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Study of heavy metal bioaccumulation in *Mytilus galloprovincialis* (Lamark 1819) from heavy metal mixture using the CCF design

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ABSTRACT

Marine pollutants such as heavy metals are well-known due to their bioaccumulative nature, toxicity and persistence in the marine environment. The present research aims to evaluate the bioaccumulation of three metals (Cd, Zn and Cu), individual mean (multimetal) bioaccumulation index (IMBI) and metal pollution index (MPI) as an assessment tool in Mediterranean mussel (*Mytilus galloprovincis*), based on the full factorial central composite face centered (CCF) design. Mussels were exposed to different sub-lethal concentrations (0-10%-72h-LC_{50}-72h-mg.L^{-1}) of studied heavy metals for three days. At the end of exposure cycle, the bioaccumulations of heavy metals content in mussels were determined. Multiple regressions were used to fit experimental data to a second-order polynomial equation, which was then examined using variance analysis (ANOVA). The results revealed that the indexes IMBI and MPI depend on the type and concentration of metals and also on the metals combination. Accordingly, the toxicological metal–metal interactions were found not linear meaning supra-additive (synergistic), infra-additive (antagonistic) or no/zero interaction. The optimization of experimental data was used to determine the optimal conditions for the heavy metal bioaccumulation. It was demonstrated that the CCF design combined with the concept of quantitative bioindicators can be used as appropriate tool in ecotoxicological evaluation and prediction of metals bioaccumulation.

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1. Introduction

Due to their non-biodegradability, bioaccumulative and toxic nature, heavy metal is one of the major environmental problems (Sharifuzzaman et al., 2016). The marine environments (estuarine/coastal) receive a large amount of heavy metals from agriculture by improper use of mineral pesticides (e.g. $CuSO_4$ in Bordo Mix), wastewater (Zn, Cu, Cd, Ni, and Pb), industrial activities such as zinc electrolysis and hydrocarbon-related activities (Tayeb et al., 2015; Boudjema et al., 2014). Their toxicity leads to the death and disappearance of mollusks (Ali et al., 2019; Gheorghe et al., 2017; Sharifuzzaman et al., 2016; Jakimska et al., 2011). Particularly, the Algerian coastline constantly receives unacceptable

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amounts of heavy metals, more specifically Zn, Cu and Cd (Belhadj et al., 2017; Bachouche et al., 2017; Benfares et al., 2015).

The assessment of bioaccumulation processes is crucial in the assessment of chemical hazards (Radomyski et al., 2018). Recently, the bioaccumulation indexes such as the individual mean (multimetal) bioaccumulation index (IMBI) and metal pollution index (MPI) were utilized as good monitoring tools for the assessment of multimetal contamination in marine ecosystem or bioaccumulation bioassay (acute or chronic) using living organisms (Abderrahmani et al., 2020; Zaghloul et al., 2020; Barath Kumar et al., 2019; Genç and Yilmaz, 2017). In this respect, various investigations were based on a quantitative bioindicator concept, using the capacity of marine organisms (such as *Mytilus galloprovincialis*) to concentrate and accumulate xenobiotics in their tissues with respect to the ambient level (Salvat-Leal et al., 2020; Ronci et al., 2016; Lacroix et al., 2015; Zaldívar, 2008; Casas and Bacher, 2006).

Besides, the definition of bioaccumulation is some what controversial in the literature; for some authors, it is a phenomenon leading to the increase of the concentration of chemicals in a biotic compartment with respect to the surrounding medium or, more precisely, is the consequence of uptake, distribution, metabolism and excretion of a substance in aquatic organism (Radomyski et al., 2018; Mackay et al., 2013). This approach is more efficient than direct measurements in water (Lacroix et al., 2015). In this fact, many experimental and modeling approaches were developed in order to estimate bioaccumulation metrics such as the bioaccumulation (BA) and the biomagnification (BM) factors (Radomyski et al., 2018).

In laboratory experiments, the number of exposure doses of chemicals (e.g. heavy metals) is limited and the bioaccumulation responses are following only in running exposure (single, multiple/mixtures: binary and ternary). Moreover, the relationship "dose–response" between the bioaccumulation and exposure concentration and their interactions (antagonistic, synergistic and zero interaction) are not yet defined. Consequently, the bioaccumulation is not continuously defined in all factor's domain of variation (between the lower limit (low level) and the upper limit (high level)) of exposure concentration (García-Navarro et al., 2017; Boudjema et al., 2014; Casas and Bacher, 2006; Prato et al., 2006; Mersch et al., 1993).

In modeling bioaccumulation approach, the toxicokinetic models (based on the compartment, physiological and energetics, respectively) have been used to investigate metal bioaccumulation in aquatic organisms – quantitatively and predict metal accumulation in aquatic invertebrates – (Landrum et al., 1992). Additionally, to predict the fate and bioaccumulation of chemicals in the food web, these toxicokinetic models use upstream quantitative knowledge of chemical uptake, metabolism, excretion, and depuration processes in organisms (Moriarty and Walker, 1987). However, all these processes are strictly related to specific physiological factors (growth, weight loss, absorption and accumulation), chemical factors (metal concentration, speciation and bioavailability) and environmental factors such as temperature and food concentration (Casas and Bacher, 2006; Zaldívar, 2008).

To our best knowledge, there are no studies reported in literature which utilize combination of heavy metal bioaccumulation process in marine biota and design of experiments (DoE) for modelization and optimization of process parameters in ecotoxicological assessment and modeling. In this particular context, it seems very important to use a statistical technique and in particular a response surface methodology (RSM) for modeling and optimizing the bioaccumulation by using the bio-sentinel organisms in ecotoxicological bioassay. This innovative application of RSM allows obtaining maximum data that needed bases on the downstream quantitative knowledge of metal concentration in tissue, which reduces the experimental trials number, time and study costs.

To ovoid the hazardous effects of heavy metals in marine ecosystem, an environmental assessment must be made. Taking into account all the constraints, this investigation has just put the point on the problems raised by the accumulation of heavy metals in the marine environment, more particularly in the molluscs.

In this study, a 2^3 full factorial central composite face centered (CCF) design was employed to evaluate and predict the effect of heavy metals (essential elements: Zn^{2+}, Cu^{2+}, no-essential element/xenobiotic: Cd^{2+}) on the bioaccumulation of heavy metals and on the bioaccumulation indexes (IMBI, MPI) in *Mytilus galloprovincialis*. The toxicological interactions (antagonistic, synergistic and no interaction) between the parameters were also evaluated and optimized.

2. Materials and methods

2.1. Mussels materials

Mussels *M. galloprovincialis* (4.5–5.5 cm shell length) were obtained from a mussel farm (Culture Marine, *N* 36°35.496′, E 002°30.669′) at Kwali 3 (Tipaza, Algeria).

After harvesting, mussels were immediately transported to the laboratory and acclimatized for a period of 15 days in tanks containing 1.5 L NSW (natural seawater) per animal under the following conditions: constant aeration 7.39 ± 0.14 mg L^{-1}, temperature 17.56 ± 0.2 °C, salinity 37 psu, pH 8.01 ± 0.2 and natural light. Similarly, the NSW was daily changed and mussels were not fed during the acclimation period except by phytoplankton, naturally presented in NSW. Knowing that no mortality was recorded during this acclimatized period.

2.2. Determination of the metals-lethal concentration (LC_{50})

Firstly, short-term toxicity tests with metals (Cd, Zn and Cu) were determined in order to establish the range of their LC_{50} during 72 h for *Mytilus galloprovincialis* used as a model organism of the marine invertebrate. Toxicity tests were carried out as described by Boudjema et al. (2014). Briefly, fifteen (15) acclimated mussels were selected for each metal exposure treatment in 20 L of NSW into polystyrene rectangular trays (42 × 28×19 cm). For each metal, different concentrations (mg L^{-1}) were used: $CdSO_4$: 0.50, 1.00, 2.00, and 4.00 ; $ZnCl_2$: 0.50, 1.00, 2.00, and 4.00 and $CuSO_4.5H_2O$: 0.025, 0.05, 0.10, 0.20, and 10.0. A control group of 15 mussels without metal exposure was simultaneously set up for each experiment. The exact values of LC_{50}-72 h were determined by probit analysis using IBM SPSS Statistics 25 software (2018, trial version) and expressed as the median lethal concentration for three independent replicates by concentration after 72 h of exposure. The medium test was kept static but constantly aerated (8.01 ± 0.14 mg L^{-1}), temperature 16.78 ± 0.004 °C, salinity 37 ± 0.004 psu and pH 7.83 ± 0.06 under natural light and with phytoplankton (naturally presented in NSW). After each 24 h exposure period, the NSW was renewed containing the same initial heavy metals concentrations in order to keep the contaminant concentration stable in the tanks until the all experiment period. Mussel mortality was detected if there is no response to a tactile stimulation.

2.3. Bioaccumulation assay using experimental design

CCF design was applied to predict the effects of initial nominal concentrations of three trace metals (experimental factors), namely Cd^{2+} (X_1), Zn^{2+} (X_2) and Cu^{2+} (X_3) and their interactions (antagonistic, synergistic and zero interaction) on the bioaccumulation responses of cadmium (Cd: mg L^{-1}), zinc (Zn: mg L^{-1}), copper (Cu: mg L^{-1}) and on the bioaccumulation indexes (IMBI and MPI). The CCF design was chosen because it provides relatively high-quality predictions over the entire design space and does not require using points outside the original factor range (Karian and Dudewicz, 2016).

Fifteen healthy mussels in seventeen groups (15 × 17) were randomly selected as inoculums, and were exposed to contaminants (heavy metals) for 72 h under similar experimental conditions ($\Delta T = +0.4196 \pm 0.2967$ °C, Δ salinity = -0.002745 ± 0.0019411 psu, $\Delta pH = +0.0823 \pm 0.05823$, $\Delta O_{diss} = -0.12005 \pm 0.0848$ mg L^{-1}). The NSW was exchanged every 24 h to keep test concentrations of metals at constant levels. Metal concentrations sub-lethal ranges used in our experiments were much lower than LC_{50}-72 h concentrations found in the toxicity experiments. The range values of the factor levels were: $CuSO_4$ [0%–10% 72 h-LC_{50}-Cd mg L^{-1}]; $CdCl_2$ [0%–10% 72 h-LC_{50}-Zn mg L^{-1}]; $CuSO_4$ [0%–10% 72 h-LC_{50}-Cu mg L^{-1}], respectively (Table 1). However, the original measurement units for the experimental factors levels (uncoded units) were coded −1 (the lower limit is the low level) (X_1 = 0 mg L^{-1}, X_2 = 0 mg L^{-1}, X_3 = 0 mg L^{-1}), 0 (middle) (X_1 = 5% 72 h-LC_{50}-Cd mg L^{-1}, X_2 = 5% 72 h-LC_{50}-Zn mg L^{-1}, X_3 = 5% 72 h-LC_{50}-Cu mg L^{-1}) and +1 (the upper limit is the high level) (X_1 = 10% 72 h-LC_{50}-Cd mg L^{-1}, X_2 = 10% 72 h-LC_{50}-Zn mg L^{-1} and $X_3$10% 72 h-LC_{50}-Cu mg L^{-1}).

The CCF design for bioaccumulation responses induced by heavy metals mixtures was generated using Matlab 7 software and was employed for graphical analyses of the obtained data. An experimental matrix was established; it contains a total of 17 runs counting three additional trials as replicates of the central point for the calculation of the pure error (Table 1). The order of the experimental run was completely randomized. The quality of the fit polynomial model, the coefficient of determination (R^2), the adjusted determination coefficient (R^2_{adj}), the student's test "t", the Fisher's F-test and the lack of fit were checked. Model terms were evaluated by the *p*-value (probability) with 95% confidence level using ANOVA.

The response equations were calculated by the second-order polynomial equation (Eq. (1)) (Goupy and Creighton, 2006; Myers et al., 2009):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X^2{}_1 + \beta_{22} X^2{}_2 + \beta_{33} X^2{}_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y is the predicted response, β represents the regression coefficients and, X_i the factors.

