

**ASSESSMENT OF CD, CU, ZN AND NI BIOACCUMULATION AND METALLOTHIONEIN CONCENTRATIONS IN *Mytilus galloprovincialis* IN SIDI IFNI COAST (SOUTH ATLANTIC OF MOROCCO)**

**Mohamad Abbassi<sup>1,2\*</sup>, Leila Aboudloul<sup>1</sup>, Imane Charioui<sup>1</sup>, Maryem Nadir<sup>1</sup>, Mustapha Agnaou<sup>1</sup>, Omar Achahour<sup>3</sup>, Samir Bari<sup>1</sup>, Jamel Jebali<sup>4</sup>, Bah Hamoudi<sup>1,5</sup>, Mohamed Bani<sup>4</sup> and Abderrazak Kaaya<sup>1</sup>**

<sup>1</sup>BioEnvironment, Health and Bioresources Team, Faculty of Sciences, University Ibn Zohr, Agadir, Morocco

<sup>2</sup>Suprior Institute of Maritime Fishing, Agadir, Morocco

<sup>3</sup>Laboratory of Materials and Renewable Energy, Faculty of Sciences, University Ibn Zohr Agadir, Morocco

<sup>4</sup>Laboratory of Biochemistry and Environmental Toxicology, Higher Institute of Agronomy, ISA, 4042, ChottMariem, Sousse, Tunisia

<sup>5</sup>Marine and Coastal Environment Study Laboratory, Mauritanian Institute for Oceanographic Research and Fisheries, Nouadibou, Mauritania

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## ABSTRACT

This paper is concerned with the assessment of Cd, Cu, Zn and Ni bioaccumulation and metallothionein concentrations in *Mytilus galloprovincialis* in four sites representative of Sidi Ifni's coastline (South Atlantic of Morocco): Mirleft site (S1), Cheikh Sidi Ali Ifni (S2), Station of wastewater eputation (STP - S3) and Tazrout site (S4).

Compared to the control site (S1), the highest concentrations of are observed in mussels originating at the S2 (in summer, autumn and winter) and in the S3 in the spring. Levels of metallothionein showed like seasonal variations with significant high values at S2 site.

**Keywords:** Bioaccumulation - Biomarker - Metal - Mettallothionein - Morocco – *Mytilus galloprovincialis* – Sidi Ifni.

## 1. INTRODUCTION

In recent years, the coasts of the city of Sidi Ifni (Southern Atlantic of Morocco) have known a significant socio-economic development (Fisheries and touristic activities). Although, part of this ecosystem received several types of pollutants which are discharged in the seawater without any treatment. Our previous studies, in which physico-chemical parameters of the coastal waters of the city ( $T^{\circ}C$ ,  $pH$ , *Dissolved O<sub>2</sub>*, *Salinity*, *Conductivity*, *Turbidity*, *Salinity*, *Chlorides*, *Sulfates*, *Phosphorus*, *Ammonia Nitrogen*, *Nitrate and Nitrite*) (Abbassi et al., 2017) and some biomarkers (*Acetylcholinesterase*, *Catalase*, *Glutathione S-transferase activities* and *Malondialdehyde level*) (Abbassi et al., 2015) revealed a real disturbance of the physical and chemical characteristics of seawater and of marine organisms (*Mytilus galloprovincialis*) living at the sites receiving domestic and industrial wastewater of this ecosystem

