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Seasonal Variations of Trace Metal Concentrations in the Soft Tissue of *Lithophaga Lithophaga* Collected from the Bizerte Bay (Northern Tunisia, Mediterranean Sea)

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Abstract

The concentration of four trace metals (Zn, Cu, Pb and Cd) was determined in the soft tissue of the date mussel *Lithophaga lithophaga* that is specifically and strictly protected by international law. Samples were collected from the Bizerte bay in the northern coast of Tunisia. Comparisons were made on the basis of season and sex. Trace metal concentration decreased in the order Zn > Cu > Pb > Cd. The average concentration was 54.15 ± 23.037, 3.429 ± 1.453 and 1.809 ± 2.252 µg g^{-1} dry weight, for Zn, Cu and Pb respectively. Cd was not detected in all samples. Significant temporal variation was observed for Zn, Cu and Pb ($P < 0.05$). There is no significant difference between females and males except for Zn in spring and Pb in winter. Analyses of the condition index (CI) revealed the presence of a single reproductive cycle per year affecting metal bioaccumulation of *L. lithophaga*. The main objective of metal analysis was to provide useful information about trace metal pollution in the Tunisian date mussel *L. lithophaga* which will enable to evaluate the potential effect of the Bizerte Marina Project on this species.

Keywords: *Lithophaga lithophaga*; Bizerte Bay; Trace metal; Condition Index; PCA

Introduction

Contamination with trace metals still remains a big problem to the coastal environment and has been intensively studied because of their toxicity, their cumulative effect and their risk for humans and marine ecosystems [1-3]. However, trace metals are natural constituents of the marine environment and some are even biologically essential [4]. They represent an important component of the contaminant load in the water column and sediments [5]. To assess trace metals contamination in marine environment, different types of organisms may be used, such as seaweeds and benthic bivalves [1]. It is well known that bivalves, especially mussels, are good filter feeders and are used in bio-monitoring programs of metal contamination, because of their ability to accumulate trace metals and various kinds of other pollutants [6,7]. Therefore, they are appropriate to act as a biological indicator of pollution [8-10]. One of the most successful examples of applied bio-monitoring used to identify contaminated areas is the Mussel Watch Program [11]. This program is based on chemical analysis of heavy metals in bivalve's soft tissues and mainly in *Mytilus* species [12]. In addition to their ability to concentrate high levels of metals, bioaccumulation of contaminants from the surrounding media or food, can lead to latent toxic responses in the affected organisms, higher in the food chain and can represent a threat to human health when used as a food [13-15]. Therefore, the determination of metal concentrations in organisms should be part of any assessment and monitoring program in the coastal zone [16]. Lagoon complex of Bizerte constitutes the only Tunisian site of mussel and oyster farming. It is located in a very important economic area in Northern Tunisia and it's continually submitted to various anthropogenic activities (urban and agriculture activities, cement works, metallurgical industry, naval and commercial ports, boatyard…). The date mussel *L. lithophaga* is abundant in this area. It's a rock-boring bivalve that can live for fifty years or more [17], by its organoleptic quality; it is greatly appreciated as seafood and frequently used for human consumption. It is often collected and sold even though it is forbidden by law. The species is considered among the threatened species in the Mediterranean Sea and has been protected by the Bern and the Barcelona Conventions and the Convention on International Trade of Endangered Species, (CITES). Studies on the Tunisian date mussel are limited to shell disturbances, biology and health status of this bivalve [18-23]. The primary aim of this study was to obtain quantitative information on the concentration of trace metals (Zn, Cu, Pb and Cd) in the Tunisian date mussel *L. lithophaga* collected from the Bizerte Bay (Northern Tunisia). Additionally, the following points were considered: i) seasonal variation of metal concentration in the *L. lithophaga* body, ii) variation of metal content in relation with sex, iii) the relationship between trace metal concentration, condition index and abiotic factors. Such data are important to determine if the chosen pollutants have potential effect on bivalve health and provide baseline information to evaluate temporal trends in metal accumulation in this species before and after installation of the Bizerte Marina project in the sampling area.