The change from the original coordinates A to the reduced centered coordinates (RCC), x, and vice versa, is given by the following formula (Goupy and Creighton, 2006; Myers et al., 2009):

$$X = \frac{A - A_0}{Step} \quad (2)$$

where A_0 is the centered value in current units.

Finally, mussels were dissected after the exposure period, and the soft tissues were weighed and frozen at −20 °C. The samples were lyophilized, weighed, and ground into a fine powder before being stored in polyethylene pots until they were analyzed. No mortality was observed during the course of the CCF design for biomarkers responses. The 10% of the 72 h-LC_{50} for Cd, Zn and Cu can be considered as a safe concentration in ternary mixture exposure.

2.4. Metals analysis

Samples were analyzed using flame and graphite furnace atomic absorption spectrometry (GF/F-AAS, Agilent Technologies, 200 Series, USA) to determine Cd, Zn and Cu contents. However, the samples treatment for the trace metal

Table 1
2^3 full factorial CCF design for experimental results.

Run	Factor			Responses									
	Coded levels			Heavy metals accumulation						Bioaccumulation indexes			
	X_1	X_2	X_3	Y_1: Cd Accumulation µg g^{-1} wet weight		Y_2: Zn Accumulation µg g^{-1} wet weight		Y_3: Cu Accumulation µg g^{-1} wet weight		Y_4: IMBI Unidimensional		Y_5: MPI Unidimensional	
				Act[a]	Pred[b]	Act[a]	Pred[b]	Act[a]	Pred[b]	Act[a]	Pred[b]	Act[a]	Pred[b]
1	−1	−1	−1	00.061	00.627	37.590	35.899	01.467	01.378	00.346	00.341	01.500	02.096
2	+1	−1	−1	26.450	26.237	38.059	37.822	01.729	02.192	00.580	00.593	12.030	11.635
3	−1	+1	−1	00.781	00.158	49.032	48.549	01.131	01.171	00.346	00.369	03.511	02.237
4	+1	+1	−1	16.850	16.639	44.981	44.933	01.413	01.196	00.468	00.450	10.231	10.719
5	−1	−1	+1	00.670	00.770	29.396	28.864	04.697	04.893	00.394	00.411	04.524	03.926
6	+1	−1	+1	23.600	24.111	22.850	22.753	03.818	03.758	00.710	00.686	12.722	13.887
7	−1	+1	+1	00.601	00.702	67.036	66.693	08.091	07.608	00.376	00.363	06.883	07.168
8	+1	+1	+1	15.591	14.914	53.933	55.044	05.616	05.685	00.464	00.468	16.778	16.072
9	−1	0	0	00.401	00.257	49.464	52.512	03.419	03.754	00.359	00.337	04.080	05.070
10	+1	0	0	19.580	20.168	48.380	47.649	03.452	03.199	00.492	00.516	14.843	14.292
11	0	−1	0	15.730	14.765	39.870	42.423	03.519	03.009	00.494	00.493	13.020	12.252
12	0	+1	0	08.522	09.931	65.130	64.893	03.278	03.869	00.393	00.397	12.208	13.415
13	0	0	−1	08.757	09.237	45.723	48.179	01.600	01.403	00.408	00.396	08.621	09.206
14	0	0	+1	08.483	08.447	49.857	49.717	05.126	05.405	00.424	00.440	12.943	12.797
15	0	0	0	11.670	10.441	58.456	55.058	03.753	03.417	00.421	00.415	13.680	12.524
16	0	0	0	11.254	10.441	55.872	55.058	03.662	03.417	00.422	00.415	13.205	12.524
17	0	0	0	09.290	10.441	55.481	55.058	03.001	03.417	00.407	00.415	11.565	12.524

Factors (mg L^{-1})	Coded symbol	Range and levels		
		−1	0	+1
Nominal concentration of Cd^{2+}	X_1	0	5% 72h-LC_{50} (0.130)	10% 72h-LC_{50} (0.260)
Nominal concentration of Zn^{2+}	X_2	0	5% 72h-LC_{50} (0.093)	10% 72h-LC_{50} (0.186)
Nominal concentration of Cu^{2+}	X_3	0	5% 72h-LC_{50} (0.005)	10% 72h-LC_{50} (0.010)

[a]Actual value.
[b]Predictive value.

determination was performed following a standardized method for analysis of AIEA (2004). After shaking sample pools, a quantity of 0.5 g was digested with 5 mL of HNO_3 (69%, Panreac, CAS: 1310037.1611) in labeled Teflon bombs (60 mL, Savillex) at 120 °C on a hot plate (Ceran 500, 22 SR) for 2 h.30 min. Blanks were prepared in a similar manner as samples, using only HNO_3. Heavy metal results are the mean of three replicate measurements of each sample. The wavelengths (nm) were 220.353 (Cd), 214.438 (Zn), and 324.754 (Cu).

Quality assurance and quality control were assessed by processing blank samples and reference standard material (SRM 2976-AIEA) (supplementary Table S1). The recovery for Cd, Zn and Cu ranged between 95.12 and 96.02% and low standard deviations (below 10%) were registered.

2.5. Indexes bioaccumulation determination

The bioaccumulation indexes, namely the individual mean (multimetal) bioaccumulation index (IMBI) and metal pollution index (MPI) were modeled to assess and predict the most potential effects of metals traces and to compare the total content of metals at the different points in the experimental design of bioaccumulation tests. In this fact, IMBI and MPI were calculated to enable the presentation of all results from the metal concentrations as a "one value" according to the works of Maes et al. (2005) and Usero et al. (2005). Succinctly, in the first method, the IMBI was calculated by dividing (standardizing) the individual concentration of heavy metal i (C_i) by the maximum observed concentration (C_{imax}) and averaging over all metals, to relate heavy metal bioaccumulation to ambient conditions. Thus, the IMBI was calculated using Eq. (3).

$$\mathrm{IMBI} = \frac{\sum_1^n C_i / C_{imax}}{n} \tag{3}$$

where n is the total number of studied metals, Ci is the individual concentration of heavy metal i, $C_i$max is the found maximal concentration of heavy metal i ($0 < \mathrm{IMBI} < 1$).

In the second method, the MPI was obtained by Eq. (4).

$$\mathrm{MPI} = (Cf_1 \times Cf_2 \ldots Cf_n)^{1/n} \tag{4}$$

where Cf_i is the concentration of the metal 'i' in mussels and, n is the total number of metals.

The cluster analysis of IMBI and MPI was performed with IBM SPSS Statistics 25 software (2018, trial version).

3. Results and discussion

3.1. Toxicity tests of metals in mussels

The results of the values of the median lethal concentrations after 72 h-exposure (LC_{50}-72 h), calculated from Probit analysis, were for Cd: 2.602 (2.054–3.610) mg L^{-1}; Zn: 1.866 (1.154–3.880) mg L^{-1} and Cu: 0.106 (0.07–0.158) mg L^{-1}.

In this study, there is a positive correlation between mortality of test mussels and increasing in concentration of metals during 72 h-exposure. However, many experimental reports showed that the mortality of animal tests were positively correlated with increasing concentration of metals as well as increasing exposure period (Ramakritinan et al., 2012; Vlahogianni and Valavanidis, 2007). In addition, high mortalities were observed in mussels at low concentrations of Cu. Whereas, Cd and Zn exposure did not induce a high mortality, even for exposures equal or higher than 0.5 mg L^{-1}. These results suggested that *M. galloprovincialis* mussels were most sensitive to the toxic effects of essential elements (Cu and Zn) when compared with no-essential element (Cd) and the decreasing order of toxicity was Cu > Zn > Cd. The obtained results about sensitivity of *Mytilus galloprovincialis* to metal exposure are comparable with those of Vlahogianni and Valavanidis (2007); they found that *M. galloprovincialis* mussels were most sensitive to the toxic effects of Cu, and the decreasing order of toxicity between copper and cadmium was Cu > Cd. In addition, for other marine molluscs, the sensibilities to metal exposure were: Cu > Cd > Zn for *Perna perna* (Yap et al., 2004), Cu > Cd > Zn for *Modiolus philippinarum* (Ramakritinan et al., 2012), Cu > Zn > Cd for *Cingulina cingulata* (Ramakritinan et al., 2012), Cu > Cd > Zn for *Perna viridis* (Chan, 1988) and Cu > Cd > Zn for *Mya arenaria* (Eisler, 1977).

Zn and Cu are essential for normal biological functions of mussels to perform various physiological and biochemical processes because they are important components of enzymes or metalloproteins, yet the hyper concentration (even a little bit more than needed) causes serious threat to life (Mustafa, 2014; Vosloo et al., 2012; Lodhi et al., 2006; Pelletier et al., 2004). On the contrary, Cd is considered as non-essential metal because it has no role in biological systems (Vosloo et al., 2012; Pelletier et al., 2004). The toxicity differences between metals suggested that their action mode at cellular and physiological levels are different (Abdelfattah and Jean-Claude, 2007). According to many researchers, the behavioral, metabolisms and physiological responses such as valve movement, mucus secretion rate, soluble nitrogen and phosphorus excretion were utilized as responses to metal toxicity levels (Boudjema et al., 2016; Ait Fdil et al., 2006; Yap et al., 2004; Sze and Lee, 1995). However in seawater, high concentrations of ions such as calcium and chloride can reduce the Cd bioavailability by competition and complexation processes, respectively. Thus, in seawater the formation of Cd chloride species reduce Cd toxicity, or through calcium competition for uptake (Marcus Zamora et al., 2015).

To date toxicity studies that conducted on marine species are limited and there are no similar bioassay for acute toxicity of heavy metals with *M. galloprovincialis* (Marcus Zamora et al., 2015; Vlahogianni and Valavanidis, 2007). In scientific literature, the toxicity of heavy metals was evaluated in bioassays by using LC_{50} values. Additionally, the differences in LC_{50} values for Cu, Cd and Zn recorded in marine molluscs are not similar and may be attributed to different collection sites and periods, species, experimental salinities, age and size test chambers, laboratory tests conditions and geographical regions (Ramakritinan et al., 2012; Chan, 1988). For these reasons, the 72 h-LC_{50} values determined in this research can be compared to varieties of LC_{50} values mentioned for marine molluscs species. For example, Vlahogianni and Valavanidis (2007) found a 96 h-LC_{50} value of 0.35 ± 0.02 mg L^{-1} and 1.7 ± 0.1 mg L^{-1} in the case of *Mytilus galloprovincialis* (4–5 cm length) exposed to Cu and Cd, respectively. Similarly, Ramakritinan et al. (2012) announced that the 72 h-LC_{50} values for *Modiolus philippinarum* (1 ± 0.2 cm length) were equal to 0.280 (0.179–0.465) mg L^{-1}, 4.040 (1.566–10.327) mg L^{-1} and 0.038 (0.023–0.061) mg L^{-1}, for Cd, Zn and Cu, respectively. Krishnakumar et al. (1987) reported that the 72 h-LC_{50} values for *Perna* viridis were found for shell lengths (15–20 mm and 30–40 mm) equal to 6.20 (4.63–8.31) and 0.225 (0.157–0.322) mg L^{-1} for Zn and, 0.088 (0.074–0.104) and 0.100 (0.083–0.120) mg L^{-1} for Cu. Additionally, Chan (1988) found that the ranged values of 96 h-LC_{50} for *Perna viridis* (3–4 cm length) were 1.570 mg L^{-1} for Cd, 6.090 mg L^{-1} for Zn and 0.620 mg L^{-1} for Cu. Furthermore, the 96 h-LC_{50} values for *Perna perna* (3–4 cm length) were 1.53 mg L^{-1} for Cd, 3.20 mg L^{-1} for Zn and 0.25 mg L^{-1} for Cu (Yap et al., 2004). Likewise, Eisler (1977) reported that the values on acute toxicities (LC_{50}) of metals in the softshell clam *Mya arenaria* (3.8–4.9 cm length) were 0.15 mg L^{-1} for Cd, 1.55 mg L^{-1} for Zn and 0.035 mg L^{-1} for Cu.

