BIO-CHEMICAL BIOMARKERS IN ALGAE SCENEDESMUS OBLIQUS EXPOSED TO HEAVY METALS CD, CU AND ZN

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ABSTRACT

Laboratory studies were conducted to determine the effects of different concentrations of Cu, Cd, Zn and mixture (equal concentrations from the three heavy metals) on growth and some oxidative stress (catalase and glutathione reductase activities) on Scenedesmus obliquus (microalgae) after exposure to 24, 48, and 96 h. In addition, the uptake of Cu, Cd and Zn were determined in the culture medium after 24, 48 and 96h of exposure. The catalase and glutathione reductase enzyme activities were used as biomarkers to evaluate the toxic effects of Cu, Cd, Zn and mixture (equal concentrations from the three heavy metals) on the microalgae. Enzymatic activities were measured in the presence of both compound alone after 24, 48 and 96 h and also in mixture after the same time of exposure. The results showed that Cu, Cd, Zn and mixture induced antioxidative enzyme activities (CAT and GR) at different concentrations. Catalase activities (CAT) in both heavy metals treated algae were significantly increased. Additionally, a decrease in Chl.a, Chl.b and carotenoids was observed in algae after 24, 48 and 96 h of exposure to both Cu, Cd, Zn and mixture (equal concentrations from the three heavy metals).

KEYWORDS

Biomarkers, Scenedesmus obliquus (microalgae), heavy metals and oxidative stress.

1. INTRODUCTION

Heavy metals group is one of the main water pollutants. In aquatic environment, heavy metals present naturally with low concentration, which are not harmful to the environment and trace amounts of some heavy metals, including manganese, copper, iron, cobalt, zinc and molybdenum may play very important roles in both growth and metabolism of the aquatic organisms [1]. The Environmental Protection Agency reported that, heavy metals have major importance in bioavailability studies due to their potential for human exposure and increased health risk [2]. The presence of heavy metals in the aquatic environment in excess reveal the occurrence of additional extra sources. Those sources could be natural "erosion, volcano and deposition" or resulted from anthropogenic activities "domestic sewage, industrial effluent and agricultural run-off" [3]. Many heavy metals are known to reduce growth and interrupt metabolic activities, while, high concentrations have ecotoxicological effects [4,5]. The toxic effects of heavy metals towards aquatic organisms depend not only on their concentrations but also on the forms of their occurrence [6]. So, contamination of the aquatic environment by non-biodegradable heavy metals has been a subject of much concern in the recent years [7,8].

Algae considered as the primary producers and the basis of most aquatic ecosystems, hence, algae have been shown to be good bioindicators to qualitative and quantitative heavy metal contamination, specially, microalgae which are sensitive to any environmental changes [9,10]. Both diatoms and green algae are the most universally used bioindicator for heavy metals, by evaluating the toxic effect of different environmental levels of heavy metals. Hence, in the present study focus on the use of microalgae as bioindicator for heavy metals, by evaluating the toxic effect of different concentration of some heavy metals on growth, photosynthesis, and some physiological activities of the microalgae Scenedesmus obliquus.

2. MATERIAL AND METHODS

2.1 Microalgae culture

The microalg Scenedesmus obliquus (SAG 276-3a; Gottingen, Germany cultures; formerly S. acutus) was maintained in batch cultures containing 200 mL of mineral growth medium [pH 6.3; [19,20]. This medium consisted of (in mg L−1): ZnSO4, 7H2O 0.0063; LiCl 0.0075; KI 0.249; NH4VO3 0.0029; KNO3 1.000; NiSO4 7H2O 0.023; MnCl2 4H2O 0.099; CuSO4 5H2O 0.0025; AI2(SO4)3 1H2O 6.58; MgSO4 24H2O 0.099; H3BO3 0.031; K2HPO4 740; KBr 0.237; (NH4)6Mo7O26 5.88; Al2(SO4)3 14H2O 0.0063; and LiCl 0.0075. This provided a mineral medium that supported algae growth and maintained the desired pH.

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The catalase (CAT) activity was measured spectrophotometrically by the following the consumption of H2O2 at 240 nm for 1 min at 25 °C, in potassium phosphate buffer (750 mL, 50 mM, pH 7.5) containing the enzyme extract (10 mg protein). An addition of 200 mM H2O2 (100 mL) started the enzymatic reaction [21]. Absorbance at 340 nm was followed for 4 min and the activity was expressed as the consumption of micromoles of CDNB per minute and per milligram of protein.

