 0.5 g of the homogenized wet sample was added to 5 ml of concentrated (65%) ultra-pure  $HNO_3$  (Merck, Germany),  $HCl$ , and  $H_2O_2$  (proportion of 2:2:1). Each sample was subjected to acid digestion in a microwave system for 40 min. A repetition of two blood samples with added standard multielement SIGMA 6000 (Perkin-Elmer) (0.06  $\mu L$ ) and another with deionized water were used to evaluate the efficiency of the method and observe whether the analysis matrix could generate interference. The digested material including residues obtained

from the blood digestions, blanks, and standard was dissolved in 25 mL of deionized water. Calibration curves were made for the spectrophotometer using SIGMA 3000 (Perkin-Elmer). The detection and reading limits for each metal were achieved using the wavelength (nm) recommended by the distributor. The limit of detection (LOD) was 0.01 µg/g for Pb, Cd, As, and Ni; 0.001 µg/g for Hg; and 0.04 µg/g for Zn, Cu, Mn, and Se. To determine the percentage of recovery and evaporation during the digestion process, replicates were used in the reference material (RM) TORT-2. Recovery of RM (TORT-2) and standard repetition were added with SIGMA 3000, and the percentage of recovery was between 85 and 97%. Concentrations of trace elements are expressed as micrograms per gram of blood on wet weight basis.

### Exploration of the data

**Univariate exploration** The distribution of each metal concentration in the blood of individuals was tested for normality using histogram visual inspection and the Shapiro test for normality.

**Multivariate exploration** Permutational MANOVA (also known as PERMANOVA) is a method used for partitioning sums of squares using semimetric and metric distance matrices (Anderson 2001). The distance matrix using Euclidean distance for all metal concentrations between individuals was regressed against sex, location, length, and CI. The advantage of this method is that it allows all metal concentrations to be tested in a single step. If a significant effect is detected, univariate analyses must be performed to determine which factors are involved. PERMANOVA has the advantage over other multivariate analysis in producing a test with a significance level. Before this analysis, the concentration of each metal  $C_i$  was standardized (named  $SC_i$ ) from 0 to 1 (see Warton et al. 2012 for justification).

**Metal concentration spatial pattern** The Mantel test was used to test for a possible relationship between metal concentration and the great-circle distances (geographic distance) between individual locations. The Harversine formula was used to produce the geographic distance matrix, and Euclidean distance with standardized metal concentrations was used for the metal concentration matrix. Distances were computed with all elements in a single metric or on an element-by-element basis.

### Statistical analysis

The same analysis was done for each element: A GLM with Gaussian distribution and identity link was fitted against sex, sites, length, and CI. All first-order interactions were initially included:

$$C \sim \text{sex} + \text{site} + \text{length} + \text{CI} + \text{sex} * \text{site} + \text{sex} * \text{length} \\ + \text{sex} * \text{CI} + \text{site} * \text{length} + \text{site} * \text{CI} + \text{length} * \text{CI}$$

Model simplification was based on the AIC. At each step, the AIC was calculated with one factor being removed, and the model was retained when the removed factor led to a better AIC. A factor could not be removed if it was used in an interaction. All statistical analyses were performed with R 3.3.0 and the MASS, visreg, HelpersMG, mapdata, fields, ppcor, vegan, fossil, and FactoMine R packages.

## Results

### Biometry of animals

Males presented a length mean ( $\pm$  SD) of  $11.78 \pm 1.78$  cm, mass mean of  $279.6 \pm 100.6$  g, and a condition index mean of  $-0.07 \pm 0.23$ . Female's biometrics in the same order were  $10.87 \pm 1.52$  cm,  $272.5 \pm 63.5$  g, and  $0.07 \pm 0.14$  CI. For both sex biometrics were  $11.36 \pm 1.71$  cm,  $273.9 \pm 83.0$  g, and  $0.0 \pm 0.20$  CI. As expected, length and mass were strongly correlated ( $r = 0.74$ ,  $p < 0.000$ ), and thus both factors cannot be simultaneously analyzed. The fit of  $\text{mass} = a \times \text{length}^b$  for males, females, and both sexes together led to AIC being 1.99,  $-18.26$ , and  $-11.11$  respectively; a single model for both sexes is thus sufficient (Akaike weight,  $p = 0.07$ ).

### Exploration of metal concentration

A visual inspection of histograms and Shapiro tests do not show a strong deviation from the Gaussian distribution for the concentration of the nine measured elements. Two elements (Ni and Hg) show a slightly significant deviation from the Gaussian distribution ( $p$  values between 0.02 and 0.05), but further analyses are fairly robust against such a difference (Atkinson 1984).

PERMANOVA was used to test for sex, sites, length, and CI effects on the standardized distribution of the nine elements in a single analysis. A very significant effect of sex ( $p = 0.002$ ) and sites ( $p = 0.008$ ) was observed. However, a Mantel test on the Euclidean distances matrix according to geographic distances between sites does not reveal any spatial structuration by distance when all elements are tested simultaneously ( $p = 0.10$ ) or separately (all  $p > 0.14$ ).

### Analysis of metal concentration

Each element was tested against sex, sites, length, and CI, including all first-order interactions. The model and graphical

representation of the significant effects and interactions is shown in Figs. 2, 3, and 4.