In general, as well as that the values of LC_{50} for various exposure times demonstrated a progressive decrease with the increase of the exposure duration (EI-Komi and Tayel Fathy, 1996). For example, for *M. philippinarum* (1 ± 0.2 cm length) exposed to Cu, the LC_{50} decreases with time of exposure: 24 h-LC_{50} (0.137 (0.080–0.499) mg L^{-1}) > 48 h-LC_{50} (0.076 (0.044–0.222) mg L^{-1}) > 72 h-LC_{50} (0.038 (0.023–0.061) mg L^{-1}) > 96 h-LC_{50} (0.023 (0.014–0.032) mg L^{-1}) (Ramakritinan et al., 2012). These findings imply that mussels have a high feeding rate and are thus more susceptible to metal toxicity in short-term toxicity tests (Vlahogianni and Valavanidis, 2007).

Finally, our objective of this part was to determine the 72 h-LC_{50} of Cd, Zn and Cu for *Mytilus galloprovincialis* and introduce them as ranges values of factor levels for *modeling and optimizing* the heavy metal bioaccumulation by using experimental design (Table 1). On the other hand, these results may be useful to deduce the marine water quality standards in the coastal waters of Bousmail Bay in Algeria.

3.2. Optimization of bioaccumulation and toxicological interactions

3.2.1. CCF bioaccumulation models

CCF bioaccumulation models proposed in this study are based on simple assumptions about the heavy metal concentration in *M. galloprovincialis* tissues under the sub-lethal exposures. For building the quadratic regression models, the CCF bioaccumulation responses in terms of coded factors for Cd accumulation (Y_1, $\mu g\ g^{-1}$ wet weight), Zn accumulation (Y_2, $\mu g\ g^{-1}$ wet weight), Cu accumulation (Y_3, $\mu g\ g^{-1}$ wet weight), IMBI (Y_4, Unidimensional) and MPI (Y_5, Unidimensional) were correlated with the three design factors using the second-order polynomial (Eq. (1)) and are given by Eqs. (5)–(9), respectively:

$$Y_1 = 10.4418 + 9.9555X_1 - 2.4167X_2 - 0.3953X_3 - 2.2824X_1X_2 - 0.5674X_1X_3 + 0.1002X_2X_3 - 0.2288X^2{}_1 + 1.9065X^2{}_2 - 1.5994X^2{}_3 \quad (5)$$

$$Y_2 = 5.0586 - 2.4316X_1 + 11.2349X_2 + 0.7688X_3 - 1.3847X_1X_2 - 2.0084X_1X_3 + 6.2949X_2X_3 - 4.9780X^2{}_1 - 1.4003X^2{}_2 - 6.1101X^2{}_3 \quad (6)$$

$$Y_3 = 3.4179 - 0.2775X_1 + 0.4298X_2 + 2.0009X_3 - 0.1971X_1X_2 - 0.4871X_1X_3 + 0.7306X_2X_3 + 0.0593X^2{}_1 + 0.0217X^2{}_2 - 0.0134X^2{}_3 \quad (7)$$

$$Y_4 = 0.4150 + 0.0892X_1 - 0.0476X_2 + 0.0219X_3 - 0.0424X_1X_2 + 0.0060X_1X_3 - 0.0189X_2X_3 + 0.0120X^2{}_1 - 0.0305X^2{}_2 - 0.0031X^2{}_3 \quad (8)$$

$$Y_5 = 12.5244 + 4.6106X_1 + 0.5815X_2 + 1.7956X_3 - 0.2643X_1X_2 + 0.1054X_1X_3 - 0.7754X_2X_3 - 2.8430X^2{}_1 + 0.3092X^2{}_2 - 1.5225X^2{}_3 \quad (9)$$

The second-order regression models obtained for the three design factors (Cd, Zn and Cu) are satisfied as the predicted versus observed value plot approximates along a straight line as shown in supplementary Figure S1. In addition, the residual dispersion was very small for the studied cases. The results showed that the values of the predicted and measured responses were very close. Since, the high R^2 value, close to 1, is desirable and the predicted R^2 must be in reasonable agreement with the adjusted R^2 for a significant model. The R^2 values for Cd, Zn and Cu, IMBI and MPI are equal to 0.992, 0.9819, 0.9647, 0.9717 and 0.9654, respectively. In this case, it indicates that only 0.8–3.53% of the total variation is not explained by the models. The adjusted determination coefficients ($R^2{}_{adj}$) equal, respectively, to 0.9834, 0.9586, 0.9193, 0.9343 and 0.9210 for Cd, Zn, Cu accumulated, IMBI and MPI responses; these values are closed to those of R^2, ensuring a satisfactory adjustment and confirming the significance of the models. Myers et al. (2009) reported that when R^2 and $R^2{}_{adj}$ differ considerably, there is a good chance that non-significant terms have been included in the model. In this fact, the obtained results indicated good agreements between the experimental and predicted values of bioaccumulation responses. According to Demim et al. (2013) and Barrick et al. (2018), the mathematical models are good tools to describe and predict the nature of relationships between abiotic factors and biochemical responses in both *in situ* and laboratory.

The adequacy of the CCF bioaccumulation models for Cd, Zn, Cu and bioaccumulation indexes (IMBI and MPI) were justified through variance (ANOVA) analysis and there results are given in supplementary Table S2. The ANOVA of these responses demonstrated that the CCF models are highly significant (p-value < 0.001) as are evident from the value of F-ratio F_0 (defined as the ratio of the respective mean square effect and the mean square error). The value of $F_{0.05,1,9}$ was equal to 5.12. It must be noted that all effects presenting F_0 higher than 5.12 have statistical significance ($F_{model(a)} = 106.54$, $F_{model(b)} = 42.096$, $F_{model(c)} = 21.26$, $F_{model(d)} = 26.51$ and $F_{model(e)} = 21.72$) . The lack of fit (p-value > 0.05, as shown in supplementary Table S2) also showed that the CCF bioaccumulation and indexes models could be used to predict the heavy metal bioaccumulation from the ambient environmental medium and used to investigate the surface responses for the optimization of the bioaccumulation process.

3.2.2. Effects of operational factors on the bioaccumulation

The student's test "t" was used to confirm the validity of the regression coefficients of the studied parameters, when the t-value is higher than the t-critic, the regression is statistically significant (Anderson and Whitcomb, 2007; Myers et al., 2009). It was found that the t-critical value is equal to 2.365 from two-tailed t-table (95% confidence level, 9 freedom degrees). The Pareto chart (supplementary Figure S2) showed the absolute values of the standardized effects of the independent variables and their interactions on the dependent variables from the largest effect to the smallest effect as already mentioned by some authors (Anderson and Whitcomb, 2007; Yetilmezsoy et al., 2009). The bars that represent the linear terms, quadratic terms and interactions (supplementary Figure S2) indicate that the models are statistically significant at the 0.05 level. In this case, these terms contributed the least in prediction of the three studied heavy metals bioaccumulation and bioaccumulation indexes (IMBI and MPI). The length of each bar in the Pareto chart represents the factor's standardized influence on the responses (Anderson and Whitcomb, 2007; Solanki et al., 2007).

The main (average) effects of factors (X_1, X_2 and X_3) on responses are defined as the change in responses produced by a change in the level from low to high (Anderson and Whitcomb, 2007; Myers et al., 2009). The one-half the values of those effects (effect/2) represent the coefficients (β_1, β_2, β_3) in the models (Anderson and Whitcomb, 2007; Goupy and Creighton, 2006). However, if the coefficients are not statistically significant (supplementary Figure S2) and different

Table 2
Optimum values of the bioaccumulation process parameters for maximum heavy metals bioaccumulation, with permissible upper limits.

Optimum values of heavy metals	Metals exposures (mg L^{-1})		
	Cd	Zn	Cu
	0.220	0.186	0.01
Bioaccumulation ($\mu g\ g^{-1}$ ww)	12.919	59.379	5.946
Composite desirability		0.653	
Permissible upper limits ($\mu g\ g^{-1}$ ww)	1	50	20

from zero, no effect condition holds (Katsnelson et al., 2015; Sereshti et al., 2012). On contrary, if the coefficients are statistically significant and different from zero, the sign of a coefficient (+ or −) defines the direction of the relationship between the related effect and response. The positive sign indicates that as the value of one effect changes, the value of the response changes in the same direction too (positive effect), while for the negative sign the response operates in the opposite direction (negative effect) (Kermet-Said and Moulai-Mostefa, 2015; Sereshti et al., 2012). The absolute value of the coefficients measures the strength of the relationship (Sereshti et al., 2012).

In the case of Cd bioaccumulation, an increase in X_1 from low to high level resulted in 20.02 $\mu g\ g^{-1}$ wet weight increase in bioaccumulation process, whereas an increase in X_2 and X_3 resulted in decreasing bioaccumulation process by 4.8837 $\mu g\ g^{-1}$ wet weight and 0.7401 $\mu g\ g^{-1}$ wet weight, respectively. In the case of Zn bioaccumulation, an increase in X_2 and X_3 from low to high level resulted in 22.469 $\mu g\ g^{-1}$ wet weight and 1.5375 $\mu g\ g^{-1}$ wet weight increase in bioaccumulation process, whereas an increase in X_1 resulted in decreasing bioaccumulation process by 4.8636 $\mu g\ g^{-1}$ wet weight. In addition in the case of Cu bioaccumulation, an increase in X_2 and X_3 from low to high level resulted in 0.42982 $\mu g\ g^{-1}$ wet weight and 2.0009 $\mu g\ g^{-1}$ wet weight increase in bioaccumulation process, whereas an increase in X_1 resulted in decreasing bioaccumulation efficiency by 0.2775 $\mu g\ g^{-1}$ wet weight.

In the case of IMBI, an increase in X_1 and X_3 from low to high level resulted in 0.08292 $\mu g\ g^{-1}$ wet weight and 0.0218 $\mu g\ g^{-1}$ wet weight increase in IMBI, whereas an increase in X_2 resulted in decreasing metal charge by 0.0475 $\mu g\ g^{-1}$ wet weight. For MPI, an increase in X_1, X_2, and X_3 resulted in increasing metal charge by 4.6106 $\mu g\ g^{-1}$ wet weight, 0.5815 $\mu g\ g^{-1}$ wet weight and 1.795 $\mu g\ g^{-1}$ wet weight, respectively.

Mussels of the species *Mytilus galloprovincialis* have a high capacity for accumulating heavy metals from the surrounding environment (water, sediments, and/or food) due to their sedentary and filter-feeding habits (García-Navarro et al., 2017; Rosioru et al., 2016). Furthermore, knowledge of heavy metal accumulation in mussels *via* laboratory and/or *in situ* exposures could provide highly useful information for use in marine ecotoxicology (Boudjema et al., 2014; García-Navarro et al., 2017; Jovic et al., 2012). In the control group (Run_1-Table 1), the concentration of the investigated metals in the soft tissues of *Mytilus galloprovincialis* decreased in the following order: Zn (37.59 $\mu g\ g^{-1}$ wet weight) > Cu (1.72 $\mu g\ g^{-1}$ wet weight) > Cd (0.0612 $\mu g\ g^{-1}$ wet weight) and were well below the two maximum permissible levels fixed by the Algerian reglementation (JORADP, 2011) (2 μg of Cd g^{-1} ww) and Turkish Food Codex (2008) (50 μg of Zn and 20 μg of Cu by gram of wet weight respectively) (Table 2). These results are comparable to the biomonitoring studies where the bioaccumulation (trace metal content) in *Mytilus galloprovincialis* followed this order Zn > Cu > Cd (Rouane-Hacene et al., 2015). Likewise, Kamaruzzaman et al. (2011) reported that metal bioaccumulation in the soft tissue of the green mussel *Perna viridis* flowed in the following order: Zn > Cu > Cd, showing that essential heavy metals are accumulated at a higher rate than non-essential metals. García-Navarro et al. (2017) declared that the concentration of oligo-elements metals detected in the control group can be considered as optimum for developing the physiological process. Indeed, increasing of Cd, Zn and Cu accumulation in mussels in bioaccumulation tests under laboratory conditions has been reported by some authors (Boudjema et al., 2014; Yap et al., 2003; García-Navarro et al., 2017; Vosloo et al., 2012). As shown for the marine invertebrates, metal toxicity is a result of bioaccumulation, which is a time-dependent process, as well as a specific exposition system (static or dynamic), physiological factors, chemical factors (e.g. bioavailability) and environmental factors (Abdelfattah and Jean-Claude, 2007; Casas and Bacher, 2006; Marcus Zamora et al., 2015; D'Silva and Kureishy, 1978).