2.7 Total chlorophyll, chlorophyll a, b and carotenoids.

After 96 hours of exposure, 4ml of 100% acetone was placed into a knownconcentrated cells titer (from each concentration of the three heavy metals) of 1 mL and homogenized at 1000 rpm for one min. The homogenate was centrifuged at 2500 rpm for 10 min. The supernatant was separated and the absorbance was read at 400-700 nm on Shimadzu UV-260 spectrophotometer. It was recorded that chlorophyll a showed the maximum absorbance at 662 nm while chlorophyll b at 646 nm and the amounts of these pigments were calculated according to the simultaneous equations [22]. Maine while the carotenoids were calculated according to Holm (1954) as follows:

\[
\text{Chl } \text{a} = 11.75 \text{ A}_{662} - 2.35 \text{ A}_{646} \\
\text{Chl } \text{b} = 18.61 \text{ A}_{646} - 3.96 \text{ A}_{662} \\
\text{Car} = 4.69 \text{ A}_{440} - 0.267 \text{ Chl } \text{a+b}
\]

2.8 Statistical analysis

All experiments were performed in four replicates and repeated three times. Data presented in this study is the mean standard deviation (SD). Significant differences between controls and contaminated samples were determined by the Mann and Whitney test and P-value was considered significant (*). All statistical analyses were performed with Sigma Stat 2.03 (SSCP Inc.) for Windows. The cluster analysis is an explicit way of multivariate analysis identifying similarity and relations between parameters in raw data [23]. Bray-Curtis dissimilarity index in cluster analysis has been used by the MVSP program (multivariate statistical package).

3. RESULTS

3.1 % Inhibition of growth rate

The values of LC10 (99.4 ± 3.8, 120.3 ± 3.4 and 75.6 ± 3.9 µg L−1 after 24 h for Cu, Cd and Zn, respectively) are shown in (Table 1). The S. obliquus was then exposed to those sub lethal concentrations and the physiological parameters were measured after 24, 48 and 96 h of exposure.

The analysis of variance (ANOVA) revealed that the rate of growth inhibition of S. obliquus in different concentration (5, 10, 50 and 100 µg L−1) of the tested heavy metals (Cu, Cd, Zn and HM mix.) have more or less the same trend, except within the mix. Cd gave the higher inhibition rate of 47.8 - 75.8 µg Cd L−1, 20.6 - 37.7 µg ZnL−1 and 54 -84.3 µg HM mix L−1. Aeration to the algal culture must be continuous with filtered air and the tests were conducted to determine the lethal concentrations (LC50) of Cd, Cu and Zn against S. obliquus as described above, a range of different concentrations of Cd, Cu and Zn (1, 5, 10, 20, 50 and 100 mg L−1) formulations were done in 100 mL of distilled water. The toxicity was evaluated after 24, 48 and 96 h in microplates. Three replicates without Cd, Cu or Zn were used as control. After 24, 48 and 96 h the LC10, LC25, and LC50 values were determined graphically according to Finney (1971). LC values from three experiments were averaged and mean standard deviations are presented.

2.3 Determination of lethal concentrations

The tests were conducted to determine the lethal concentrations (LC50) of Cd, Cu and Zn against S. obliquus as described above, a range of different concentrations of Cd, Cu and Zn (1, 5, 10, 20, 50 and 100 mg L−1), formulations were done in 100 mL of distilled water. The toxicity was evaluated after 24, 48 and 96 h in microplates. Three replicates without Cd, Cu or Zn were used as control. After 24, 48 and 96 h the LC10, LC25, and LC50 values were determined graphically according to Finney (1971). LC values from three experiments were averaged and mean standard deviations are presented.

2.4 Growth rate determination

The growth rate of S. obliquus was determined by counting cell number with Malassez's cell. The cell growth rate was calculated for 24, 48 and 96 h after addition of the heavy metals Cu, Cd and Zn with different concentrations (1, 5, 10, 20, 50 and 100 mg L−1) to the medium.

2.5 Metal Uptake

To determination at metal uptake by the algae; the salts of respective metal like copper sulphate (CuSO4), cadmium chloride (CdCl2) and Zinc chloride (ZnCl2) were dissolved in double distilled water in different concentration like 1, 5, 10, 20, 50 and 100 ppm. (Control without metal concentration used). Heavy metals like cadmium (Cd), Zn and copper (Cu) estimation of samples were done digesting with HClO4, HNO3 (1:4 V/V) and diluted with double distilled water. The various concentration of metals was measured by using Inductively Couple Plasma spectrophotometer, Perkin Elmer Corporation (ICP optima 3300RL).

Uptake of heavy metals by isolated microorganism. The isolated microorganism was cultured in YPG broth at 30 °C for 4 days and was then harvested by centrifugation at 2000g for 15 min. Next, 100mg (dry weight) of harvested cells were suspended in 100ml of 10 mM Tris-HCl buVer (pH 7.0) containing 1-5mg of either Cd2+, Zn2+ or Cu2+, or 2.5mg each of Cd2++ Zn2+, Cu2++ Zn2+, and Cd2++ Cu2+. These suspended solutions were shaken at 100 rpm at 30 °C. Residual heavy metals in the upper phase following centrifugation at 2000g for 5min were quantWed by atomic absorption spectrophotometry (Shimadzu, Japan).