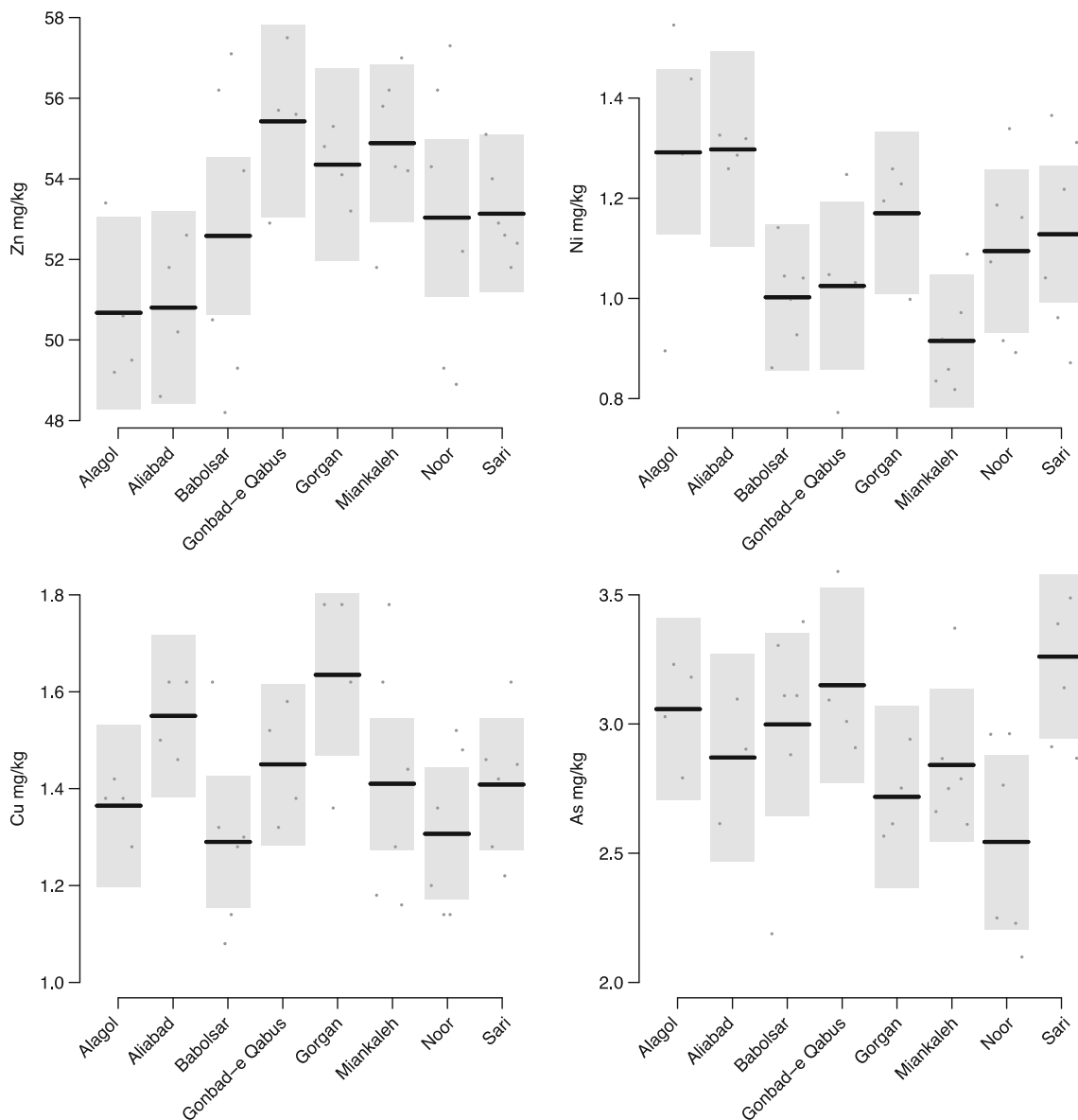
Figure 2 shows the element concentrations by site for Zn, Cu, Ni, and As. Partial correlations for the concentrations of these four elements at the eight sites show only a significant relationship for Zn and Ni ( $p = 0.001$ ) with a negative correlation ( $r = -0.84$ ). Figure 3 shows the two significant effects found for Ni and Cd with the turtle's length and describes sex effect observed for Cd ( $M > F$ ) and Se ( $M < F$ ).

An interaction between sex and length was observed for As (Fig. 4), with the concentration being higher for larger males and lower for larger females. Pb and Hg showed an effect for sex, length, and CI but in the opposite direction: Pb concentration was higher for smaller males with a high CI and for

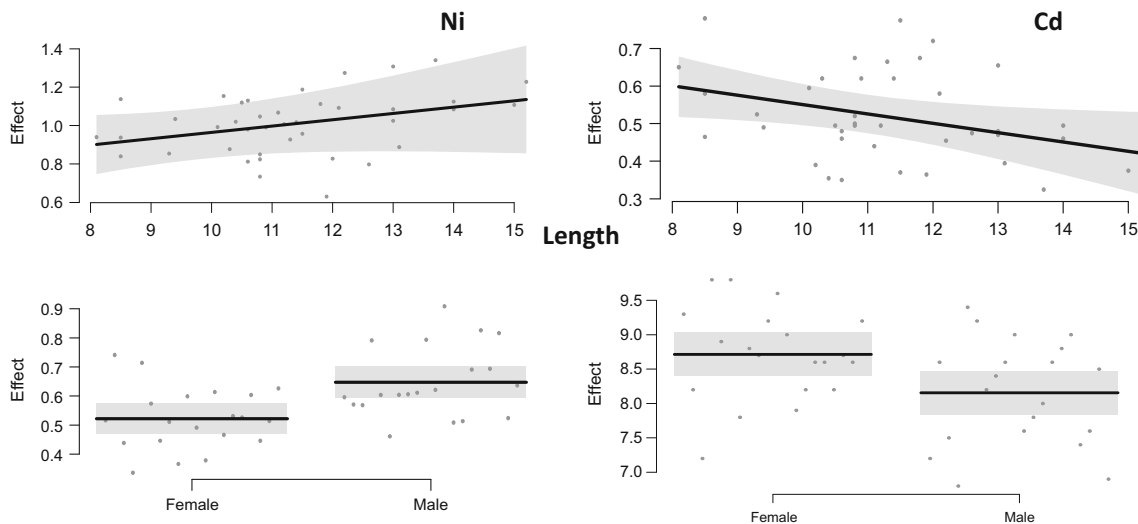
larger females with a low CI (Fig. 4), whereas Hg concentration was higher for smaller males with a low CI and for larger females with a high CI (Fig. 4). Only Mn showed no significant effect for any of the tested factors.