It can be concluded that Zn was the most important single-factor of the overall bioaccumulation process since its coefficient were the largest (11.2349 $\mu g\ g^{-1}$ wet weight). Anna Jakimska et al. (2011) declared that the level of zinc will be higher in the soft tissues of mollusc because it is an important element in metabolic processes and constituent of hemocyanin in mollusc.

The bioaccumulation of Cd, Zn and Cu was a linear coloration; it depends on the metal type and metal concentration in the experimental medium. This observation is an agreement with that of Mersch et al. (1993); they found a highly significant linear correlation between Cu and Cd concentrations in the medium and the metal levels in the mussel "*Dreissena polymorpha*". Similarly, Boudjema et al. (2014) observed that the Cd, Pb and Cu bioaccumulations in *Perna perna* were linear and depend on the concentration of heavy metals in the experimental medium in acute toxicity tests during 72 h.

Based on the mean effect of factors, the bioaccumulation selectivity was as following: Zn > Cd > Cu. The presence of the previous heavy metals was well over the two maximum permissible levels fixed by the Algerian reglementation

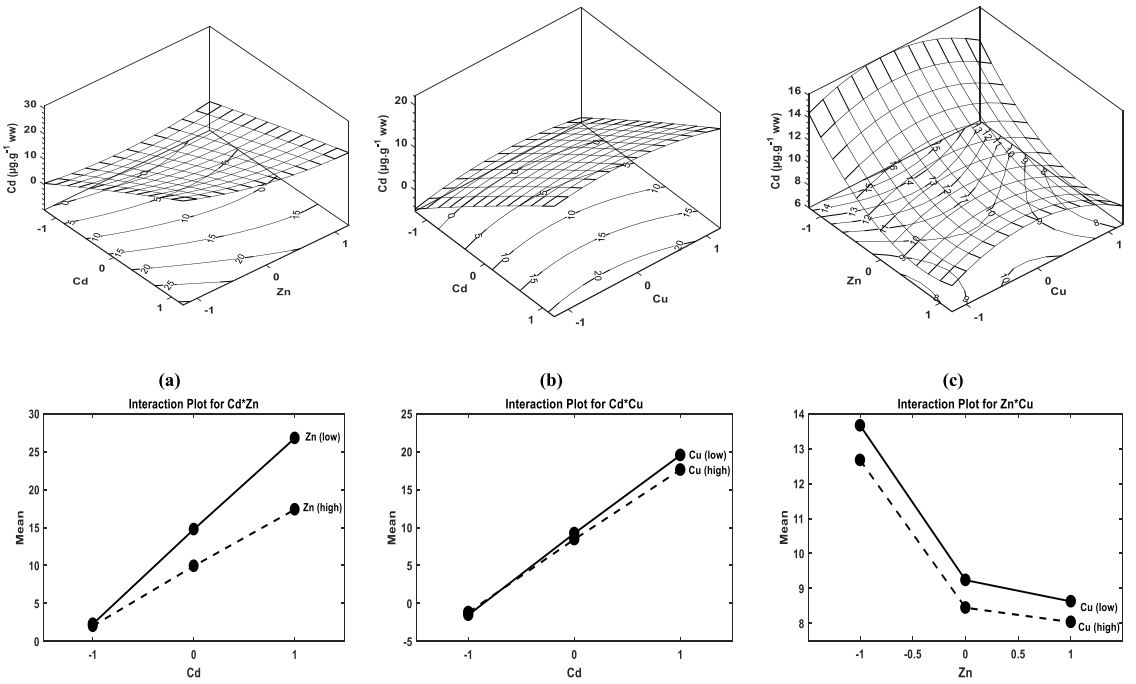


Fig. 1. Effect of Cd, Zn and Cu on Cd bioaccumulation (µg g^{-1} ww); 3D surface graphs associated 2D contour curves and interaction plots: (a) Cd–Zn, (b) Cd–Cu,(c) Zn–Cu.

(JORADP, 2011) and the Turkish Food Codex (2008) (Table 2). In addition, they were not safe within the limits for human consumption. These different levels of metal accumulation and selectivity might be due to the competition between metal ions during uptake and different mechanisms of metal binding and regulation (Pelletier et al., 2004; Yap et al., 2003). Thus, the criterion of ability to accumulate heavy metals, mussels as seafood can induce health risk to consumers.

3.2.3. Optimization of operating conditions and metal–metal interactions identification

The 3D surface graphs associated with the 2D contour curves of the bioaccumulation (heavy metals and indexes) and interaction graphs (Figs. 3 to 5) of the two tested variables were generated from the regression models to describe the toxicological interactions between the independent variables and the optimal biological process parameters.

RSM is one of the most important general methods used in the analysis of combined effects produced by mixtures of heavy metals (Varaksin et al., 2018; Demim et al., 2013). The toxicological interactions occur when the effect of one independent variable changes depending on the level of another independent variable (Rahman, 2019). The one-half of the different values of those effects represent the interaction effect coefficients (β_{12}, β_{13}, β_{23}) in the models (Eqs. (5)–(9)). An interaction is described as synergistic when the exposure to one independent variable causes a dramatic increase in the effect of another. Contrary, an interaction is described as antagonistic when the exposure to one independent variable results in a reduction in the effect of another (Moeller, 2021). Also, in plots for interaction effects (Figs. 3–5) the non-parallel lines are an indicator of the existence of interactions between two factors (synergistic or antagonistic) and parallel lines (or almost parallel) indicate no (zero) interactions between the factors (Antony, 2014). For mild interaction, the lines on the plot do not cross each other (Antony, 2014). In contrast, for strong interaction, the lines on the plot cross each other (Antony, 2014; Hoerl and Snee, 2020). In this study, the metals could interfere and result in synergistic, antagonistic or zero interaction on Cd, Zn and Cu bioaccumulation by mussels. Based on the Pareto chart (supplementary Figure S2), the model coefficients and 2D contour curves (Figs. 3 to 5) lead to identify the types of combined toxicological interactions of independent variables on the bioaccumulation of Cd, Zn and Cu and, on the bioaccumulation indexes (IMBI and MPI).

In the case of Cd bioaccumulation, Fig. 1-a showed antagonistic interaction between X_1 and X_2. The Cd bioaccumulation (in µg g^{-1} wet weight) is maximum when the Cd concentration in experimental medium is at high level and Zn at low level. Moreover, Fig. 1-b and -c showed no interaction effects between X_2 and X_3 and X_1 X_3. In the case of Zn bioaccumulation, Fig. 2-f showed antagonistic interaction between X_1 and X_3, synergetic interaction between X_2 and X_3 (Fig. 2-e) and no interaction effect between X_1 and X_2 (Fig. 2-d). The Zn bioaccumulation (in µg g^{-1} wet weight) is maximum when the Cd concentration in experimental medium is at high level and Zn at middle level ; Cu concentration in experimental medium is at high level and Zn at high level. For Cu bioaccumulation, Fig. 3-k showed antagonistic interaction between X_1 and X_3, synergetic interaction between X_2 and X_3 (Fig. 3-h) and no interaction effect between X_1 and X_2 (Fig. 3-g) . The Cu bioaccumulation (in µg g^{-1} wet weight) is maximum when the Cd concentration in experimental

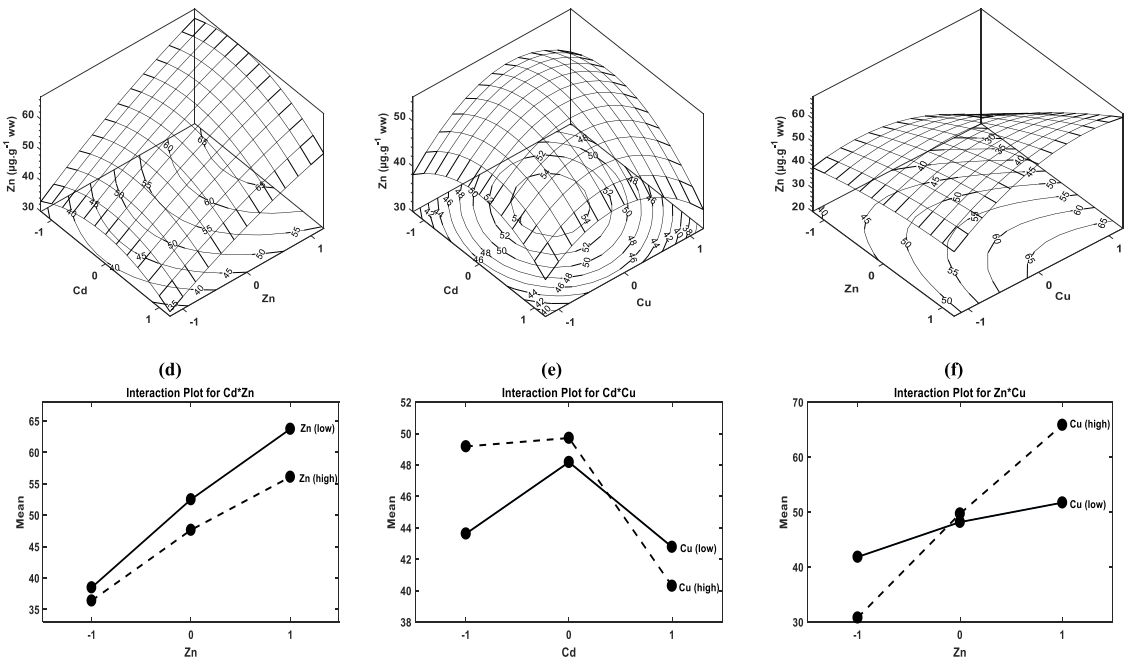


Fig. 2. Effect of Cd, Zn and Cu on Zn bioaccumulation (µg g^{-1} ww); 3D surface graphs associated 2D contour curves and interaction plots: (d) Cd–Zn, (e) Cd–Cu (f) Zn–Cu.

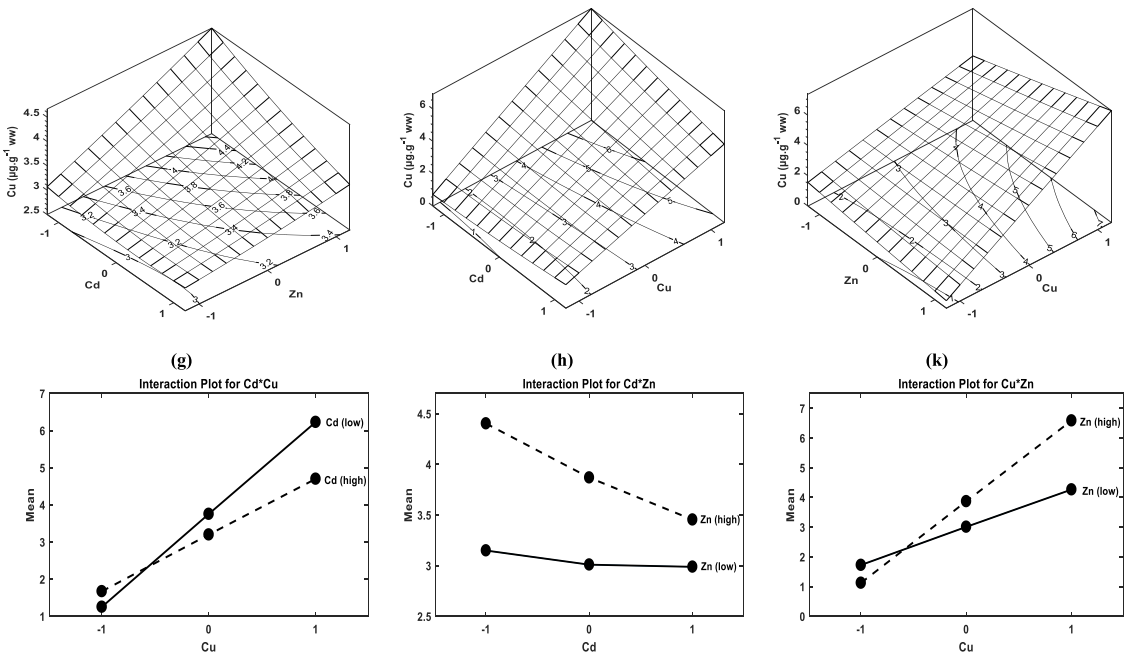


Fig. 3. Effect of Cd, Zn and Cu on Cu bioaccumulation (µg g^{-1} ww); 3D surface graphs associated 2D contour curves and interaction plots: (g) Cd–Zn, (h) Cd–Cu (k) Zn–Cu.

medium is at low level and Cu at high level; Cu concentration in experimental medium is at high level and Zn at high level.