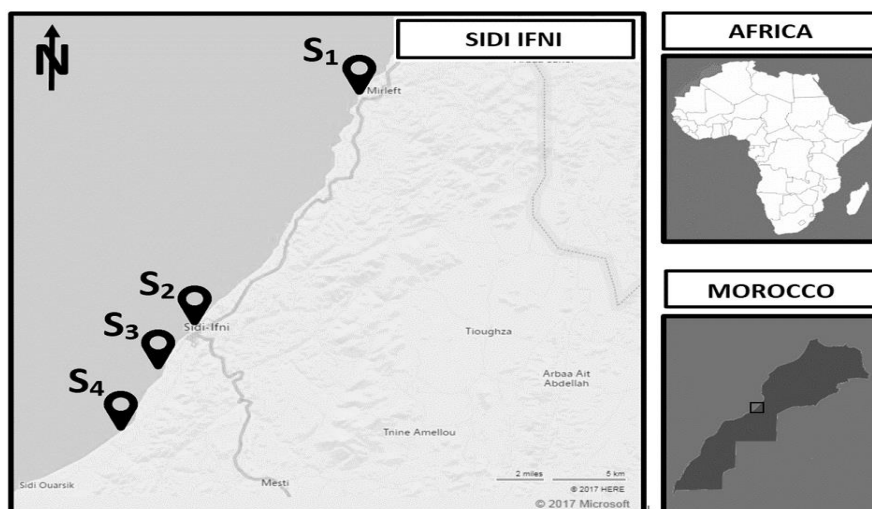
In the present work about metallic pollution, Cd, Cu, Zn and Ni bioaccumulation and metallothionein (MT) concentrations were evaluated in *Mytilus galloprovincialis*. These environmental pollutants are well known to their toxicity, persistence in the environment, and bioaccumulative nature (Hazrat et al., 2019).

Indeed, data about heavy metals in marine organisms from Moroccan coasts were rare and limited to the north Atlantic coasts in Morocco (El Hraiki et al., 1992; Cheggour et al., 1999; Kaimoussi 2001; Kaaya, 2002; Eddaoudi et al., 2014). The accumulation and transformation capabilities of pollutants make these sentinel organisms suitable for marine pollution monitoring (Lower and Kendall, 1990).

Otherwise, this work constituted also a contribution in the assessment of the heavy metal level in the African South-West Moroccan coasts, which were characterized by an important upwelling responsible for the wealth of marine resources.

## 2. MATERIALS AND METHODS

### *Sampling sites (Figure 1)*



**Figure 1:** Map of sampling sites in coastline of Sidi Ifni: Mirleft site (MIR - S1), Cheikh Sidi Ali site (CHIKH - S2), Sidi Ifni wastewater treatment plant (STP - S3) and Tazrout site (TZR - S4)

Sampling was conducted in four sites representative of Sidi Ifni's coastline : *i*) Mirleft site (S1) (Coordinated : 29°58'41.68''N, 10°07'40.82''W) , which is considered as reference site, located at 30 Km to the north of Sidi Ifni, far from any source of pollution, *ii*) Cheikh Sidi Ali Ifni (S2) (Coordinated : 29°38'70.55''N, 10°17'29.54''W) located at the entrance of Sidi Ifni at the rejection of the Oued Sidi Ifni and receives untreated wastewaters of the city, *iii*) Station of wastewater eparation (S3) (Coordinated : 29°34'20.80''N, 10°20'12.13''W) which receives treated wastewaters of a part of Sidi Ifni city and *iv*) Tazrout site (S4) (Coordinated: 29-17'37.39"N, 10-14'11.27"W), located at the southern end of the city, is 15 km from the crude sewage discharge point and 10 km from S2. It is far from any source of pollution and considered a clean site.

### ***Biological material***

Assessment of metal bioaccumulation and MT level were conducted in Mediterranean mussel (*Mytilus galloprovincialis*). 30 individuals animals (40 to 60 mm) were collected monthly in

each sites and washed in fresh seawater in order to free any impurities then they are placed in cooler containing seawater from sampling site. In the laboratory, animals were kept in confinement for 48 hours to remove the contents of the digestive tract and frozen at  $-30^{\circ}\text{C}$  until their use for the mineralization step or for MT extraction.

### ***Metals bioaccumulation analysis***

For each sample, lyophilized mussel tissue aliquot (200 mg) was placed in polyethylene pipes to which 4 ml of pure nitric acid of analytical grade is added. They are then allowed to stand overnight at room temperature for 24 hours. After that they are placed in a heating block at a temperature of  $80^{\circ}\text{C}$ . After the tubes cooled, freshly deionized water is added (MilliQ Water) to get a final volume of 50 ml. The solution thus obtained was analyzed by atomic absorption spectrometry (SAA) (Amiard *et al.*, 1987).

Cadmium and nickel concentrations were determined by atomic absorption spectrometry (SAA) with Zeeman correction (SAA type VARIAN 220 Zeeman) using the UNEP/IOC/IAEA (1994) method. The device is equipped with an automatic sample smuggler (VARIAN AA 400), the atomization of the metal is done in a pyrolytic graphite furnace (Model GTA96).