Materials and Methods

Monthly sampling of calcareous rocks at approximately three meters depth was carried between September 2002 and October 2003 by diving in the infra-littoral zone of the Bizerte Bay (Northern Tunisia) (Figure 1). Surface seawater temperature, salinity, and oxygen were measured monthly in the study site at the sampling time, while the pH value was measured only once per season. In the laboratory, rocks were broken by a hammer and bivalves were extracted. The size of specimens varied between 40 and 60 mm. The shell was opened and the soft parts of the organism were carefully removed. Sexing was based on gonad

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color and microscopic observations of gonad smears. The reproductive cycle was investigated in both sexes using the bio-physiological index: the condition index (CI) according to Walne and Mann [24] and calculated as CI = (soft tissues dry weight/shell dry weight) x 100. Soft tissues of five males and five females were used, seasonally, to analyze metals after preserving lyophilized tissue. The soft tissue was finely grinding with a T 18 basic Ultra-Turrax® disperser at 6,000 rpm. After being freeze-dried, soft tissues were maintained at -20°C in the dark until chemical analysis. Soft tissue samples were digested on a hot plate with concentrated nitric acid in Erlenmeyer flasks of 25 ml until total digestion of the tissues was observed. During digestion, flasks were covered with glass balls. Digestion was considered finished when there were no organic remains in suspension in the acid. Erlenmeyer flasks (without the glass balls) were kept in a hot plate at 90°C inside a cabinet until all there maining acid evaporated and watching them so that they did not get burnt. The inorganic residue was re-suspended with 10 ml of HNO_3 0.1 M for 48 h and then centrifuged at 4000 rotations for 5 min. Supernatants were moved to clean glass tubes and stored at 4°C till they were ready for chemical analysis. The measurements (two replicates) were performed using the Perkin Elmer Pinaacle 900T (USA) atomic absorption spectrophotometer. The method was approved using CRM artificial saline water NIST1643e [20]. The detection limits were (μg ml^{-1}): 0.8 (Cd), 5 (Cu), 15 (Pb) and 1.5 (Zn).

Analysis of variance (ANOVA) was performed using the software Statistica 8.0. After testing ANOVA assumptions, statistical significance was evaluated through one way ANOVA. Whenever ANOVA detected significant differences, post-hoc comparisons were made using the Tukey's HSD test. In all statistical analyses, significance level was considered at P<0.05.

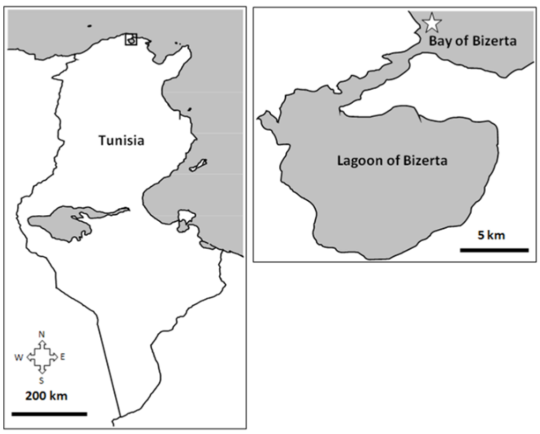

Figure 1: Map of Tunisia showing the sampling site (marked by a star) of *Lithophaga lithophaga* (Bizerta Bay).

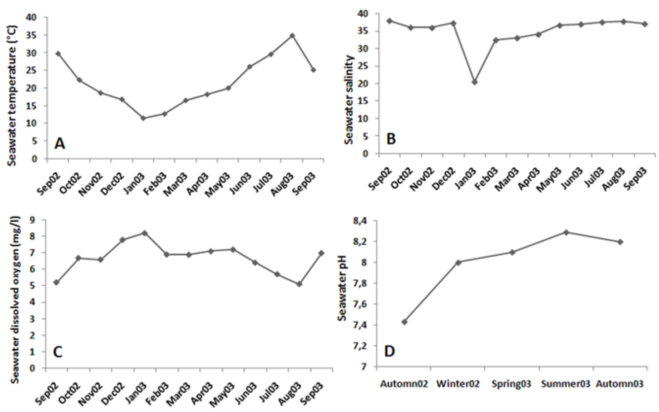

Figure 2: Monthly variation in surface seawater temperature (A), salinity (B) and dissolved oxygen (C) and season variation of seawater pH (D) recorded in Bizerta Bay during the study period (September 2002 – September 2003).