In the case of IMBI bioaccumulation, Fig. 4-l showed antagonistic interaction between X_1 and X_2 and no interaction effect between X_1 and X_2 (Fig. 4-m) and X_2 and X_3 (Fig. 4-n). The IMBI (Unidimensional) is maximum when the Cd

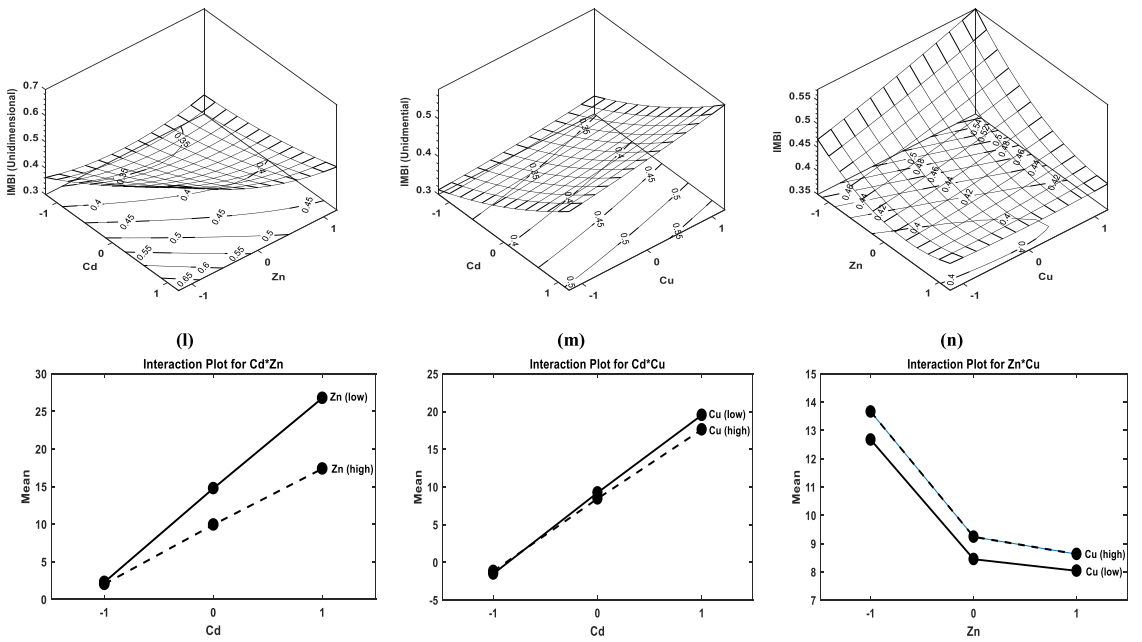


Fig. 4. Effect of Cd, Zn and Cu on IMBI (Unidimensional); 3D surface graphs associated 2D contour curves and interaction plots: (l) Cd–Zn, (m) Cd–Cu (n) Zn–Cu.

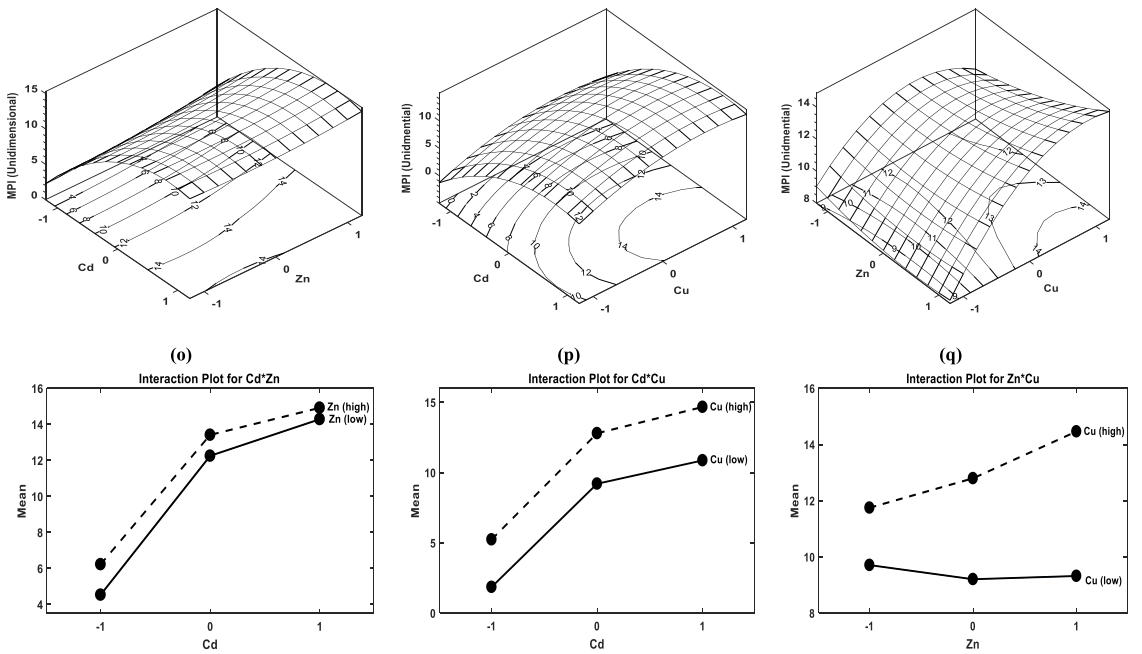


Fig. 5. Effect of Cd, Zn and Cu on MPI (Unidimensional); 3D surface graphs associated 2D contour curves and interaction plots: (o) Cd–Zn, (p) Cd–Cu (q) Zn–Cu.

concentration in experimental medium is at high level and Zn at low level. For MPI bioaccumulation, Fig. 5-o, -p and -k showed no interaction effects between combined factors ($X_1 * X_2$), ($X_1 * X_3$) and ($X_2 * X_3$).

However, the 3D response surfaces of heavy metal bioaccumulation (Cd, Zn and Cu) and bioaccumulation indexes showed clear peaks or optimum zones, suggesting that optimum conditions for maximum Cd, Zn and Cu bioaccumulation and bioaccumulation indexes are well inside the design boundary (Figs. 1–5). Automatic searches for optimal settings of three variables and responses were conducted by running the optimizer through Minitab Statistical Software Trial

Table 3
Optimum values of the bioaccumulation process parameters for maximum bioaccumulation indexes (IMBI and MPI).

	Bioaccumulation indexes					
	IMBI (Unidimensional)			MPI (Unidimensional)		
	Metals exposures (mg L^{-1})					
	Cd	Zn	Cu	Cd	Zn	Cu
	0.260	0	0.01	0.231111	0.186	0.009394
Optimum values	0.6858			16.2312		
Composite desirability	0.933981			0.964204		

version 20 (Minitab Inc., State College, PA, USA). The maximum desirability is found by a Nelder–Mead Simplex, with the fitted response functions to optimize an overall desirability function combining the individual desirability of each response (Kermet-Said and Moulai-Mostefa, 2015; Montgomery, 2013). It was found that the optimal values of the test variables in coded units were: $X_1 = 0.699$, $X_2 = 1$, and $X_3 = 1$ with the corresponding $Y_1 = 12.9191$ μg g^{-1} wet weight, $Y_2 = 59.3794$ μg g^{-1} wet weight and $Y_3 = 5.9467$ μg g^{-1} wet weight for Cd, Zn and Cu, respectively. The original coordinates values were then determined to be $[Cd^{2+}] = 0.2205$ mg L^{-1}, $[Zn^{2+}] = 0.186$ mg L^{-1} and $[Cu^{2+}] = 0.01$ mg L^{-1}. For IMBI, the original coordinates values were then determined to be $[Cd^{2+}] = 1.809438$ mg L^{-1}, $[Zn^{2+}] = 0.186$ mg L^{-1} and $[Cu^{2+}] = 0.01$ mg L^{-1} with the corresponding $Y_4 = 16.2313$. For MBI, the original coordinates values were then determined to be as following: $[Cd^{2+}] = 0.231$ mg L^{-1}, $[Zn^{2+}] = 0.186$ mg L^{-1} and $[Cu^{2+}] = 0.009$ mg L^{-1} with the corresponding $Y_5 = 16.231$. The details of optimization conditions are shown in Tables 2 and 3. As it can be seen, these results confirmed that the RSM could be effectively used to optimize the bioaccumulation process parameters and bioaccumulation indexes in mussels using the combination of bioassay tests and statistical design of experiments.

The bioaccumulation of metals is a natural process required by all living organisms in order to meet their basic needs (Pelletier et al., 2004). Furthermore, the accumulation mechanism of heavy metals occurs as a result of a combination of uptake, metabolism, and excretion processes (Casas and Bacher, 2006). Metals can become toxic when their uptake is not balanced by excretion processes and detoxifying systems (Pelletier et al., 2004; Castaldo et al., 2020). However, most of the laboratory studies about the accumulation of heavy metals were devoted to the impacts induced by each heavy metal acting alone, and less has been done to assess the toxicological interactions (antagonistic, synergistic and no interaction) caused by the mixture of heavy metals (Boudjema et al., 2014; Castaldo et al., 2020; Freitas et al., 2016; García-Navarro et al., 2017; Mersch et al., 1993; Rocha et al., 2015; Vlahogianni and Valavanidis, 2007).

As well, metals are primarily accumulated in two organs in mollusks: the hepatopancreas and kidney; however some elements can be found in other tissues including gills and mantles (Azizi et al., 2018). Furthermore, the uptake and accumulation of metals such as Cd, Cu, and Zn by mussels is linked to the binding of these metal ions to specific ligands where the metals have a higher affinity (Bebianno and Langston, 1999). Two of the best-studied intracellular structures involved in metal sequestration and storage are the metallothioneins (MTs) and the intracellular vesiclebound granules, classified as granules (Azizi et al., 2018). Metallothioneins (MTs) are cysteine-rich metal-binding (storage) proteins with a wide range of biological activities, including essential metal homeostasis, heavy metals (Zn and Cu) and non-essential metal (Cd) detoxification, and cellular antioxidative defense (free radical scavenging) (Anna Jakimska et al., 2011; Mourgaud et al., 2002; Viarengo et al., 1999).

The capacity for metallothionein induction is the greatest in tissues that are active in metal uptake, storage and excretion (Mkandawire et al., 2017). In *Mytilus galloprovincillis*, MTs have been identified in the gills (Salvatore et al., 2008). Binding of metals to metallothionein enhances bioaccumulation in these organs, as well as in the liver and kidney (Azizi et al., 2018). Several studies have shown that interactions between Cd and Zn in the organism stem to a large extent from both metals' affinity for MT and their capacity to stimulate its synthesis in various tissues, including the intestine, liver, and kidney (Brzóska and Moniuszko-Jakoniuk, 2001).

Additionally, experiments performed in vivo on *Mytilus galloprovincialis* mussels or Sprague Dawley rats confirmed the rank of the affinity is not full respected between metal and sulfhydryl groups in vitro that decreases in the hierarchical sequence $Hg^{2+} > Cu^{+}$, Ag^{+}, $Bi^{3+} \gg Cd^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+}$. Also, it was demonstrated that in vivo Cd^{2+}, Cu^{+} or Hg^{2+} may displace Zn^{2+} from MTs. However, in mussel digestive gland, Cu^{2+} is able to displace Zn^{2+}, but not Cd^{2+}, from cadmium- or zinc-thioneins (Funk et al., 1987; Sigel et al., 2015; Vašák, 1991; Viarengo et al., 1999). Bebianno and Langston (1999) reported that, when the mussels were exposed to the same metal concentrations individually Cd and Cu induced metallothionein, while Zn exposure did not. Whereas, Cd accumulated more Cu in the gills of mussels (*Mytilus galloprovincialis*) exposed to single metals (García-Navarro et al., 2017).