### Discussion

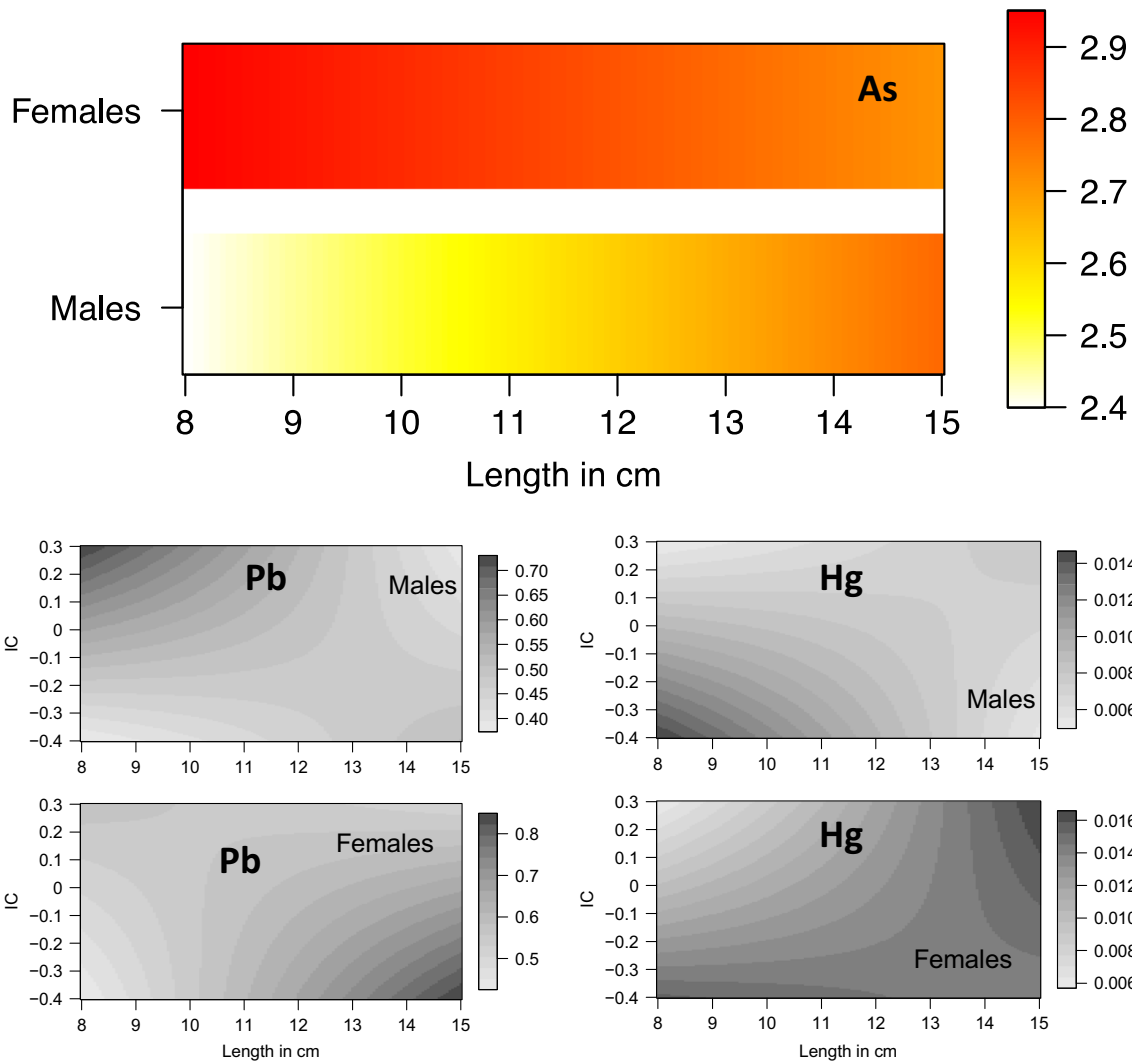
There is an important lack of information about the concentration of trace elements in freshwater turtles and even less on their effects. A very high concentration of inorganic elements was reported in two previous studies in *M. caspica* from Iran (Adel et al. 2015; Yadollahvand et al. 2014). However, these results were difficult to interpret because of the lack of comparison with other sites. We therefore sampled the same



**Fig. 2** Significant effects of Zn, Cu, Ni, and As concentrations (y-axis) in the different sampling sites



**Fig. 3** Positive significant effect for Ni and negative for Cd concentrations (above) and turtle length and significant effect for Cd and Se concentrations and sex (below). The y-axis shows the scale effect



**Fig. 4** Significant effects of As concentrations against male and females length (at the right, being darker color is equal to higher As concentration) against male and female length. And for Pb and Hg concentrations against turtle IC (y-axis) and length (x-axis)