Then, comparison of differences in metal burden between season and between genders was determined by the Pearson's principal component analysis (PCA). Pearson correlation analysis was performed between the metal body concentrations and between them and season and environmental parameters (T°, S°°, pH and O_2). Statistical analysis was conducted using the software Xlstat® version 7.5.2 for Windows

Results

Environmental parameters

The seawater temperature ranged between 11.5°C in January and 35.0°C in August. The annual average was 21.8°C (Figure 2A). Salinity was high for most of the year, reaching 37.9 in September, and low in rainy season with 20.5 in January (Figure 2B). The annual average was 34.82. The average content of dissolved oxygen was 6.64 mg/l (Figure 2C). The extreme values were recorded in August (5.1 mg/l) and January (8.25 mg/l). The pH measured only once per season, was rather constant, ranging between 7.43 in autumn and 8.29 in summer (Figure 2D).

Variation of the condition index (CI)

Analysis of the condition index in both sexes showed the presence of a single reproductive cycle per year in *L. lithophaga.* In females, CI increased from 17.28 ± 4.64 to 30.56 ± 12.79 from September 2002 to April 2003; in males, an increase from 19.7 ± 6.57 to 28. 12 ± 10.5 was recorded from November 2002 to May 2003 (Figure 3). During this period, *L. lithophaga* increased in weight which could be partly or totally related to the gonad development. In the following months, the CI values showed only moderate variations (Figure 3). A decrease in CI was recorded from August 2003 to September 2003 for females (27.31 ± 7.54–20.59 ± 12.09), and from August 2003 to October 2003 for males (27.5 ± 10.56–20.5 ± 7.86). Probably, gametes were released during this period. Pairwise comparisons did not show significant differences between sexes (Tukey's HSD test; P < 0.05).

Concentration of Zn, Cu, Pb and Cd in the soft tissue

Confounded sexes

Inter-season concentrations of Zn, Cu, Pb and Cd in the soft tissues of *L. lithophaga* are presented in Table 1. Results revealed that metal concentrations varied notably depending in the season for Zn, Cu and Pb (Tukey's HSD test; P < 0.05). Cd concentrations was below detection limit (<DL) in all samples considered. Metal concentration decreased in the following order Zn > Cu > Pb > Cd. The highest concentration of trace metals in the soft tissues of *L. lithophaga* for Zn and Cu was registered in Winter (71.4 ± 8.591 and 5.095 ± 2.27 μg g^{-1}dw respectively), while for Pb the peak was recorded in Summer (4.395 ± 3.094 μg g^{-1}dw). The lowest concentrations are found during the fall season for the three metals (Figure 4A).

Separated sexes

Analysis of variance for both males and females showed no significant differences for Zn and Cu concentrations (Tukey's HSD test; p > 0.05). Mean concentrations are 49.733 ± 18.768 and 58.568 ± 27.318 μg Zn g^{-1} dw for females and males respectively (Table 1). Zn body burdens in female tissues ranged from 20.54 in autumn to 72.4 μg g^{-1} dw in winter (Figure 5), however for males, Zn concentrations varied between 88.5 μg g^{-1}dw in summer and 17.44 μg g^{-1}dw in autumn (Figure 4B).