According to Gundarker (1999), the differences in the rates of accumulation and depuration indicate that they are the result of internal metal treatment and regulation. In addition, García-Navarro et al. (2017) declared that the bioaccumulation of heavy metals increased significantly in the group exposed to the ternary mixture (Cd + Pb + Cu). On contrary, there was no common response pattern to exposure in single (Cd, Pb and Cu) and binary mixtures (Cd–Pb, Cd–Cu and Pb–Cu).

Table 4
Intervals of variation IMBI and MPI.

Numbers	Color codes	Metal charge	IMBI	MPI
1	Green	Low	[0.34–0.42]	[1.50–4.52]
2	Yellow	Moderate	[0.46–0.49]	[6.62–6.88]
3	Orange	High	[0.49–0.58]	[11.57–13.68]
4	Red	Very high	[0.58–0.71]	[14.84–16.78]

Komjarova and Blust (2009) reported that heavy metals interactions can affect bioaccumulation and toxicity. Further, competition between chemically similar ions (e.g. Cd^{2+} and Zn^{2+}) for binding sites can significantly affect ion bioaccumulation by mussels (Azizi et al., 2018). When the exposure process is simultaneous, Tao et al. (1999) suggested that copper and lead uptake by fish have a synergistic effect, which means that Pb appears to facilitate Cu uptake and vice versa. Hemelraad et al. (1987) found that Zn inhibited Cd uptake (Antagonistic interaction Zn–Cd) in freshwater mussels' gills and accelerated Cd redistribution from the gills to internal organs. Furthermore, the presence of Cu can decrease Cd uptake (Komjarova and Blust, 2009).

The toxic effects of Cd action are caused by interactions with essential components such as Zn. These interactions can occur at various phases of metal uptake, distribution, and excretion, as well as at the stage of Zn biological functions (Brzóska and Moniuszko-Jakoniuk, 2001). In addition, Cd and Zn have a high affinity to biological structures (proteins, enzymes) containing -SH (sulphydryl) groups and a similar electron configuration on their outer shells. Therefore, a competition between Zn^{2+} and Cd^{2+} ions is predictable for the uptake (Brzóska and Moniuszko-Jakoniuk, 2001). Farther, the results of Fowler and Benayoun (1974) do not show evidence of any influence of Zn on the uptake of Cd by *Mytilus galloprovincialis*. In this work, the toxicological interaction between heavy metals on bioaccumulation may be interpreted by the competition effects at different phases of metal uptake, accumulation, distribution and excretion of metal with the presence of other metals. In addition, Stewart (2011) found an antagonistic effect of heavy metals (Ni, Cu, Pb and Cu) on Cd accumulation in freshwater mussel *P. grandis*.

According to Pelletier et al. (2004), the toxicological action of a metal, once accumulated, profit from its abnormal interaction with an essential biomolecule. In our results, the toxicity of Cd, Zn and Cu may be interpreted by cellular cytotoxicity (or spillover) models. Giguère Couillard et al. (2003) declared that, at low chronic exposure concentrations, the presence of Cd in the low molecular weight (LMW) and high molecular weight (HMW) fractions, indicates that metal detoxification is incomplete in *P. garandis*; it is subjected to some Cd related stress.

The acute toxicity may be due to the imperfect detoxification of metals, which suggests that the *Mytilus galloprovincialis* is subjected to some Cd, Zn and Cu related oxidative stress at acute exposure concentrations. The optimal conditions of bioaccumulation may be correspond to the saturation of detoxification mechanism of metallothionein (MT) followed by metal "spillover" into the HMW and LMW pools above a threshold free-ion activity (Table 2).

The bioaccumulation indexes (IMBI and MPI) values are used *in situ* and laboratory experiments to estimate the variation of trace metal pollution based on the metal content (metal charge) in tissues of aquatic organisms, which makes it possible to differentiate between contaminated and unpolluted ecosystems (Kontas et al., 2021; Mohamed Harris et al., 2019; Esteve et al., 2012; Teodorović et al., 2000; Öğlü et al., 2015). According to Maes et al. (2005), the low and high polluted individuals have the IMBI lower 0.22 and upper 0.25, respectively. Whereas, according to Usero et al. (2005), the MPI values in the bivalve *Donax trunculus* (based on Cu, Pb, Zn, Ni, Cr, and Cd) varied from roughly 3.4 to 3.8 and from approximately 6.8 to 7.8 for unpolluted and contaminated regions respectively.

The relationship between the total content of metals in mussel soft tissues and the level of ternary metal mixtures exposure was highlighted by the bioaccumulation indexes (IMBI and MPI) (Run1 to Run 17, Table 1). The IMBI values of mussel tissues ranged between 0.346 and 0.710, which showed a small range of variations (Table 1). High IMBI values were recorded in the mussels tissues at Run 6 ($X_1 = +1$, $X_2 = -1$, $X_3 = +1$ which correspond to Cd = 0.260 mg L^{-1}, Zn = 0 mg L^{-1}, Cu = 0.01 mg L^{-1}, Table 1) indicating higher metal content, while low values were found in the mussels tissues at Run 1 ($X_1 = X_2 = X_3 = -1$ which corresponded to Cd = Zn = Cu = 0 mg L^{-1}, Table 1) and Run 3 ($X_1 = -1$, $X_2 = +1$, $X_3 = -1$ which corresponded to Cd = 0 mg L^{-1}, Zn = 0.1866 mg L^{-1}, Cu = 0 mg L^{-1}, Table 1) indicating lower metal content .

Mussel tissues MPI values ranged from 1.500 to 16.77, which showed a large range of variations (Table 1). The lowest MPI value was found in control (unexposed) mussels as a baseline level (Run 1: $X_1 = X_2 = X_3 = -1$ which correspond to Cd = Zn = Cu = 0 mg L^{-1}, Table 1) indicating lower metal content. The highest MPI was found in mussels exposed to ternary metal mixtures of Cd = 0.260 mg L^{-1}, Zn = 0.186 mg L^{-1} and Cu = 0.01 mg L^{-1} which correspond to $X_1 = X_2 = X_3 = +1$ (Run 8, Table 1), indicating higher metal content in the tissues of mussels *Mytilus galloprovincialis*. Overall, the IMBI values followed the same pattern of the MPI variations and both can differentiate polluted (Run 2 to Run 17, Table 1) from unpolluted mediums (Run 1, Table 1).

As shown in Table 4, IMBI and MPI (based on three metals) can be used as good tools usable for risk assessment to identify metal charge in *Mytilus galloprovincialis* under laboratory conditions by employing color codes: low metal charge (green), moderate metal charge (yellow), high metal charge (orange), very high metal charge (Red).

4. Conclusion

The evaluation of the short-term sublethal effects of cadmium (Cd), zinc (Zn) and copper (Cu) was carried out *via* the bioaccumulation in *Mytilus galloprovincialis* tissues and the determination of the bioaccumulation indexes (IMBI and MPI) using RSM and in particular the CCF design. The obtained results showed clearly that RSM was one of the suitable methods to characterize and predict the bioaccumulation of heavy metals under laboratory conditions. In addition, the bioaccumulation of heavy metals and the indexes IMBI and MPI were found to be dependent on the type and concentration of metals, and also on their combination. Under optimal conditions, the maximum values of Cd, Zn and Cu bioaccumulation were respectively, 12.9191 $\mu g\ g^{-1}$ wet weight, 59.3794 $\mu g\ g^{-1}$ wet weight, 5.9467 $\mu g\ g^{-1}$ wet weight. In addition, the values of IMBI and MPI were found equal to 0.6858 units and 16.2312 units. These statements are still valid in all level factor ranges of initial concentrations: Cd^{2+} (0%–10% 72 h-LC_{50}), Zn^{2+} (0%–10% 72 h-LC_{50}) and Cu^{2+}(0%–10% 72 h-LC_{50}). The developed quadratic models showed the presence of high correlations between experimental and predicted values. ANOVA showed also high coefficients of determination values, thus ensuring a satisfactory adjustment of the second-order regression model with the experimental data.

Finally, the bioaccumulation indexes (IMBI and MPI) models approach associated with the bioaccumulation of heavy metals in mussels are very promising for future coastal sites monitoring, classification and heavy metals risk assessment .

CRediT authorship contribution statement

Kamel Boudjema: Conceptualization, Writing – original draft, Analysis and interpretation of data, Software, Modelization. **Abdelmalek Badis:** Supervision, Methodology, Review and editing. **Nadji Moulai-Mostefa:** Validation, Methodology, Review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2021.102202.

References

Abdelfattah, M., Jean-Claude, P., 2007. Le test comportemental de dreissena polymorpha: un outil biologique de prévision et d'évaluation de la toxicité en milieu d'eau douce. Hydroécol. Appl. 5, 97–109. http://dx.doi.org/10.1051/hydro:1993105.

Abderrahmani, K., Boulahdid, M., Bendou, N., Aissani, A., 2020. Seasonal distribution of cadmium, lead, nickel, and magnesium in several tissues of mussels from the Algerian coasts. Environ. Sci. Pollut. Res. 27, 22547–22567. http://dx.doi.org/10.1007/s11356-020-08682-8.

AIEA, 2004. Training workshop on the analysis of trace metals in biological and sediment samples. In: Laboratory Procedure Book. International Atomic Energy Agency marine environment Laboratory, p. 45.

Ait Fdil, M., Mouabad, A., Outzourhit, A., Benhra, A., Maarouf, A., Pihan, J.C., 2006. Valve movement response of the mussel mytilus galloprovincialis to metals (Cu, Hg, Cd and Zn) and phosphate industry effluents from Moroccan Atlantic coast. Ecotoxicol 15, 477–486. http://dx.doi.org/10.1007/s10646-006-0083-3.

Ali, H., Khan, E., Ilahi, I., 2019. Environmental chemistry and ecotoxicology of hazardous heavy metals: environmental persistence, toxicity and bioaccumulation. J. Chem. 4, 1–14. http://dx.doi.org/10.1155/2019/6730305.

Anderson, M.J., Whitcomb, Patrick J., 2007. DOE Simplified: Practical Tools for Effective Experimentation. CRC Press, New York.

Anna Jakimska, P.K., Skóra, Krzysztof, Namieśnik, Jacek, 2011. Bioaccumulation of metals in tissues of marine animals, part I: the role and impact of heavy metals on organisms. Pol. J. Environ. Stud. 20, 1117–1125.

Antony, J., 2014. Understanding key interactions in processes. In: Antony, J. (Ed.), Design of Experiments for Engineers and Scientists, second ed. Elsevier, Oxford, pp. 19–32.

Azizi, G.M.A., Baghour, M., Mostafa, L., Moumen, A., 2018. The use of mytilus spp. mussels as bioindicators of heavy metal pollution in the coastal environment. A review. J. Mater. Environ. Sci. 9, 1170–1181. http://dx.doi.org/10.26872/jmes.2018.9.4.129.

Bachouche, S., Houma, F., Gomiero, A., Rabah, B., 2017. Distribution and environmental risk assessment of heavy metal in surface sediments and red mullet (Mullus barbatus) from Algiers and BouIsmail Bay (Algeria). Environ. Model. Assess. 22, 473–490. http://dx.doi.org/10.1007/s10666-017-9550-x.

Barath Kumar, S., Padhi, R.K., Satpathy, K.K., 2019. Trace metal distribution in crab organs and human health risk assessment on consumption of crabs collected from coastal water of south east coast of India. Mar. Pollut. Bull. 141, 273–282. http://dx.doi.org/10.1016/j.marpolbul.2019.02.022.

Barrick, A., Marion, J.M., Perrein-Ettajani, H., Châ, A., Mouneyrac, C., 2018. Baseline levels of biochemical biomarkers in the endobenthic ragworm hediste diversicolor as useful tools in biological monitoring of estuaries under anthropogenic pressure. Mar. Pollut. Bull. 129, 81–85. http://dx.doi.org/10.1016/j.marpolbul.2018.02.006.