For Zinc element (Zn), copper (Cu), the determination of concentrations was performed by atomic absorption spectrophotometry with flame (SAAF, flame: air-acetylene, Varian AA20) using the UNEP/FAO/IOC/IAEA method (1984). The correction of spectral background noise for specific wavelengths (Zn - 213.9 nm, Cu - 324.8 nm) is done with a deuterium lamp.

Quality control was applied to all metal analyses. A standard white and two certified materials (IAEA-407/IAEA-437 and IAEA-405/IAEA-433) of known concentration are included in the analytical series in systematic manner. Reference materials are essential tools available to laboratories to monitor and evaluate the performance of their chemical testing procedures. Control samples are processed under the same analytical conditions as the samples to be analyzed.

### ***Metallothionein analysis***

Mussels collected at each site were used for MT determination. They were weighed (total weight), then the soft tissues were separated from the shell and drained with absorbent paper, and then dissected in order to recover branchias and digestive gland of each individual. These tissues are cold-crushed in three volumes of a lysis solution consisting of a Tris-HCl 20 mM tampon; pH 8.6; sucrose 0.5M; PMSF 0.5M; 0.01 mM  $\beta$ -mercaptoethanol as a reducing agent. The subsequent homogenate is centrifuged to 30000 g for 20 minutes at  $-4^{\circ}\text{C}$ . In order to precipitate the high molecular weight proteins, the resulting supernatant was treated with 1.05 ml of cold absolute ethanol ( $-20^{\circ}\text{C}$ ) and 80 l of chloroform and underwent again a further centrifuge at 9000 g for 10 minutes. A volume of 40  $\mu\text{l}$  of HCl 37%, 10  $\mu\text{l}$  of an RNA solution (1mg/10 l) and three volumes of absolute cold ethanol were added to the supernatant which was centrifuged at 9000g for 10 minutes and incubated for an hour at  $-20^{\circ}\text{C}$ . The pellet obtained and again centrifuged after washing with a solution of Ethanol/Chloroform homogenization buffer (87:1:12 V/V) was then cold at  $-20^{\circ}\text{C}$ . A final centrifugation at 9000 g for 10 minutes is realized. Resulting pellets containing MT were suspended in 150  $\mu\text{l}$  of NaCl 0.25 M solution and 150  $\mu\text{l}$  a solution consisting of HCl 1 N containing 4 mM of EDTA (destabilization solution).

Metallothionein level in sample was followed by a reaction with DTNB (5,5 dithiobis 2 benzoic nitro acid) (Ellman, 1959). Sample absorbance was measured at 412 nm and the MT concentration was quantified using the reduced glutathione (GSH) as a reference standard (Viarengo et al., 1997). MT concentration was expressed as  $\mu\text{g}/\text{mg}$  of proteins of analysed tissues

The Bradford method (1976), using the Coomassie Brilliant Blue, was used for quantitative determination of proteins using bovine serum albumin as the standard.