The concentrations of copper were 3.012 ± 0.612 and 3.845 ± 1.937 µg g-1dw in female and male tissues respectively (Table 1). The highest concentrations were recorded during winter for both sexes (3.98 and 8.5 µgg^{-1}dw in females and males respectively) (Figure 4C), while the lowest concentrations were registered in the fall season for males (2.36 µg g^{-1}dw) and spring for females (2.34 µg g^{-1}dw) (Figure 4C).

Results showed significant differences in Pb accumulation between females and males (Tukey's HSD test, P <0.05). The mean values were 0.53 ± 0.929 and 3.087 ± 2.503 µg g^{-1}dw in female and male tissues respectively (Table 1). Males accumulate Pb more than females. Maximum concentrations of Pb were recorded in summer for both sexes (females: 2.6 µg g^{-1}dw and males: 8.08 µg g^{-1}dw) (Figure 4D). The lowest values were recorded in winter for females (0.08µg g^{-1}dw) and autumn for males (0.72µg g^{-1}dw) (Figure 4D). Cd concentrations was undetectable (<DL) in both females and males.

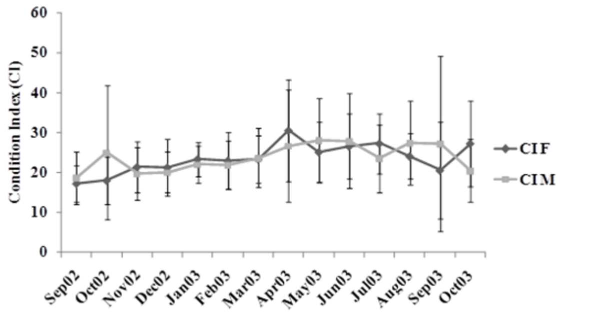

Figure 3: Monthly variation in the bio-physiological index CI during the study period. Error bars represent standard deviation. (n = 50 – 100).

	Zn	Cu	Pb	Cd
Females	49.733	3.013	0.53	ND
± SD	± 18.768	± 0.612	± 0.929	ND
Males	58.568	3.845	3.088	ND
± SD	± 27.318	±1.937	± 2.503	ND
Mixed	54.15	3.429	1.809	ND
± SD	± 23.037	±1.453	± 2.252	ND

Table 1: Zn, Cu, Pb and Cd concentrations (µg g-1 dw) with standard deviation (± SD) in the soft body of *L. lithophaga* collected from Bizerte Bay.

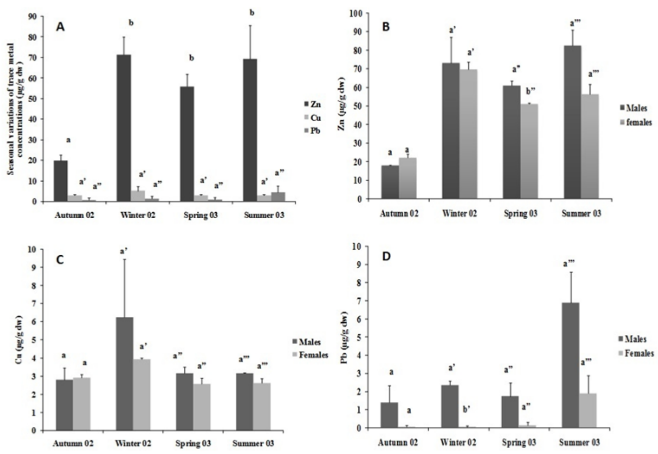

Figure 4: Trace metal concentrations sexes confounded (A). Seasonal variations of trace metal concentrations in the soft tissues of both males and females of *L. lithophaga*: Zn (B), Cu (C), Pb (D).