Bebianno, M.J., Langston, W.J., 1999. Metallothionein induction in mussels exposed to a metal mixture. In: Klaassen, C.D. (Ed.), Metallothionein IV. Birkhäuser Basel, Basel, pp. 187–194. http://dx.doi.org/10.1016/j.aquatox.2015.10.001.

Belhadj, H., Aubert, D., Dali Youcef, N., 2017. Geochemistry of major and trace elements in sediments of Ghazaouet Bay (western Algeria): An assessment of metal pollution. C.R. Geosci. 8, 412–421. http://dx.doi.org/10.1016/j.crte.2017.09.013.

Benfares, R., Seridi, H., Belkacem, Y., Inal, A., 2015. Heavy metal bioaccumulation in brown algae cystoseira compressa in Algerian coasts, mediterranean sea. Environ. Processes 2, 429–439. http://dx.doi.org/10.1007/s40710-015-0075-5.

Boudjema, K., Kourdali, S., Bounakous, N., Meknachi, A., Badis, A., 2014. Catalase activity in brown mussels (perna perna) under acute cadmium, lead, and copper exposure and depuration tests. J. Mar. Biol. 2014, 1–10. http://dx.doi.org/10.1155/2014/830657.

Boudjema, K., Meknachi, A., Kourdali, S., Bounakous, N., Badis, A., 2016. Effect of sublethal concentrations of heavy metals (cadmium, lead, and copper) on the soluble nitrogen and phosphorus excretion of marine brown mussel (Perna perna) (Linnaeus, 1758) (Mollusca; Bivalvia). Int. J. Fish. Aquat. Stud. 4, 455–462.

Brzóska, M.M., Moniuszko-Jakoniuk, J., 2001. Interactions between cadmium and zinc in the organism. Food and Chem. Toxicol. 39, 967–980. http://dx.doi.org/10.1016/s0278-6915(01)00048-5.

Casas, S., Bacher, C., 2006. Modelling trace metal (Hg and Pb) bioaccumulation in the Mediterranean mussel, Mytilus galloprovincialis, applied to environmental monitoring. J. Sea Res. 56, 168–181. http://dx.doi.org/10.1016/j.seares.2006.03.006.

Castaldo, G., Pillet, M., Slootmaekers, B., Bervoets, L., Town, R.M., Blust, R., De Boeck, G., 2020. Investigating the effects of a sub-lethal metal mixture of Cu, Zn and Cd on bioaccumulation and ionoregulation in common carp, Cyprinus carpio. Aquat. Toxicol. 218, 105363. http://dx.doi.org/10.1016/j.aquatox.2019.105363.

Chan, H.M., 1988. Accumulation and tolerance to cadmium, copper, lead and zinc by the green mussel Perna viridis. Mar. Ecol. Prog. Ser. 48, 295–303.

Demim, S., Drouiche, N., Aouabed, A., Semsari, S., 2013. Ccd study on the ecophysiological effects of heavy metals on Lemna gibba. Ecol. Eng. 57, 302–313. http://dx.doi.org/10.1016/j.ecoleng.2013.04.041.

D'Silva, C., Kureishy, T.W., 1978. Experimental studies on the accumulation of copper and zinc in the green mussel. Mar. Pollut. Bull. 9, 187–190. http://dx.doi.org/10.1016/0025-326X(78)90176-5.

EI-Komi, M.M., Tayel Fathy, T., 1996. Acute toxicity of four heavy metals to Sphaeroma walkeri and Ciroiana bovina (Crustacea:Isopoda). Pak. J. Mar. Sci. 5, 15–23.

Eisler, R., 1977. Acute toxicities of selected heavy metals to the softshell clam, Mya arenaria. Bull. Environ. Contam. Toxicol. 17, 137–145. http://dx.doi.org/10.1007/BF01685540.

Esteve, C., Alcaide, E., Ureña, R., 2012. The effect of metals on condition and pathologies of European eel (Anguilla anguilla): In situ and laboratory experiments. Aquat. Toxicol. 109, 176–184. http://dx.doi.org/10.1016/j.aquatox.2011.10.002.

Fowler, S.W., Benayoun, G., 1974. Experimental studies on cadmium flux throught marine. biota. Proc. Symp. Comp. Studies Food Environ. Contamin. IAEA, Vienna.

Freitas, R., Salamanca, L., Velez, C., Wrona, F.J., Soares, A.M.V.M., Figueira, E., 2016. Multiple stressors in estuarine waters: Effects of arsenic and salinity on ruditapes philippinarum. Sci. Total Environ. 541, 1106–1114. http://dx.doi.org/10.1016/j.scitotenv.2015.09.149.

Funk, A.E., Day, F.A., Brady, F.O., 1987. Displacement of zinc and copper from copper-induced metallothionein by cadmium and by mercury: In vivo and ex vivo studies. Comp. Biochem. Physiol. C: Part C: Comp. Pharmacol. 86, 1–6. http://dx.doi.org/10.1016/0742-8413(87)90133-2.

García-Navarro, J.A., Franco, L., Romero, D., 2017. Differences in the accumulation and tissue distribution of Pb, Cd, and Cu in Mediterranean mussels (Mytilus galloprovincialis) exposed to single, binary, and ternary metal mixtures. Environ. Sci. Pollut. Res. 24, 6599–6610. http://dx.doi.org/10.1007/s11356-016-8349-7.

Genç, T.O., Yilmaz, F., 2017. Metal accumulations in water, sediment, crab (Callinectes sapidus) and two fish species (Mugil cephalus and Anguilla anguilla) from the köyceğiz lagoon system-turkey: an index analysis approach. Bull. Environ. Contam. Toxicol. 99, 173–181. http://dx.doi.org/10.1007/s00128-017-2121-7.

Gheorghe, S., Stoica, C., Vasile, G.G., Nita-Lazar, M., Stanescu, E., Lucaciu, I.E., 2017. Metals toxic effects, aquatic ecosystems: modulators of water quality. In: Hlanganani, T. (Ed.), Water Quality. IntechOpen, London, pp. 60–89.

Giguère Couillard, Y., Campbell, P.G.C., Perceval, O., Hare, L., Pinel-Alloul, B., Pellerin, J., 2003. Steady-state distribution of metals among metallothionein and other cytosolic ligands and links to cytotoxicity in bivalves living along a polymetallic gradient. Aquat. Toxicol. 64, 185–200. http://dx.doi.org/10.1016/S0166-445X(03)00052-3.

Goupy, J., Creighton, L., 2006. Introduction Aux Plans D'Expériences, 3e édition avec applications, Dunod, Paris.

Gundarker, C., 1999. Tissue-specific heavy metal (Cd, Pb, Cu, Zn) deposition in a natural population of the zebra mussel Dreissena polymorpha pallas. Chemosphere 38, 3339–3356. http://dx.doi.org/10.1016/S0045-6535(98)00567-0.

Hemelraad, J., Kleinveld, H.A., de Roos, A.M., Holwerda, D.A., Zandee, D.I., 1987. Cadmium kinetics in freshwater clams. III. Effects of zinc on uptake and distribution of cadmium in Anodonta cygnea. Arch. Environ. Contam. Toxicol. 16, 95–101. http://dx.doi.org/10.1007/BF01055364.

Hoerl, R.W., Snee, R.D., 2020. Statistical Thinking Improving Business Performance, third ed. CRC., New York.

Jakimska, A., Konieczka, P., Skóra, K., Namieśnik, J., 2011. Bioaccumulation of metals in tissues of marine animals, part I: the role and impact of heavy metals on organisms. Pol. J. Environ. Stud. 20, 1117–1125.

JORADP, 2011. Arrêté interministériel du 30 moharram 1432 correspondant au 5 janvier 2011 fixant les seuils limites de présence de contaminants chimiques, microbiologiques et toxicologiques dans les produits de la pêche et de l'aquaculture. J. Off. Republ. Alger. 25, 23.

Jovic, M., Onjia, A., Stankovic, S., 2012. Toxic metal health risk by mussel consumption. Environ. Chem. Lett. 10, 69–77. http://dx.doi.org/10.1007/s10311-011-0330-6.

Kamaruzzaman, B.Y., Mohd Zahir, M.S., Akbar John, B., Jalal, K.C.A., Shahbudin, S., Al-Barwani, S.M., Goddard, J.S., 2011. Bioaccumulation of some metals by green mussel Perna viridis (Linnaeus 1758) from Pekan, Pahang, Malaysia. Int. J. Biol. Chem. 5, 54–60. http://dx.doi.org/10.3923/ijbc.2011.54.60.

Karian, Z.A., Dudewicz, E.J., 2016. Handbook of Fitting Statistical Distributions with R. First Ed. Chapman and Hall, New York.

Katsnelson, B.A., Minigaliyeva, I.A., Panov, V.G., Privalova, L.I., Varaksin, A.N., Gurvich, V.B., Sutunkova, M.P., Shur, V.Y., Shishkina, E.V., Valamina, I.E., Makeyev, O.H., 2015. Some patterns of metallic nanoparticles combined subchronic toxicity as exemplified by a combination of nickel and manganese oxide nanoparticles. Food Chem. Toxicol. 86, 351–364. http://dx.doi.org/10.1016/j.fct.2015.11.012.

Kermet-Said, H., Moulai-Mostefa, N., 2015. Optimization of turbidity and COD removal from pharmaceutical wastewater by electrocoagulation. Isotherm modeling and cost analysis. Pol. J. Environ. Stud. 24, 1049–1061. http://dx.doi.org/10.15244/pjoes/32334.

Komjarova, I., Blust, R., 2009. Multimetal interactions between cd, cu, Ni, pb, and Zn uptake from water in the zebrafish danio rerio. Environ. Sci. Technol. 43, 7225–7229. http://dx.doi.org/10.1021/es900587r.

Kontas, A., Alyuruk, H., Bilgin, M., Uluturhan, E., Ünlüoğlu, A., Darilmaz, E., Altay, O., 2021. Metal bioaccumulation and potential health risk assessment in different tissues of three commercial fish species (merluccius merluccius, Mullus barbatus, and Pagellus erythrinus) from Edremit Bay (Aegean Sea), Turkey. Biol. Trace Elem. Res. 1, 1–13. http://dx.doi.org/10.1007/s12011-021-02683-5.

Krishnakumar, P.K., Damodaran, R., Nambisan, P.N.K., 1987. Acute toxicity of selected heavy metals to green mussel Perna viridis. Indian J. Geo. Mar. Sci. 16, 263–264.

Lacroix, C., Richard, G., Seguineau, C., Guyomarch, J., Moraga, D., Auffret, M., 2015. Active and passive biomonitoring suggest metabolic adaptation in blue mussels (Mytilus spp.) chronically exposed to a moderate contamination in Brest harbor (France). Aqua. Toxicol. 162, 126–137. http://dx.doi.org/10.1016/j.aquatox.2015.03.008.

Landrum, P.F., Lee, H., Lydy, M.J., 1992. Toxicokinetics in aquatic systems: model comparisons and use in hazard assessment. Environ. Toxicol. Chem. 11, 1–17. http://dx.doi.org/10.1002/etc.5620111205.

Lodhi, H.S., Khan, M.A., Verma, R.S., Sharma, U.D., 2006. Acute toxicity of copper sulphate to fresh water prawns. J. Environ. Biol. 27, 585–588.

Mackay, D., Arnot, J.A., Gobas, F.A.P.C., Powell, D.E., 2013. Mathematical relationships between metrics of chemical bioaccumulation in fish. Environ. Toxicol. Chem. 32, 1459–1466. http://dx.doi.org/10.1002/etc.2205.

Maes, G.E., Raeymaekers, J.A.M., Pampoulie, C., Seynaeve, A., Goemans, G., Belpaire, C., Volckaert, F.A.M., 2005. The catadromous European eel Anguilla anguilla (L.) as a model for freshwater evolutionary ecotoxicology: Relationship between heavy metal bioaccumulation, condition and genetic variability. Aquat. Toxicol. 73, 99–114. http://dx.doi.org/10.1016/j.aquatox.2005.01.010.