**Statistical analysis**

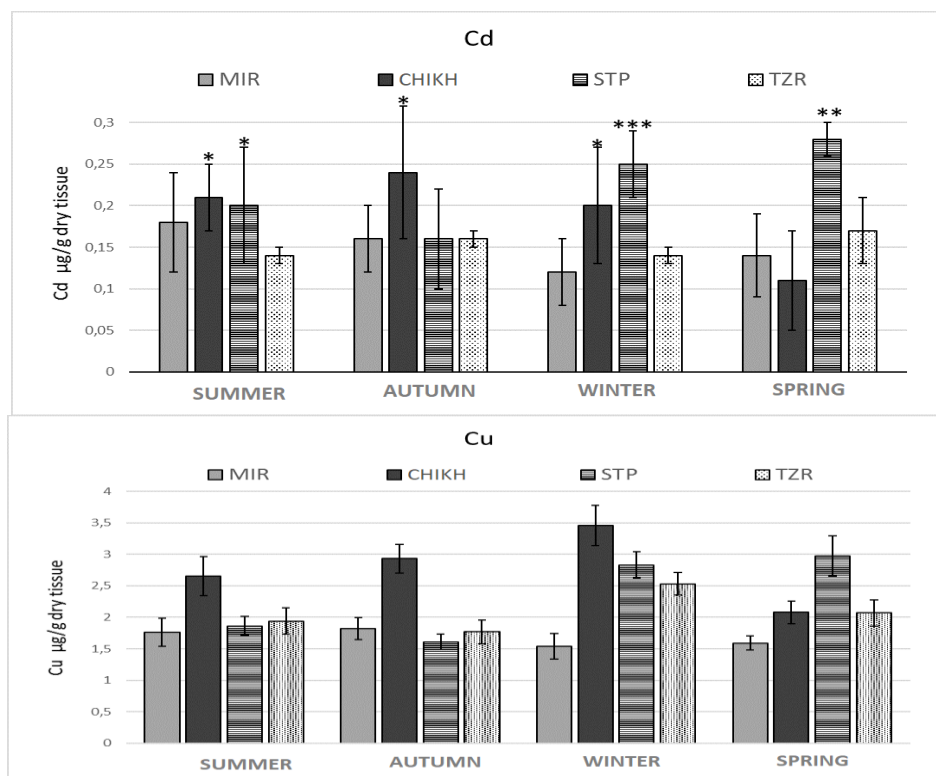
Differences in variables investigated among sampling studied sites stations were evaluated by the analysis of variance (ANOVA). Values were expressed on average  $\pm$  standard deviation (SD). All statistical tests were conducted using Statistica10 (StatSoft).

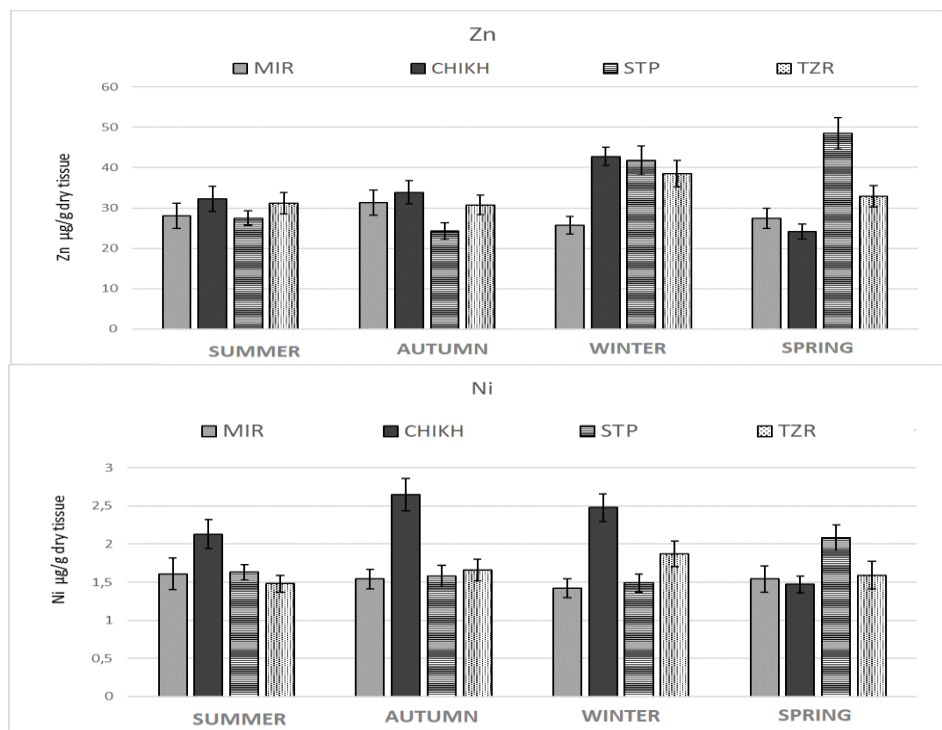
**3. RESULTS**

**Trace metals**

The inter-site comparison of seasonal variations in trace metal content in *Mytilus galloprovincialis* from the MIR, CHIKH, STP and TZR sites is shown in Figure 2.

The temporal variations in Cd concentrations are different in the sites studied. In the CHIKH site, the Cd shows very large fluctuations with a significant increase in autumn. At STP site, these are dominated by two peaks noticeable in winter and spring. In TZR, Cd levels are generally low and not very fluctuating.