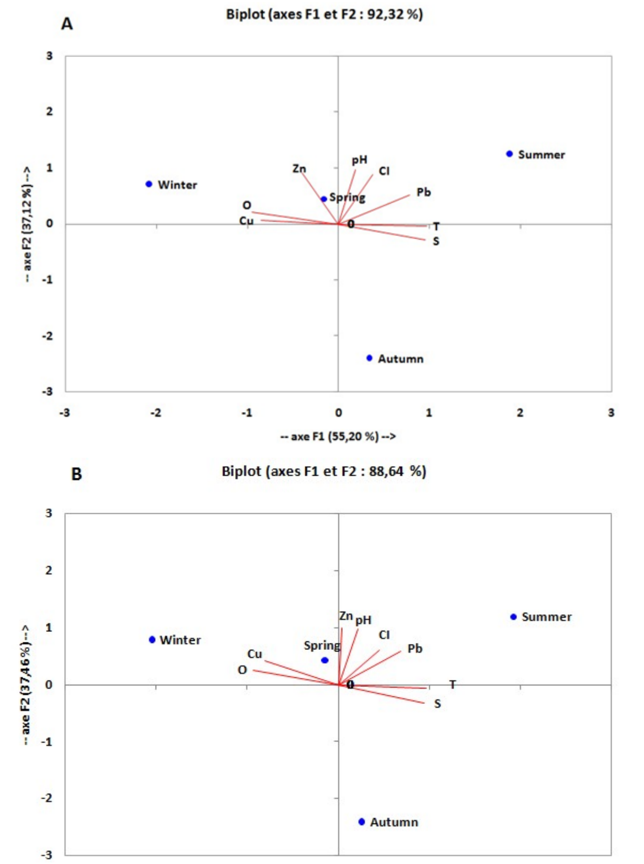

Figure 5: Principal component analysis using variables (Zn, Cu, Pb, Cd, CI, T°C, S, pH O_2 and seasons) in females (A) and males (B) of *L. lithophaga*.

PCA

The Principal Component Analysis resulted in a biplot graph that explained 92.32% of the variability for females (Figure 5A). The F1 axis (55.2% of the variability) was defined (significant squared cosines) by water temperature (0.944), water salinity (0,910), O_2 (0.901), winter (0.896), summer (0.754), Cu (0.715) and Pb body concentration (0.610). The F2 axis (37.12% of the variability) was defined mainly by the fall season (0.967), pH (0.956), Zn (0.822), IC (0.786) and spring (Figure 5A).

PCA explained 88.64% of the variability for males (Figure 5B). The F1 axis (51.18% of the variability) was defined by temperature (0.942), salinity (0.890), O_2 (0.888), winter (0.854), summer (0.752), and lesser extent by Pb and Cu concentration body (Figure 5B).

The F2 axis (37.46% of the variability) was defined by Zn (0.984), fall season (0.977), pH (0.941) and the condition index (0.378).

Discussion

Marine bivalves have the ability to accumulate various types of pollutants. Therefore, they have been widely used in bio-monitoring programs of marine ecosystems such as the "Mussel Watch Program" [25-27]. Among these species, mytilids are considered as useful bio-indicators of metal contamination for their ability to accumulate metals at high concentrations [28,29]. The present study aims to add further insight about the bioaccumulation ability of four metals (Zn, Cu, Pb and Cd) in the date mussel *L. lithophaga* collected from the Bizerte Bay. Our results showed that *L. lithophaga* concentrate three metals among the four analyzed metals. Cd was undetectable for all the samples examined. Zinc was the most abundant element. Metal concentration values decreased in the following order Zn > Cu > Pb > Cd which was in agreement with the results of Deudero et al. [30] and Ozsuer and Sunlu [31]. Comparison of Zn and Cu tissue concentrations showed no significant difference between sexes, while Pb is more accumulated by males.

Zn and Cu are two essential elements at low doses. They are regulated by aquatic organisms due to their implication in many biological processes [32-34]. Zinc plays a variety of role in biochemical processes like regulatory, structural and enzymatic composition [14,35,36]. This metal is usually associated with suspended particles which are used by mussels in their filter-feeding diet [35]. According to Páez-Osuna et al. [37], suspended particles constitute a major source for several metals. In addition, for the essential metals such as Zinc and Copper, there are two major routes by which suspension-feeding bivalves can uptake metals: dissolved uptake or food ingestion [38], which can explain the high content of Zn in date mussel tissues.