**Table 1** Concentration of trace elements in the blood of Caspian pond turtles in wet and dry mass

	Metal	Zn	Cu	Cd	Ni	Mn	Pb	As	Se	Hg
Wet mass										
Sex										
Female	Mean ± SD	53.01 ± 2.60	1.39 ± 0.16	0.52 ± 0.11	1.10 ± 0.21	2.06 ± 0.24	0.55 ± 0.08	2.95 ± 0.38	8.66 ± 0.80	0.011 ± 0.003
	Median	52.60	1.38	0.49	1.10	2.04	0.56	2.90	8.80	0.012
	2.5–97.5%	49.28–57.31	1.14–1.63	0.37–0.72	0.84–1.40	1.77–2.36	0.45–0.68	2.19–3.50	7.77–9.80	0.006–0.016
	Min–max	48.90–57.30	1.14–1.80	0.35–0.78	0.79–1.42	1.60–2.42	0.40–0.69	2.10–3.50	6.90–9.80	0.006–0.018
Male	Mean ± SD	59.27 ± 2.72	1.44 ± 0.20	0.63 ± 0.13	1.11 ± 0.22	1.99 ± 0.21	0.49 ± 0.07	2.65 ± 0.36	8.22 ± 0.73	0.009 ± 0.002
	Median	53.40	1.42	0.62	1.02	1.96	0.49	2.68	8.40	0.008
	2.5–97.5%	48.58–56.20	1.13–1.78	0.47–0.81	0.84–1.43	1.67–2.31	0.39–0.62	2.18–3.40	6.89–9.21	0.005–0.014
	Min–max	48.20–56.20	1.07–1.78	0.42–0.90	0.89–1.63	1.62–2.35	0.38–0.62	1.90–3.40	6.80–9.40	0.004–0.014
Dry mass										
Female	Mean ± SD	15.9 ± 0.78	0.42 ± 0.04	0.15 ± 0.03	0.33 ± 0.06	0.61 ± 0.07	0.16 ± 0.02	0.88 ± 0.11	2.59 ± 0.24	0.0033 ± 0.0009
	Median	15.78	0.414	0.14	0.33	0.61	0.16	0.87	2.64	0.0036
	2.5–97.5%	14.78–17.19	0.34–0.49	0.11–0.21	0.25–0.42	0.53–0.70	0.13–0.20	0.65–1.05	2.33–2.94	0.0018–0.0048
	Min–max	14.67–17.19	0.34–0.54	0.10–0.23	0.23–0.42	0.48–0.72	0.12–0.20	0.63–1.05	2.07–2.94	0.0018–0.0054
Male	Mean ± SD	17.78 ± 0.81	0.43 ± 0.06	0.18 ± 0.03	0.33 ± 0.06	0.59 ± 0.06	0.14 ± 0.02	0.79 ± 0.10	2.46 ± 0.21	0.0027 ± 0.0006
	Median	16.02	0.426	0.18	0.30	0.58	0.14	0.80	2.52	0.0024
	2.5–97.5%	14.57–16.86	0.33 ± 0.53	0.14–0.24	0.25–0.42	0.50–0.69	0.11–0.18	0.65–1.02	2.06–2.76	0.0015–0.0042
	Min–max	14.46–16.86	0.32 ± 0.53	0.120.27	0.260.48	0.48–0.70	0.11–0.18	0.57–1.02	2.04–2.82	0.0012–0.0042

Values are expressed in µg/g. Concentrations on dry mass were estimated taking into account the humidity in this study of 70%. The 2.5 and 97.5% quantiles are based on the Hyndman and Fan (1996) method. *N* = 20 for both males and females

species in eight locations around the sampled site used in those previous studies. For ethical reasons, we sampled trace elements just in the blood, which is a physiologic medium of exchange and transport of substances among the tissues of organisms; its flux in the different organs and tissues varies significantly (Chang 1996). When trace metals are ingested in organisms, they are rapidly distributed from the gastrointestinal tract by the sanguineous torrent, and the final concentration in organs and tissues depends on the relative velocities of the distribution processes and elimination (Chang 1996). Day et al. (2005) proposed the use of blood for monitoring trace metal exposure in turtles because it is possible to predict the load in internal tissues; moreover, sanguineous material is considered a good parameter for diagnostic indexes of recent exposure in organisms.

**Elements concentrations**

We were able to detect the nine trace elements in all samples (Table 1), in males and females. Element concentrations (µg/g wm) in decrease order were as follows: Zn (53.01 ± 2.66) > Se (8.66 ± 0.80) > As (2.95 ± 0.38) > Mn (2.06 ± 0.24) > Cu (1.39 ± 0.16) > Ni (1.10 ± 0.21) > Pb (0.55 ± 0.08) > Cd (0.52 ± 0.11) > Hg (0.011 ± 0.003). This elements order is

relatively normal since the first five elements are considered as *essential elements* in trace concentration; all these elements are essential and involved in growth, proper functioning of the immune system, and reproduction of all vertebrates and have been described as elements that are relatively easily absorbed and physiologically important in tissues of reptiles (Grillitsch and Schiesari 2010). In the other hand, Ni, Pb, Cd, and Hg are considered as *non-essential* and potential toxic elements in very low concentrations in different species (ATSDR 2003, 2007a, b, 2012, 2015; Cortés-Gómez et al. 2014; da Silva et al. 2016).

The comparison of trace elements is complicated. Firstly, there are very few works about these pollutants in freshwater turtles. Secondly, when different species are used, their ecology and physiology could have an effect, because trophic levels or ingested food differ or the excretion pattern varies (Meyers-Schöne 1989). Even within a species, the situation can be complicated, because CI, age, and sex can differ depending on the location. Furthermore, the number of eggs or nesting events per female could vary for different populations, thus changing the excretion concentration in eggs (Guirlet et al. 2010). Even if we found more than 30 studies about these elements in turtles (Table 2), it was very difficult to compare our results with all of them because the different

tissues analyzed (most of them in organs than in the blood). Moreover, no two studies use the same tissues, species, trace elements, and protocols. Meta-analysis is impossible in such a situation, or very strong hypotheses must be done to convert one value (species, tissue, and dry or wet weight) to another metric. Even more, the values below the detection limit are sometimes not included in the final estimates, thus creating a bias in the reported metal concentration. Often, the standard error is reported without indicating the sample size, and thus the standard deviation cannot be estimated.

In Table 3, all the works found regarding inorganic elements in the blood of freshwater turtles and selected works in marine turtles to be compared with the present study in wet and dry mass are shown. Regarding just freshwater turtles and tortoises, we found six representative works: two from Rio Negro in Brazil (Cd, Pb, As, Se, and Hg (Burger et al. 2010) and Hg (Schneider et al. 2011)), three from the USA (Hg (Bergeron et al. 2007); Zn, Cu, As, Se, and Pb (Allender et al. 2015), both in four different species; and Pb (Bishop et al. 2010)), and one work from Spain in freshwater turtles (Zn, Cu, Cd, Pb, and Hg (Martínez-López et al. 2017)).