**Figure 2:** Seasonal variations in Cadmium (Cd), Copper (Cu), Zinc (Zn) and Nickel (Ni) bioaccumulation in *Mytilus galloprovincialis* at the sites of MIR, CHIKH, STP and TZR. Significant differences from the control site (MIR): ANOVA -LSD (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ )

In each site, the Cu and Zn levels show the same trends. Cu and Zn show significant fluctuations with a peak in winter and a fall in the spring in site S1, while in the CHIKH site, clear seasonal variations with high concentrations in winter and autumn were obtained. However, they are almost stable in the STP site, except for a slight increase in winter.

The seasonal variations of the Ni are markedly different between the study sites. Ni concentrations at the Sheikh site fluctuate widely with well-individualized peaks in summer, autumn and winter. However, in other sites, seasonal variations are not significant if a slight increase in winter and spring concentrations is excluded, respectively at TZR and STP sites.

Compared to the control site (MIR), the highest concentrations of the four metals measured, including copper (Cu), cadmium (Cd), zinc (Zn) and nickel (Ni), were observed in mussels originating at the CHIKH station (in summer, autumn and winter; and the STP station in the spring, with highly significant differences (\*\*\*,  $p < 0.001$ ). However, on the TZR station, only one significant difference in Cu, Zn and Ni was detected in the winter.

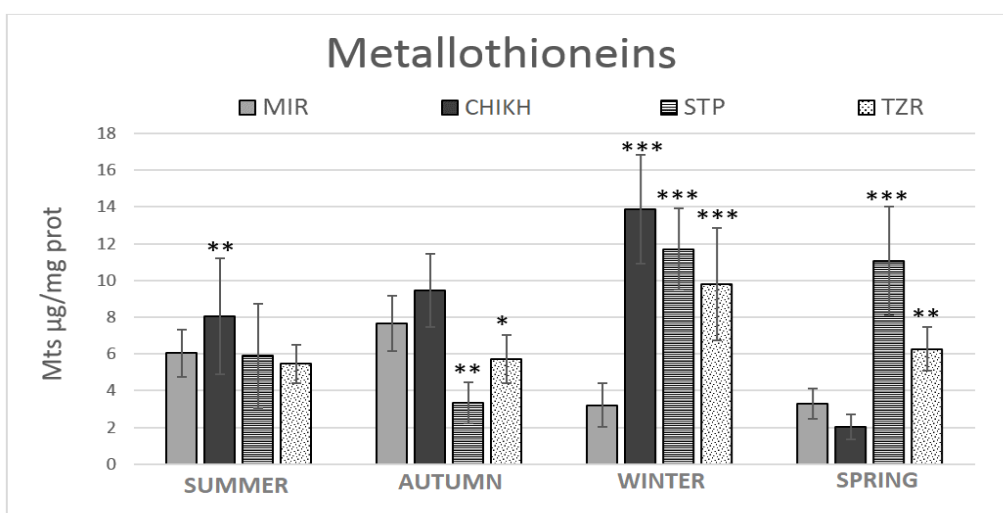
### **Metallothioneins**

Figure 3 shows the evolution of the level of metallothioneins (MT) in *Mytilus galloprovincialis* from the MIR, CHIKH, STP and TZR sites during the period of stoppage of wastewater discharges at the CHIKH site and the start of STP.

With the exception of spring 2014, the rate of MT accumulation is mostly higher in mussels living in Cheikh site with a maximum in winter ( $13.87 \pm 2.94$  g/mg of protein).

In STP and TZR sites the level of MT shows that the seasonal variation in 2014 (winter and spring) is greater than that of 2013 (summer and autumn). The highest values ( $11.70 \pm 2.21$  and  $11.05 \pm 2.95$  mg/mg of protein) are recorded in winter and spring 2014 respectively in mussels from the STP site. During this period study, the rate of MT accumulation is significantly higher in mussels at this site compared to mussels at the control site.

At the TZR site, seasonal variations in metallothionein appear more in the spring and winter when the maximum level of MT induction was observed with a significantly high value ( $9.79 \pm 3.07$  g/mg of protein,  $p < 0.05$ ).