Cadmium and lead are non-essential trace metals that have a tendency to be accumulated in aquatic organisms. These elements are less concentrated by *L. lithophaga*. Both lead and cadmium are toxic trace metals, even in very low concentrations. Pb is toxic, bioaccumulative trace metal with no known biological function [39,40], its absorption may constitute a serious risk to public health (EC, 2001). Cd absorption constitutes a risk to humans because it is may be accumulated in the human body and can compete with Zn and displace this essential cellular metal from sulfhydryl groups of enzymes, altering their functions and inducing toxic effects [36].

Comparison of metal tissue levels showed seasonal fluctuations. Zn and Cu showed similar trend in whole soft tissue. The highest concentrations occurred in rainy season while the lowest concentrations were recorded in the fall season. For Pb, maximum concentration was registered in summer, however lower tissue level was observed in autumn. A number of explanations for seasonal variations of trace metals have been proposed. According to Paez-Osuna et al. [37] and Ferreira et al. [14], seasonal fluctuations of trace metals can be associated to the food supply and to changes in run-off of metal particulate to the sea due to high precipitation. The seasonal variations are also associated with local phytoplankton productivity. Thus, an increase in phytoplankton efficiency implies an increase in bivalve nutritional status which leads to increase metal concentration in observed organisms [14].

Boening [13] considered that metal uptake and subsequent bioavailability, are highly dependent on biological and geochemical factors. Accumulation can be a function of age, size, sex, feeding activity and reproductive state. Geochemical factors that influence bioaccumulation are organic carbon, water hardness, temperature, pH, dissolved oxygen, sediment grain size and hydrologic features.

Tissue levels of several trace metals depend on various environmental parameters such as salinity and water temperature [41]. Ali & Taylor [42] noted that the uptake of Cd by *M. edulis* was at its maximum at low salinity and high temperature, while at high salinity, temperature did not significantly affect the uptake rate. Salinity had also a significant effect on Zn uptake. Zn uptake is higher at low salinity. Moreover, Jiann and Presley [43] and Szefer et al. [41], demonstrated that low salinity, promote accumulation of Zn, Cu and Cd. In the present study, maximum levels of Zn and Cu coincides with minimum salinity. According Jiann and Presley [43], salinity plays important role in the Cu and Zn bioaccumulation. For Pb, the highest concentrations were registered in summer when temperature is at its maximum. These results are similar to those found by Frias-Espericurta et al. [44] and Ferreira et al. [14] which recorded maximum Pb levels in summer and minimum Cu and Zn levels in winter. The concentration of trace metals accumulated by the date mussel *L. lithophaga* seems to be depending on seasonal factor, temperature and salinity variation.

Variation of metal concentrations can also be explained by the life cycle of the bivalve which influences food intake, storage and excretion [4,29]. Páez-Osuna et al. [37] reported high concentrations of Cd, Cu and Zn in *Crassostrea iridescens* at the end of the breeding season and during the sexual rest period. Similarly, Ali et al. [45] recorded significant levels of metals in *Crassostrea belcheri, Crassostrea glomerata* and *Crassostrea rivularis* (Pakistan) during winter. According to the same authors, seasonal variation of metals is due to phytoplankton bloom, reproductive cycle and fluctuations of the body weight. In addition, the penetration of gonadic tissues into the digestive gland during gametogenesis may biologically dilute metal concentrations in mussels and therefore decreases in body metal concentration [45]. For some metals, animal growth acts as a dilution factor for levels of incorporated contaminant and weight loss as a concentration factor [46-49,32,50-52].

Metal levels depend on environmental factors and physiology but also on discharge coming from human and industrial activities. According DGEQV [53] and Yoshida [54], metallic contamination of Bizerte lagoon is mainly of industrial origin (Figure 6). Trace metals are originated from the activities of nine industrial plants classified as major sources of pollution in the Bizerte lagoon [53] (Table 2).

The respective estimated rate for Cd, Pb and Zn from industrial sources, are respectively 69.62%, 68.89% and 63.58% [53].