Marcus Zamora, L., King, C.K., Payne, S.J., Virtue, P., 2015. Sensitivity and response time of three common Antarctic marine copepods to metal exposure. Chemosphere 120, 267–272. http://dx.doi.org/10.1016/j.chemosphere.2014.07.051.

Mersch, J., Morhain, E., Mouvet, C., 1993. Laboratory accumulation and depuration of copper and cadmium in the freshwater mussel Dreissena polymorpha and the aquatic moss Rhynchostegium riparioides. Chemosphere 27, 1475–1485. http://dx.doi.org/10.1016/0045-6535(93)90242-W.

Mkandawire, E., Mierek-Adamska, A., Stürzenbaum, S.R., Choongo, K., Yabe, J., Mwase, M., Saasa, N., Blindauer, C.A., 2017. Metallothionein from wild populations of the african catfish clarias gariepinus: from sequence, protein expression and metal binding properties to transcriptional biomarker of metal pollution. Int. J. Mol. Sci. 18, 1–10. http://dx.doi.org/10.3390/ijms18071548.

Moeller, D.W., 2021. Environmental Health, third ed. Harvard University Press, London.

Mohamed Harris, J., Vinobaba, P., Kularatne, R.K.A., Kankanamge, C.E., 2019. An assessment of heavy metal levels in brackish water shrimps: Impact on sexes and the relationship between metal pollution index and Fulton's K condition indices. Hum. Ecol. Risk Assess. 25, 1968–1979. http://dx.doi.org/10.1080/10807039.2018.1478717.

Montgomery, D.C., 2013. Design and Analysis of Experiments, eighth ed. Wiley, Hoboken, New Jersey.

Moriarty, F., Walker, C.H., 1987. Bioaccumulation in food chains-A rational approach. Ecotoxicol. Environ. Saf. 13, 208–215. http://dx.doi.org/10.1016/0147-6513(87)90009-1.

Mourgaud, Y., Martinez, E., Geffard, A., Andral, B., Stanisiere, J.Y., Amiard, J.C., 2002. Metallothionein concentration in the mussel Mytilus galloprovincialis as a biomarker of response to metal contamination: validation in the field. Biomarkers 7, 479–490. http://dx.doi.org/10.1080/1354750021000034528.

Mustafa, K., 2014. Elimination of essential (Cu, Zn) and non-essential (Cd, Pb) metals from tissues of a freshwater fish Tilapia zilli. Turk. J. Zool. 4, 429–436.

Myers, R.H., Anderson-Cook, C.M., Montgomery, D.C., 2009. Response Surface Methodology Process and Product Optimization using Designed Experiments, first ed. Wiley, Hoboken, New Jersey.

Öğlü, B., Yorulmaz, B., genç, T., Yılmaz, F., 2015. The assessment of heavy metal content by using bioaccumulation indices in European chub, squalius cephalus (linnaeus, 1758). Carpathian J. Earth Environ. Sci. 10, 85–94.

Pelletier, E., Denizeau, F., Campbell, P.G.C., 2004. écotoxicologie Moléculaire : Principes Fondamentaux Et Perspectives de Développement, first ed. Presses de l'université de Québec., Québec.

Prato, E., Biandolino, F., Scardicchio, C., 2006. Test for acute toxicity of copper, cadmium, and mercury in five marine species. Turk. J. Zool. 30, 1–6.

Radomyski, A., Giubilato, E., Suciu, N.A., Critto, A., Ciffroy, P., 2018. Modelling bioaccumulation in aquatic organisms and in mammals. Handb. Environ. Chem. 57, 191–214. http://dx.doi.org/10.1007/978-3-319-59502-3-9.

Rahman, A.S., 2019. Effects of nanofibers on properties of geopolymer composites. In: Pacheco-Torgal, F., Diamanti, M.V., Nazari, A., Granqvist, C.G., Pruna, A., Amirkhanian, S. (Eds.), Nanotechnology in Eco-Efficient Construction, second ed. Woodhead Publishing, pp. 123–140.

Ramakritinan, C.M., Chandurvelan, R., Kumaraguru, A.K., 2012. Acute toxicity of metals: Cu, Pb, Cd, Hg and Zn on marine molluscs, Cerithedia cingulata G. Modiolus philippinarum H. Indian. J. Geo Mar. Sci. 41, 141–145.

Rocha, T.L., Gomes, T., Mestre, N.C., Cardoso, C., Bebianno, M.J., 2015. Tissue specific responses to cadmium-based quantum dots in the marine mussel Mytilus galloprovincialis. Aquat. Toxicol. 169, 10–18. http://dx.doi.org/10.1016/j.aquatox.2015.10.001.

Ronci, L., Meccoli, L., Iannilli, V., Menegoni, P., De Matthaeis, E., Setini, A., 2016. Comparison between active and passive biomonitoring strategies for the assessment of genotoxicity and metal bioaccumulation in echinogammarus veneris (Crustacea: Amphipoda). Ital. J. Zool. 83, 162–172. http://dx.doi.org/10.1080/11250003.2016.1169321.

Rosioru, D.M., Oros, A., Lazar, L., 2016. Assessment of the heavy metals contamination in bivalve Mytilus galloprovincialis using accumulation factors. J. Environ. Prot. Ecol. 17, 874–884.

Rouane-Hacene, O., Boutiba, Z., Belhaouari, B., Guibbolini-Sabatier, M.E., Francour, P., Risso-de Faverney, C., 2015. Seasonal assessment of biological indices, bioaccumulation and bioavailability of heavy metals in mussels Mytilus galloprovincialis from Algerian west coast, applied to environmental monitoring. Oceanologia 57, 362–374. http://dx.doi.org/10.1016/j.oceano.2015.07.004.

Salvat-Leal, I., Verdiell, D., Parrondo, P., Barcala, E., Romero, D., 2020. Assessing lead and cadmium pollution at the mouth of the river Segura (SE Spain) using the invasive blue crab (Callinectes sapidus Rathbun, 1896, Crustacea, Decapoda, Portunidae) as a bioindicator organism. Reg. Stud. Mar. Sci. 40, 101521. http://dx.doi.org/10.1016/j.rsma.2020.101521.

Salvatore, F., Mauceri, A., Giannetto, A., Maisano, M., Bianchi, N., Parrino, V., 2008. Expression of metallothionein mrnas by in situ hybridization in the gills of Mytilus galloprovincialis, from natural polluted environments. Aquat. Toxicol. 88, 62–68. http://dx.doi.org/10.1016/j.aquatox.2008.03.009.Epub2008Mar21.

Sereshti, H., Heravi, Y.Entezari., Samadi, S., 2012. Optimized ultrasound-assisted emulsification microextraction for simultaneous trace multielement determination of heavy metals in real water samples by ICP-OES. Talanta 97, 235–241. http://dx.doi.org/10.1016/j.talanta.2012.04.024.

Sharifuzzaman, S.M., Rahman, H., Ashekuzzaman, S.M., Islam, M.M., Chowdhury, S.R., Hossain, M.S., 2016. Heavy metals accumulation in coastal sediments. In: Hasegawa, H., Rahman, I.M.M., Rahman, M.A. (Eds.), Environmental Remediation Technologies for Metal-Contaminated Soils. Springer Japan, Tokyo, pp. 21–42.

Sigel, A., Sigel, H., Sigel, R.K.O., 2015. Metallothioneins and Related Chelators. RSC publishing, Cambridge, United Kingdom.

Solanki, A.B., Parikh, J.R., Parikh, R.H., 2007. Formulation and optimization of piroxicam proniosomes by 3-factor, 3-level box-behnken design. AAPS. Pharm. Sci. Tech. 8, 43. http://dx.doi.org/10.1208/pt0804086.

Stewart, A., 2011. Accumulation of Cd by a freshwater mussel (Pyganodon grandis) is reduced in the presence of Cu, Zn, Pb, and Ni. Can. J. Fish. Aquat. Sci. 56, 467–478. http://dx.doi.org/10.1139/f98-180.
Sze, P.W.C., Lee, S.Y., 1995. The potential role of mucus in the depuration of copper from the mussels Perna viridis (L.) and Septifer virgatus (Wiegmann). Mar. Pollut. Bull. 31, 390–393. http://dx.doi.org/10.1016/0025-326X(95)00140-I.
Tao, S., Liang, T., Cao, J., Dawson, R.W., Liu, C., 1999. Synergistic effect of copper and lead uptake by fish. Ecotoxicol. Environ. Saf. 44, 190–195. http://dx.doi.org/10.1006/eesa.1999.1822.
Tayeb, A., Chellali, M.R., Hamou, A., Debbah, S., 2015. Impact of urban and industrial effluents on the coastal marine environment in Oran, Algeria. Mar. Pollut. Bull. 98, 281–288. http://dx.doi.org/10.1016/j.marpolbul.2015.07.013.
Teodorović, I., Djukic, N., Maletin, S., Miljanović, B., Jugovac, N., 2000. Metal pollution index: Proposal for freshwater monitoring based on trace metal accumulation in fish. Tiscia 32, 55–60.
Turkish Food Codex, 2008. Turkish food codex communique on determining the maximum levels of certain contaminants in foodstuffs. In: The Official Gazette, Vol. 17. p. 26879.
Usero, J., Morillo, J., Gracia, I., 2005. Heavy metal concentrations in molluscs from the Atlantic coast of southern Spain. Chemosphere 59, 1175–1181. http://dx.doi.org/10.1016/j.chemosphere.2004.11.089.
Varaksin, A.N., Panov, V.G., Katsnelson, B.A., Minigalieva, I.A., 2018. Using various nonlinear response surfaces for mathematical description of the type of combined toxicity. Dose-Response: Int. J. 16. http://dx.doi.org/10.1177/1559325818816596.
Vašák, M., 1991. Metal removal and substitution in vertebrate and invertebrate metallothioneins. Methods Enzymol. 205, 452–458. http://dx.doi.org/10.1016/0076-6879(91)05130-n.
Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., Blasco, J., 1999. Role of metallothionein against oxidative stress in the mussel Mytilus galloprovincialis. Am. J. Physiol 277, R1612–1619. http://dx.doi.org/10.1152/ajpregu.1999.277.6.R1612.
Vlahogianni, T.H., Valavanidis, A., 2007. Heavy-metal effects on lipid peroxidation and antioxidant defence enzymes in mussels Mytilus galloprovincialis. Chem. Ecol. 23, 361–371. http://dx.doi.org/10.1080/02757540701653285.
Vosloo, D., Sara, J., Vosloo, A., 2012. Acute responses of brown mussel (Perna perna) exposed to sub-lethal copper levels: Integration of physiological and cellular responses. Aquat. Toxicol. 106–107, 1–8. http://dx.doi.org/10.1016/j.aquatox.2011.10.001.
Yap, C.K., Ismail, A., Omar, H., Tan, S.G., 2004. Toxicities and tolerances of Cd, Cu, Pb and Zn in a primary producer (Isochrysis galbana) and in a primary consumer (Perna viridis). Environ. Int. 29, 1097–1104. http://dx.doi.org/10.1016/S0160-4120(03)00141-7.
Yap, C.K., Ismail, A., Tan, S., Omar, H., 2003. Accumulation, depuration and distribution of cadmium and zinc in the green-lipped mussel perna viridis (Linnaeus) under laboratory conditions. Hydrob 498, 151–160. http://dx.doi.org/10.1023/A:1026221930811.
Yetilmezsoy, K., Demirel, S., Vanderbei, R.J., 2009. Response surface modeling of Pb (II) removal from aqueous solution by Pistacia vera L.: Box-behnken experimental design. J. Hazard. Mater. 171, 551–562. http://dx.doi.org/10.1016/j.jhazmat.2009.06.035.
Zaghloul, A., Saber, M., Gadow, S., Awad, F., 2020. Biological indicators for pollution detection in terrestrial and aquatic ecosystems. Bull. Natl. Res. Cent. 44, 127. http://dx.doi.org/10.1186/s42269-020-00385-x.
Zaldívar, J.-M., 2008. A General Bioaccumulation DEB Model for Mussels, fist ed. Office for official publications of the european communities, Luxembourg.