**Figure 3:** Evolution of the level of metallothionein in *Mytilus galloprovincialis* recolted during the period 2013-2014 at the sites of MIR, CHIKH, STP and TZR

Significant Difference Limit (LSD-Fisher) with the control site (MIR), \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$

In general et comparatively to the control site (MIR), the inter-sites comparison of seasonal variations in metallothionein levels in *Mytilus galloprovincialis* recolted from different sites showed significantly very high winter values at all: CHIKH sites, STP and TZR ( $p < 0,001$ ), in the spring on the sites STP ( $p < 0,001$ ), TZR ( $p < 0,01$ ) and in the summer at the CHIKH site ( $p < 0,01$ ), while a significant decrease in MT accumulation rates was recorded in the autumn on the sites STP ( $p < 0,01$ ) and TZR ( $p < 0,05$ ).

#### 4. DISCUSSION

The Mediterranean mussel, *Mytilus galloprovincialis*, is a bioindicator widely recognized for its ability to bioaccumulate metals. This phenomenon depends on the availability of these pollutants in the biotope. Furthermore, the mussel is a species that has the ability to concentrate metals in water.



In the present study, the assess of trace metal bioaccumulation (Cd, Cu, Zn and Ni) was conducted during a seasonal cycle (summer 2013 to spring 2014) at different points of the Sidi Ifni coast.

At CHIKH Site, most metals show high concentrations in autumn and/or winter. Indeed, several studies have described higher metal concentrations in winter than in summer in *Mytilus galloprovincialis* and *Perna perna* (Bouthiret *et al.*, 2004; Tahiri *et al.*, 2005; Benbrahim *et al.*, 2006). Other authors have observed the highest levels rather in autumn (Essedaoui and Sif 2000 and 2001) in the same mussel. Variations in the results between the different authors may be related to the biology of bivalves in the study sites and especially to the quality of the biotope.

The bioaccumulation of metals in bivalves could be linked to other biological factors such as metabolism, development, age and especially the reproductive cycle. The phenomenon remains maximum before reproduction and minimal after spawning when reserves are depleted during gametogenesis (Radenac *et al.*, 1997). This trend is confirmed by our study, which describes the highest rates of bioaccumulation in the winter before the spawning period in the spring and coinciding with the spawning period of *Mytilus galloprovincialis*, as described by IdHalla (1997) in Agadir Bay and Cheggour (1989) in the Bouregreg estuary.

Our study showed also that metal bioaccumulation in *Mytilus galloprovincialis* may have different profiles depending on the sampling site. Indeed, spatial-temporal variation in the level of metal bioaccumulation in mussels may also be related to environmental factors, such as temperature, salinity and nutrients. The winter period is often accompanied by nutrients that could lead to a rich phytoplankton potentially contaminated with trace metals from wastewater discharges and stormwater.

Cajaraville *et al.*, 2000; Funeset *et al.*, 2006 report that trace metals can have adverse effects on aquatic organisms after they accumulate. However, not all of them have the same degree of toxicity and the same impact on the health of bivalve molluscs. Copper and zinc (essential metals) are considered harmful only after high-dose ingestion, while cadmium is harmful even at low doses (Vander Velde *et al.*, 1992).

Sensi and Jeng (2004) have demonstrated that excess zinc becomes a pro-oxidant element by inducing indirect formation of free radicals and inhibiting the enzyme activity of certain antioxidant enzymes such as reductase and peroxide glutathione (Splittgerber and Tappel, 1979). Other studies have shown that the accumulation of copper in the cell is the cause of cytotoxicity, which manifests itself by an enzymatic inhibition of the pyruvate-oxidase system, glucose-6-phosphodeshydrogenase and glutathion-reductase are inhibited proportional to the intracellular concentration of copper (Barceloux, 1999). Therefore, the accumulation of cadmium in organisms increases the formation of reactive oxygen species and lipid peroxidation by interaction with antioxidant systems. This can cause damage to proteins, DNA and lipids (Regoliet *et al.*, 2004). It is also described as an inhibitor of DNA damage repair (Waisberg *et al.*, 2003).