Concentrations of Cd, Pb, As, and Se reported by Burger et al. (2010); Zn, Cu, Cd, Pb, and Hg concentrations reported by Martínez-López et al. (2017); and Zn, Cu, Se, As, and Pb concentrations reported by Allender et al. (2015) were low and even very low compared with those found in this study (Table 3). The locations selected for this study also present a high agriculture activity. Moreover, Martínez-López et al. (2017) studied mostly adults *Mauremys leprosa* turtles (more than 140 mm), species closely related to *M. caspica*, and turtles in this study were mostly juveniles (108–117 mm (Tok 1999)). Then, these higher concentrations in our turtles cannot be explained only for bioaccumulation, but for a higher exposition to most of these elements. Pesticides and fertilizer are commonly and widely used in the areas where turtles were collected; this could be an important source of metal pollution in aquatic environments in these locations. Additionally, Mazandaran wood, paper industry, antibiotic companies, cattle and poultry industries, MDF factories, and fisheries farms, among others, are developed in these locations. All these factors combined may be the reason of these high concentrations found in the blood of these turtles compared with other studies (pers. comm.).

Regarding potentially toxic elements, Pb is considered one of the most toxic heavy metals, causing alterations in vascular, nervous, renal, immune, reproductive, and hematological systems, as well as behavioral abnormalities (Bishop et al. 2010; Martínez-López et al. 2017). Martínez-López et al. (2010) suggested blood Pb levels above 15 µg/dL (0.15 µg/g ww) as inductors of sublethal effects on tortoises (*Testudo graeca*). Turtles from this study had 0.54 µg/g ww, high compared with those concentrations and compared with other freshwater turtles but similar to those found in some sea turtles (Table 3).

Similar results were found for Cd, and concentrations on this study were high (0.58 µg/g ww) compared with other freshwater species, but similar or lower compared with sea turtles. This could mean that marine turtles are highly exposed to those metals, but Caspian turtles from the north of Iran are highly exposed than other freshwater species. Since turtles are from different areas, the concentration of these elements seems to be quite widespread in the north of Iran.

On the other hand, Hg in this study presented low concentrations compared with other species (Table 3). This element was especially high for the four species studied for Bergeron et al. (2007), presenting one of the highest concentrations found in turtles (ranking from 0.080 to 0.960 against 0.01 µg/g ww found in the present work). Bergeron et al. (2007) mention that these high concentrations are due to the very high persistence of this metal in the environment (more than 50 years since the use of mercuric sulfate was banned in the river where the turtles were found). He also found that Hg concentrations vary among the sites (more or less contaminated) and among the species (due to their feeding ecology). We can then suppose that the environment of the turtles from this study is not very highly contaminated with Hg.

We also compared our results with studies in marine turtles (Table 3); even if their environment is very different, they occupy a similar ecological niche depending on their feeding habits than freshwater turtles. Zn was the element with the higher concentration, and one of the highest compared with other species, but it was also highly variable among species and populations. Cu, Ni, Mn, and As tend to be in low concentrations in most turtle species, and concentrations of these elements found in this study are medium compared with other populations (Table 3). Although, Se was among the highest concentrations among other species (8.44 µg/g ww). Se is a necessary detoxifying element that can become toxic in high concentrations; in some species, Se (along with Hg) has been related with reduced reproductive success (Perrault et al. 2013). Higher Se concentrations have been reported in carnivorous than in herbivorous turtles (Aguirre et al. 1994). Caspian pond turtle are carnivorous as juveniles becoming more omnivorous as adults (Fritz and Havaš 2007); this could explain, at least in part, these high Se concentrations found in this study.

On the other hand, we have a previous work (Adel et al. 2015) in the same species but in different locations. This work showed a very high Pb concentration in the three tissues they analyzed (liver, muscle, and shell) with higher concentration in the liver (35.46 µg/g dm) but also high in the other organs (muscle, 23.06; shell, 29.5 µg/g dm). This means that this population is exposed to Pb in a chronic but also a constant form (in the liver, Pb accumulates in short–medium term and calcified tissue in a chronic exposure where it can accumulate for many years; ATSDR 2007a). Thus, and the concentration we found in the blood in this study, we hypothesized that the season could be another important variable to consider when



**Table 2** Synthesis of the turtle species analyzed for trace elements, the tissues analyzed, and the reported units for metal concentrations. (dm) and (wm) are dry and wet mass, respectively