Principal component analysis varied with abiotic variables (temperature, salinity, pH and dissolved oxygen), biotic (CI), seasonal factor and metals (Pb, Cu and Zn). Distribution of the different parameters analyzed depended in both season and sexes.

Results for abiotic variables indicated that dissolved oxygen levels are negatively correlated with water temperature and salinity. The increase of temperature and salinity, decreases dissolved oxygen solubility [55], which can explain low levels of dissolved oxygen during summer. Furthermore, due to the water agitation under the effect of wind and waves, water became oxygenated and cold (low values of temperature). In addition, turbidity may play an important role in dissolved oxygen content variation. Suspended particulate can also increase oxygen demand, which is required for the organic matter degradation in the water column [56].

Trace metal distributions in *L. lithophaga* body, showed seasonal fluctuations. A maximum is registered in summer and winter, while the minimum is registered in autumn and spring. Our results are in agreement with those of Maanan [3], Merzouki et al. [33] and Rouhane-Hacene [57], who noted seasons influence on metal accumulation in *M. galloprovincialis* from Moroccan and Algerian coasts. Moreover, Claisse [58] considered that metal levels in aquatic organisms are depending on seasons. The main factors influencing the metallic elements rate in bivalves are environmental factors (pH, salinity, temperature) that affect the shape of the metal (dissolved and / or particulate) in the water column [44].

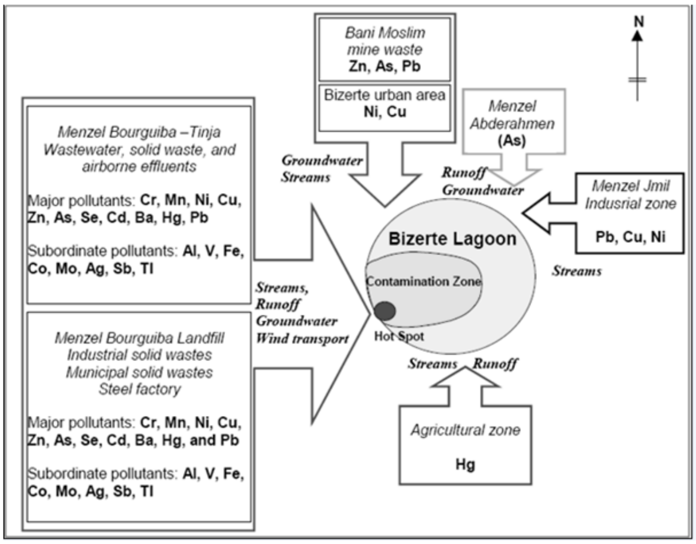

Figure 6: Main areas of Bizerte lagoon contamination by trace metals (by Yoshida 2012).

Industrial plants	Principal discharges
Menzel Jemil: MAFILTA	Suspended matter, chemical pollution, contaminated sludge and trace metals
Bizerte: TunisAcier, STABYL, Mondher El Ghoul, cemant factory of Bizerte	Suspended matter, trace metals, nutritional elements
El Azib: FUBA	Trace metals and contaminated sludge
Menzel Bourguiba: EL Fouledh, SOCOMENA and STIP	Suspended matter, trace metals, hydrocarbons and contaminated sludge

Table 2: Principal discharges of industrial plants near the Bizerte lagoon.

Zn and Cu showed negative correlation with temperature and salinity as they are positively correlated with O_2, indicating the presence of an influence of abiotic variables on Zn and Cu bioavailability.

Positive correlation is recorded between Zn and pH (r = 0.958) in males. According to Basraoui et al. [59], pH has a major influence on the speciation of metals in water.

Thus, Metal availability increased when the environment is alkaline, while it is reduced at low pH values [60]. In general, physical, chemical and biological mechanisms interact and transform contaminants into forms more or less available to organisms in the environment [61]. This showed that metal accumulation in marine organisms is largely influenced by speciation and therefore, chemical form in which it exists [62,63].