Nickel has been shown to have long-term toxic effects for aquatic invertebrates (Pane *et al.*, 2003b), fish (Pane *et al.*, 2003a; Banniet *et al.*, 2010) and in particular bivalves (Attiget *et al.*, 2010; Donderoet *et al.*, 2011). In addition to direct production of free radicals, this metal has been suspected of causing depletion of the antioxidant enzyme system (Denkhaus and Salnikov, 2002) and is considered a pro-oxidant agent.

Finally, it should be noted that the high rates of metal bioaccumulation recorded at the TZR site in winter may be related to the presence of metals resulting from the degradation (corrosion) of the hull of the boat that has failed since 1994 on the beach of this site.

For metallothioneins, results of the present study responded significantly to the levels of pollution observed at sites diagnosed on the Sidi Ifni coast. These metalloproteins, which have been found in most marine animals, play a role in detoxifying by capturing metal cations of exogenous origin in the body and protecting against the effects induced by these metals (Roesijadi, 1996; Viarengo *et al.*, 2000). Several authors have described their presence in tissues of bivalves exposed to trace metals such as Cu, Cd, Hg, Mg and Zn. However, metallothioneins are regularly considered in the literature as a parameter for assessing metal contamination (Pedersen *et al.*, 1998; Guanand Wang, 2004; Martin-Diaz *et al.*, 2005; Amiard *et al.*, 2006; Liberge and Bartholomew, 2007; Erket *et al.*, 2008; Wen-Hong *et al.*, 2009; Khan *et al.*, 2010; Pytharopoulou *et al.*, 2011; Hauser-Davis *et al.*, 2012; Asselman *et al.*, 2013; Pedersen *et al.*, 2014; Oaten *et al.*, 2015). Metallothionein induction levels has been proposed as a specific biomarker of toxic metal exposure in marine environments (Langston *et al.*, 1998; Amiard *et al.*, 2006) and is usually applied in international environmental biomonitoring programs (Viarengo *et al.*, 1999 and 2007; Banniet *et al.*, 2007).

In the present study, the level of induction of MT at Cheikh site is higher than the values observed at the other sites. In addition, the induction during wastewater discharges may be due to the presence of heavy metal contamination in wastewater discharged at this site during this period. This increase in MT would certainly be related to the inflows of domestic discharges from the town of Sidi Ifni.

The heavy inductions recorded in winter and spring at the STP site are likely due to the inputs from discharges from the treatment plant specifically originating from leaching water, which coincides with the rainy season. These results lead to conclude that treated wastewater is loaded with heavy metals. Research has shown that despite improved primary and secondary wastewater treatments, discharges from the treatment plant still retain a significant amount of organic materials and metal compounds (Speir *et al.*, 2003).

As mentioned above, with respect to the bioaccumulation of trace metals studied, the induction of MT observed in *Mytilus galloprovincialis* at the TZR site would be probably related to the presence of heavy metals resulting from the degradation (corrosion) of the hull of the boat stranded on this beach since 1994. Indeed, correlation between metal bioaccumulation and biosynthesis of metallothionein was described in mussels and many animals in aquatic ecosystems (Fabrício *et al.*, 2020). However, an organism's exposure to heavy metals leads to overproduction of metallothioneins accompanied by cellular damage (Cavaletto *et al.*, 2002). However, oxidative stress alone can cause a build-up of metallothioneins in mussel tissues. Significant increases in metallothionein isoforms are observed in *Mytilus galloprovincialis* mussels not exposed to heavy metals, but with a state of oxidative balance imbalance. (Dondero *et al.*, 2005).

## 5. CONCLUSION

The study of the bioaccumulation of Cd, Cu, Zn and Ni at *Mytilus galloprovincialis* at the coast of Sidi Ifni, allowed to compare the process of bioaccumulation in Mediterranean mussels in different sites. This part of study allowed to confirm the polluted state of CHIKH site which



received, for long periods, untreated wastewater from Sidi Ifni city. These results were then reinforced by those of metallothioneins.

Our study supports the use of *Mytilus galloprovincialis* in environmental biomonitoring context in marine ecosystems. The evaluation of metal bioaccumulation and metallothionein can be useful as biochemical biomarkers in marine organisms in assessing chemical exposure of different contaminants in this area.

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