Trace elements	Tissues	Units	Reference	Notes
<i>Chelydra serpentina</i>				
Cd, Cr, Cu, Hg, Ni, Pb, Zn	Liver, kidney	ppm wm	Albers et al. (1986b)	1
Hg	Muscle	µg/g wm	Meyers-Schöne (1989)	1
<i>Clemmys marmorata</i>				
Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, Zn	Eggs	µg/g dm	Henny et al. (2003)	1
<i>Trachemys scripta</i>				
Hg	Muscle	µg/g wm	Meyers-Schöne (1989)	1
Al, Cd, Cr, Cu, Mn, Ni, Pb, Sn, V, Zn	Egg content, egg shell	µg/g	Tryfonas et al. (2006)	1, 2
Cd, Cr, Cu, Hg, Pb	Liver, muscle, kidney	mg/kg dm	Yu et al. (2011)	3
<i>Aspideretes gangeticus</i>				
Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn	Liver, heart, kidney, carapace	µg/g	Malik et al. (2013)	1, 2
<i>Mauremys caspica</i>				
Cd, Cu, Pb, Zn	Liver, heart, shell (?), muscle	µg/g dm	Yadollahvand et al. (2014)	1, 4
As, Cd, Cu, Hg, Mn, Ni, Pb, Se, Zn	Blood	µg/g dm	This study	8
<i>Eretmochelys imbricata</i>				
As, Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Sr, V, Zn	Liver, kidney, bone	µg/g dm	de Macêdo et al. (2015)	1
Cd, Cu, Fe, Mn, Ni, Pb, Zn	Liver, kidney, adipose, muscle	µg/g dm	Gardner et al. (2006)	1, 5
<i>Natator depressus</i>				
As, Bi, Cd, Cr, Cu, Ge, Hg, In, Li, Ni, Pb, Sb, Sc, Se, Sn, Tl, Y, Zn	Blood	µg/L	Ikonomopoulou et al. (2011)	1
“	Eggs	mg/kg wm	“	1
<i>Chelonia mydas</i>				
As, Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Sr, V, Zn	Liver, kidney, bone	µg/g dm	de Macêdo et al. (2015)	1
Ag, Al, As, Ba, Cd, Co, Cr, Cs, Cu, Fe, Hg, Mo, Mn, Ni, Pb, Rb, Sb, Se, Sr, Tl, V, Zn	Egg yolk, albumen, shell	ng/g wm	Lam et al. (2006)	6
As, Cd, Co, Cu, Hg, Pb, Se, Zn	Blood, liver, muscle, kidney	µg/g wm	van de Merwe et al. (2010)	1
Cd, Cu, Mn, Ni, Se, Zn	Blood	µg/g wm	Ley-Quinonez et al. (2013)	1
Cd, Cu, Fe, Mn, Ni, Pb, Zn	Liver, kidney, adipose, muscle	µg/g dm	Gardner et al. (2006)	1, 5
<i>Caretta caretta</i>				
Cd, Cu, Hg, Mn, Ni, Pb, Se, Zn	Blood	µg/g wm	Ley-Quinonez et al. (2011)	6
Cd, Cr, Hg, Pb	Liver, lung, kidney, muscle	µg/g wm and dm	Storelli et al. (1998)	1
Cd, Cu, Pb, Zn	Liver, kidney, muscle, brain	µg/g dm	Garcia-Fernandez et al. (2009)	7
Cd, Cu, Fe, Mn, Ni, Pb, Zn	Liver, kidney, adipose, muscle	µg/g dm	Gardner et al. (2006)	1, 5
<i>Lepidochelys kempii</i>				
Cd, Cu, Se, Zn	Plasma, liver, kidney, fat, keratin, brain	ng/g wm	Innis et al. (2008)	1
Ag, Cd, Cr, Cu, Hg, Pb, Zn	Blood, carapace (keratin?)	ppb dm	Wang (2005)	1
<i>Lepidochelys olivacea</i>				
Pb	Blood, shell, albumen, egg yolk	µg/g dm	Paez-Osuna et al. (2010a)	1
Cd, Cu, Ni, Zn	Blood, shell, albumen, egg yolk	µg/g dm	Paez-Osuna et al. (2010b)	1
Hg	Blood, shell, albumen, egg yolk	µg/g dm	Paez-Osuna et al. (2011)	1
Zn, Se, Mn, Ni, As, Cd, Cu	Blood	µg/g wm	Zavala-Norzagaray et al. (2014)	1
Cd, Cu, Fe, Mn, Ni, Pb, Zn	Liver, kidney, adipose, muscle	µg/g dm	Gardner et al. (2006)	1, 5
<i>Dermochelys coriacea</i>				
Cd, Cu, Hg, Pb, Se, Zn	Blood, eggs	µg/g dm	Guirlet et al. (2008)	8
Hg, Cd, Cu, Ni, Pb, Se, As, Zn	Liver, muscle, blubber	mg/kg dm	Davenport et al. (1990)	9
Cd, Hg, Pb	Whole blood	ppm	Harris et al. (2011)	2, 10

1 = no indication of how the values were included below the detection limit. 2 = no indication if the concentration was indicated for dry or wet mass. 3 = values below the detection limit were replaced with values derived from an unpublished model. 4 = The publication indicated shell, but it was probably carapace. Shell is used for calcareous egg membrane. 5 = only the geometric mean was reported. 6 = values below the detection limits were excluded to estimate the mean. 7 = samples with non-detected values were assigned half the detection limit. 8 = no values below the detection limits. 9 = the measurement error was shown as ± without any indication of what it represents. 10 = median value was reported for n = 3 without any indication of how it was estimated

you make a biomonitoring program. Especially for blood sampling as the rain or temperatures could influence on the bio-availability of the metals for the turtles (for the reason that food could be more or less contaminated).

**Site differences**

The places where turtles were collected (Fig. 1) are characterized by the dominance of platform-type sediments, with