For Pb, maximum concentrations were recorded during summer for both sexes. This can be explained by the increasing of the urban pollution (wastewater discharge) and the traffic release of atmospheric Pb which is higher during summer. In addition, it is known that lead origin is steel discharges [58]. According to Yoshida [54], the major sources of lead in the lagoon complex is the metallurgic industry (steel complex El Fouledh) Menzel Bourguiba, urban waste of Bizerte city, mining inputs of Bani Moslim and Menzel Jemil (Figure 6).

PCA results showed that Pb contents and condition index of the date mussel evolve in the same way for both sexes during summer (Figures 5A and 5B). This can be due to the reproductive cycle of the bivalve. During gonad development, animal is submitted to various physiological conditions which are certainly linked to abiotic parameters and can affect its bioaccumulation [64]. Bivalve condition index (CI) is widely employed in environmental monitoring programs as it integrates physiological responses to stress with changes in somatic growth. Although, according to Mercado-Silva [65], the use of (CI) as an environmental monitoring tool is based on the effect that environmental conditions and different pollutants have on the oyster growth.

PCA results exhibit that seasonal fluctuation of contaminants in *L. lithophaga* could be the result of both biological factors (sexual cycle), environmental conditions (temperature, salinity, dissolved oxygen, pH) and pollutant bioavailability.

Comparison of levels recorded in *L. lithophaga* body with those found in literature (Table 3), showed that date mussel from Bizerte Bay accumulates metals much less than date mussel from Izmir Bay [31] and the Balearic Islands [30]. Similarly, the ability to concentrate metals is variable between *L. lithophaga* and *M. galloprovincialis.* Mussels from Bizerte lagoon accumulates Zinc much than *L. lithophaga* (Table 3). According to Claisse [58] and Ferreira et al. [14], the rate of metal accumulation varies from one species to another in significant proportions. This is due to the physiology of each species [62]. According to the same authors, oysters accumulate Cd three times more than mussels and accumulate 10-25 folds Cu and Zn, in comparison of the same species.

At present, date mussels consumption is increasing in some countries like Croatia, Morroco and Turkey, despite of its consideration as threatened species. In Turkey, particular concern was given to the high metal accumulation that exceeded the EC guideline values for several elements [31]. For the case of Tunisia, although no big consumption is found, the comparison with the permissible limits of EC [66,67] and FAO [68] for Zn (40 µg/g), Cu (30 µg/g), Pb (1.5 µg/g) and Cd (1 µg/g), did not show any health risk.

Finally, the lagoon complex of Bizerte constitutes the only site where *L. lithophaga* occurs. However, due to the demand increase, date mussels are submitted to highly destructive and uncontrolled harvesting activity which needs application of more legislative measurements to the protection of this species. Thus, total prohibition

References	Location	Species	Zn	Cd	Pb	Cu
Present study	Bizerte Bay	*L. lithophaga*	54.15	ND	1.81	3.43
Ozsuer & Sunlu (2013)	Izmir Bay	*L. lithophaga*	293.16	2.23	9.48	64.65
Deudero et al. (2007)	Spain (Menorca)	*L. lithophaga*	212.2	2.21	9.2	14.9
Deudero et al. (2007)	Spain (Mallorca)	*L. lithophaga*	341.9	1.73	7.9	18.4
Marasabessy (2002) in Ozsuer & Sunlu (2013)	Indonesia	*L. obesa*	539.76	ND	35.98	8.99
Mzoughi & Chouba (2012)	Bizerte Lagoon	*M. galloprovincialis*	299	1.79	0.65	3.32
Maanan (2008)	Morocco	*M. galloprovincialis*	292	7.2	9.6	26.8

Table 3: Comparison of total trace metal concentrations with those in other studies as mean μg g^{-1}dw.

of both date mussel fishing and marketing should be applied; especially that restoration of communities destroyed during the extraction of *L. lithophaga* is very slow and often impossible due to the fact that this species is rather long-lived with a growth rate which is one of the slowest among bivalves.

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