**Table 3** Concentration of trace elements in different studies in the blood in  $\mu\text{g/g}$  (mean  $\pm$  SD)

Species	REF	Zn	Cu	Cd	Ni	Mn	Pb	As	Se	Hg
Wet mass										
<i>Mauremys caspica</i>	1	56.14 $\pm$ 2.66	1.42 $\pm$ 0.18	0.58 $\pm$ 0.12	1.11 $\pm$ 0.22	2.03 $\pm$ 0.23	0.52 $\pm$ 0.08	2.8 $\pm$ 0.37	8.44 $\pm$ 0.77	0.01 $\pm$ 0.002
<i>Mauremys leprosa</i>		3.9 $\pm$ 1.7	0.34 $\pm$ 0.15	0.002 $\pm$ 0.003	NA	NA	0.09 $\pm$ 0.06	NA	NA	0.008 $\pm$ 0.005
<i>Podocnemis erythrocephala</i>	3	NA	NA	0.001	NA	NA	0.025	0.003	0.164	0.002
<i>Podocnemis sextuberculata</i>	3	NA	NA	0.001	NA	NA	0.009	0.003	0.276	0.007
<i>Peltocephalus dumerilianus</i>	3	NA	NA	0.0001	NA	NA	0.013	0.001	0.538	0.028
<i>Chelus fimbriatus</i>	3	NA	NA	0.0001	NA	NA	0.016	0.004	0.463	0.158
<i>Pseudemys rubriventris</i>	4	NA	NA	NA	NA	NA	NA	NA	NA	0.080
<i>Chrysemys picta</i>	4	NA	NA	NA	NA	NA	NA	NA	NA	0.450
<i>Sternotherus odoratus</i>	4	NA	NA	NA	NA	NA	NA	NA	NA	0.920
<i>Cheylra serpentina</i>	4	NA	NA	NA	NA	NA	NA	NA	NA	0.960
<i>Chelus fimbriatus</i>	5	NA	NA	NA	NA	NA	NA	NA	NA	0.158
<i>Peltocephalus dumerilianus</i>	5	NA	NA	NA	NA	NA	NA	NA	NA	0.028
<i>Podocnemis sextuberculata</i>	5	NA	NA	NA	NA	NA	NA	NA	NA	0.007
<i>Podocnemis erythrocephala</i>	5	NA	NA	NA	NA	NA	NA	NA	NA	0.002
<i>Terrapene carolina carolina</i>	6	2.86	0.66	NA	NA	NA	0.02	0.02	0.29	NA
<i>Lepidochelys olivacea</i>	7	10.4 $\pm$ 4.1	0.60 $\pm$ 0.11	0.17 $\pm$ 0.10	0.04 $\pm$ 0.02	0.59 $\pm$ 0.09	0.02 $\pm$ 0.01	1.19 $\pm$ 0.70	5.7 $\pm$ 2.5	NA
<i>Chelonia mydas</i>	8	63.5 $\pm$ 17.1	1.71 $\pm$ 0.73	0.99 $\pm$ 0.35	1.03 $\pm$ 1.01	1.22 $\pm$ 0.99	ND	ND	7.6 $\pm$ 3.2	ND
<i>Caretta caretta</i> <sup>a</sup>	9	44.8 $\pm$ 17.5	2.8 $\pm$ 0.64	1.8 $\pm$ 0.63	1.5 $\pm$ 2.4	0.61 $\pm$ 0.55	ND	4.1 $\pm$ 2.5	6.1 $\pm$ 3.5	ND
<i>Chelonia myda</i> <sup>b</sup>	10	7.5 $\pm$ 2.5	0.97 $\pm$ 0.36	NA	NA	NA	ND	NA	2.3 $\pm$ 2.4	NA
Dry mass										
<i>Mauremys caspica</i>	1	16.84 $\pm$ 0.79	0.42 $\pm$ 0.05	0.17 $\pm$ 0.03	0.33 $\pm$ 0.06	0.60 $\pm$ 0.06	0.15 $\pm$ 0.02	0.84 $\pm$ 0.11	2.53 $\pm$ 0.22	0.003 $\pm$ 0.0007
<i>Dermochelys coriacea</i>	11	11.10 $\pm$ 0.28	1.34 $\pm$ 0.28	0.08 $\pm$ 0.03	NA	NA	0.18 $\pm$ 0.05	NA	9.98 $\pm$ 0.05	0.011 $\pm$ 0.003
<i>Lepidochelys olivacea</i>	12	58.4 $\pm$ 4.7	2.28 $\pm$ 0.40	0.45 $\pm$ 0.20	2.8 $\pm$ 1.3	NA	0.95 $\pm$ 0.18	NA	NA	0.0006 $\pm$ 0.0005
<i>Caretta caretta</i>	13	1.41 $\pm$ 0.57	NA	0.02 $\pm$ 0.04	NA	NA	0.06 $\pm$ 0.06	1.40 $\pm$ 1.86	0.56 $\pm$ 0.26	0.004 $\pm$ 0.002

Published SE were converted into SD by  $\text{SD} = \text{SE} \cdot 19^{0.5}$ . References are 1: this study; 2: Martínez-López et al., 2017; 3: Burger et al., 2010; 4: Bergeron et al., 2007; 5: Schneider et al., 2011; 6: Allender et al., 2015; 7: Cortés-Gómez et al., 2014; 8: Ley-Quinonez et al., 2013; 9: Ley-Quinonez et al., 2011; 10: van de Merwe et al., 2010; 11: Guirlet et al., 2008; 12: Paez-Osuna et al., 2010a, Paez-Osuna et al., 2010b, Paez-Osuna et al., 2011; 13

NA no analyzed, ND no detected

<sup>a</sup>. The values were incorrectly multiplied by 100 in Table 1 of the original publication

<sup>b</sup>. Original data are in  $\mu\text{g/L} \pm \text{SE}$ . The conversion into  $\mu\text{g/g dm} \pm \text{SE}$  is based on Zavala-Norzagaray et al. (2014) without any precision about the methodology. Published SE were converted into SD by  $\text{SD} = \text{SE} \cdot 16^{0.5}$

volcanic rocks rich in trace elements and particularly potentially toxic metals (Maanijou et al. 2013; Moazzen and Oberhänsli 2008; Movahedi et al. 2005). Agriculture is also observed at all sampling sites with the use of pesticides like organophosphates. In addition, the Sari, Noor, and Gorgan regions have high industrial activities. Fossil fuel diffusion from the Shahid Salimi (Neka) power plant, oil storage reservoirs, merchant vessels in Amir Abad port, and agricultural activities could increase the presence of inorganic elements in Sari region.

For Zn, Cu, Ni, and As, we found significant differences among the sampling sites (Fig. 2). Two major sources of metals are natural (e.g., volcanoes, erosion, and natural cycling of elements) and anthropogenic (e.g., mining, industry, agriculture, wastes, nuclear plants or smelters, and fossil fuels). When these significant effects for sites were observed, individuals from the Gorgan and Gonbad-e Qabus sites were always among the most contaminated (especially with Cu and As). Gorgan is an industrial site close to the Gharehsu River sampled by Yadollahvand et al. (2014) and Gonbad-e Qabus City located at 70 km on the same river basin. Here, Aliabad, at 3 km from Gorgan City, showed high concentrations of Ni and Cu. This observation highlights the heterogeneity of the environment for trace elements. This heterogeneity could derive not only from the geology of drainage basin but also from human activities.

### Size, sex, and CI effects

Factors influencing the concentration of trace elements differ according to the element, thus reflecting the complexity of the metabolism for these elements. Ni had a significant positive relationship with the length of individuals (Fig. 3). Ni is an essential micronutrient with increased bone strength in birds as one proposed positive effect (Wilson et al. 2001). Some authors have showed that both low and high concentrations of Ni can affect bone development; it has been also noticed that the absorption of Ni increases during reproductive processes (Berglund et al. 2011; Wilson et al. 2001). We also observed a concentration of Ni in the blood within the rates compared with other turtle species (Table 3); thus, we can suggest that elder turtles absorb more Ni due to reproduction stages and/or bone physiological needs.

On the contrary, Cd had a negative significant relationship with the turtle's length (Fig. 3), meaning that older turtles have less Cd concentration in the blood. Since Cd intake in turtles is mainly through the food (Andreani et al. 2008; Gardner et al. 2006; Maffucci et al. 2005), this decrease could be due to the change of diet during these turtles life span, passing from carnivorous to omnivorous (Fritz and Havaš 2007). Compared with the other four freshwater species (Table 3), Cd concentration in this work is higher, but compared with marine turtles (Table 3), these concentrations can be

considered medium/low; this difference must be due to the difference in their ecosystem and Cd bioavailability. Cd is a very toxic element that can cause many physiological alterations; many authors also suggest that it has a very low metabolic regulation in turtles (Barbieri 2009; Camacho et al. 2013; Cortés-Gómez et al. 2014). Cd also showed a significant relationship between males and females (Fig. 2), males being more contaminated with Cd than females. This could be explained with the fact that turtles tend to transfer metals to eggs during the reproduction processes (Aguirre et al. 2006; Ehsanpour et al. 2014; Guirlet et al. 2008).

Selenium had a significant effect between males and females (Fig. 3); females showed significantly higher concentrations of this element than males. Se concentration in the blood of these turtles was relatively high compared with other species (Table 3). However, it has been reported that turtles seems to be more tolerant against high Se concentrations compared with other animal species (Hopkins et al. 2005). The full role of Se in reproduction has yet to be established; though, it also appears to be important in reproduction and oxidative stress protection (Perrault et al. 2013). With this in mind, this higher Se concentration in females could be whether females have more demand and then more absorption of this element for reproductive processes or their intake is higher than their excretion capacities than males.

Finally, we found three significant differences between sexes after the GLM-AIC selection in As, Pb, and Hg (Fig. 4). Arsenic showed significant relationships among the size and sex against this element concentration in the blood (Fig. 4). These relationships showed that smaller (younger) female turtles had higher As concentration; on the contrary in males, bigger (older) turtles had higher As concentration. This decrease in bigger females could be explained due to maternal transfer during egg formation (Martínez-López et al. 2017). On the other hand, Pb and Hg concentrations had significant relationships with sex, index condition (IC), and size. Males with higher Pb concentrations were smaller and with higher IC, and larger females with lower IC had higher Pb concentration in the blood (Fig. 4). Regarding Hg, shorter males with lower IC had higher concentration in the blood, and larger females with higher IC had higher Hg concentration (Fig. 4). Different from As, in Pb and Hg, it seems that maternal transfer to eggs is not an important detoxification pathway in these turtles. Moreover, the accumulation of these two metals seems to be related to the size in females but not in males.

All these above-observed differences among male and females can reflect the divergence in element intake, taking into account the difference in the amount of food ingested between males and females, as it has been previously observed by Lovich (1996). In this case, the relationship with CI would be also expected (Fig. 4). These relationships with CI can also reflect the effect of hematocrit (ratio between red cells and

plasma). Hematocrit can indicate a number of factors that may influence chemical concentrations in the blood, such as dehydration, malnutrition, and chronic disease (Frye 1991). Hematocrit can affect blood metal concentrations as red cells preferentially bind metals, compared to plasma (Chang 1996). The difference could also arise from the elimination of some elements in the eggs of nesting females (Guirlet et al. 2008; Paez-Osuna et al. 2010b). In this case, we should observe lower concentrations in females with a greater length (this effect was observed with As and Cd; Figs. 3 and 4). Of course, all of these factors could influence the concentration of elements and produce complex patterns (e.g., Hg and Pb).

The conclusion of Witkowski and Frazier (1982) about metal concentration in turtles was thus: “it is difficult to interpret the significance of these findings because so little is known about baseline concentrations and physiological effects of potentially toxic metals in these animals.” Indeed, this is still true after more than 30 years! These authors called for more “basic descriptive studies.” Yet we now have more than 30 descriptive studies (Table 2) from which we can conclude that we still know very little. Thus, more experimental studies with precise protocol, as being done for the impact of cadmium on reproduction (Kitana and Callard 2008), or more ecosystem studies are needed. It is also very important to standardize work protocols to be able to compare the results with others. Hence, the importance to always publish the humidity percentage found in samples, either if the results are published in wet or dry weight, could be even better if publications includes both results (e.g., Table 3).

We also observed that many researchers still consider that high dispersion in their data indicates that they have done poor laboratory work, which they try to hide using SE rather than SD. A contrario, high standard deviations indicate more interesting patterns that reveal complex individual life history. For instance, comparing two different studies often involve choosing, from among all of the combinations available, those that allow the initial hypotheses to be confirmed. Furthermore, the shape of the distribution of concentrations cannot be inferred from the mean and SD when the distribution is not symmetrical. In such a situation, the best is to report median and 2.5–97.5% centiles (see Table 1). The range of minimum and maximum values is also not informative, because these are not robust statistics for dispersion since they are very sensitive to aberrant values.

## Conclusions

This is the first work on assessing inorganic elements in Caspian pond turtles' blood. This work can be used as baseline dataset for future works in this species that has been scarcely studied. We can see that these turtles are useful as bioindicators of the environmental pollution. Most of the

studied elements were found in high concentrations compared with other locations and species. The importance of sex in the accumulation of inorganic elements in this species was also observed. For Cd, males showed higher concentrations than females; on the contrary, for Se, females showed significantly higher concentrations than males; this may be for reproductive processes. Regarding As, smaller females had higher As concentrations; on the contrary, bigger males had higher As concentrations. Males with higher Pb concentrations were smaller and with higher IC, and larger females with lower IC had higher Pb concentration in the blood. Regarding Hg, shorter males with lower IC had higher concentration in the blood, and larger females with higher IC had higher Hg concentration. All these above-observed differences among male and females can reflect the divergence in their reproductive behavior and elements intake, taking into account the difference in the amount of food ingested. Additionally, the importance of standardizing sampling and statistical protocols for biomonitoring studies is also highlighted in this study. In the future, capture-mark-recapture to estimate the survivorship of individuals and follow their load in different trace elements could allow us to decipher the impact of inorganic elements and particularly those possible toxic metals for turtles in natural conditions.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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