2.6 Enzyme assays

For determination the enzyme activities, algal suspensions were incubated for 24, 48 and 96 h in 20 mL of medium supplied with different concentrations of Cd, Cu and Zn under the conditions described above. After 24, 48 and 96 h of exposure, the cultures were collected, and the enzyme extracts were obtained after centrifugation (5 min, 2000 g, 8 °C) of an algal suspension containing 300-420 mg of chlorophyll. The algal pellet was resuspended in 250 mL of sodium phosphate buffer (0.1 M, pH 7) and was ground in a porcelain mortar with some Fontainebleau sand. For 5 min, the extract was washed in 200 mL of potassium phosphate buffer (50 mM, pH 7.5). Enzyme extracts were then collected and centrifuged for 25 min at 15000 g (5 °C). Both extracts were centrifuged again for 20 min at 25000g (2 °C).

The enzyme (CAT) activity was measured spectrophotometrically by

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\text{CAT Activity} = V_{400} - V_{0} = \frac{A_{400} - A_{680}}{A_{400}}
\]
3.3 CAT activity

An extract of the CAT activity of Scenedesmus cell in different concentrations of the tested heavy metals and there HM mixture reveal variation in response, where the catalase activity which increase by low concentrations, started to decrease with the higher concentrations (50 -100 µg mL-1). As it was illustrated in (Figure 2) both Cu and Zn have a more or less the same effect on CAT activity, where except within the high concentration (71- 63 mg L-1 and 7-8 58 mg L-1 within 100µgL-1 Cu and Zn respectively), the CAT activity more than the control (82 -86 mg L-1) during the period of the experiment, giving its maximum activity within 30 µg L-1 in case of cupper (110-118 mg L-1) and within 5 µg L-1 of case of Zn (116-120 mg L-1). In contrast to the above, Cd and HM mix. suppressed CAT activity than that recorded in the control throughout the experiment period except within concentration 5 µg mL-1 only after 24h (85 mg L-1 within Cd and 96 mg L-1 within HM mix.).

3.4 GR activity

It is of interest to mention that, in contrast to the above CAT activity, the tree heavy metals support the GR activity within all concentrations and during the entire period of experiment (Figure 3). Nevertheless, GR activity within HM mix. followed the same trend only after 24 hours, meanwhile after 48h only the low concentrations enhance GR activity (129 and 139 mg mL-1 within 5 and 10 µg mL-1 respectively), however high concentrations significantly inhibit its activity (65 and 52 mg mL-1 within 50 and 100 µg mL-1 respectively). Except within 10 µg mL mix. L-1 (77 mg L-1) a more or less the same phenomena were recorded after 96 hours.

3.5 Up Take of heavy metals

A glance of table (4) reveal that, the three heavy metals tended to accumulate in the algal cell but with different concentrations during the time of experiment. The tested heavy metals (Cu, Cd and Zn) show a significant increase in up take within concentrations. Where, the Cu concentration in the algal cell fluctuated from 0.05 mg/g at 5 µg Cu L-1 after 24h to 2.6 mg/g at 100 µg Cu L-1 after 96 h, A more or less phenomena recorded in Zn up take ranged from 0.07 mg/g at concentration 5 µg L-1 after 24h to 3.4 mg/g at 100 µg L-1 after 96h. It is noticeable that, the range of uptake in case of Cd (0.09 mg L-1 at 5 µg L-1 after 24h to 4.24 mg/g at 100 µg L-1 after 96 h) is significantly higher than that of Zn and Cu during the period of experiment (24, 48 and 96h). Among this, the difference in algal uptake for the three heavy metals within the high concentrations (50 and 100 mg L-1) were impressive high.

3.5.1 Canonical corresponding analysis (CCA)

Overlying fig. (4), by using the Canonical Corresponding Analysis (CCA) the relations between the effect of the three tested heavy metals (Cu, Cd, and Zn) and their related effects will be more obvious. A high similarity between Cu and Zn in their effect on the algal growth which present in the same quarter (side). However, a weaker relation between above effect and that of the HM mixture present in the same side appeared as a dotted line. In contrast to the above, Cd negatively affected the growth with high dissimilarity, except to certain limit, with the effect of the HM mixture.

3.5.2 Cluster analysis

It is of interest to mention that, as shown in cluster analysis (Fig. 4), the effect of the tested heavy metals (Cu, Cd and Zn) on the production of both chlorophyll b and carotenoids were closely related in a minor subgroup. While, chlorophyll a respond differently to the same heavy metals and tended to be highly dissimilar with the other parameters, especially when treated with copper. Regarding (Figure 4) showed that, the algal cell had a more or less the same ability to uptake Cu and Zn (high similarity) followed by the ability to uptake Cd (with less similarity). Again, concerning the effect of the tested heavy metals on the growth, both copper and zinc tended to relate with each other in minor subgroup. The most noticeable result in (Figure 4) is the high dissimilarity between the effect of the two heavy metals and the activity of the two enzymes (CAT and GR) with especial highlight on their dissimilarity between the effect of each heavy metal alone and the mixture of them on the activity of GR enzyme, which related by low similarity with effect of both Cd and HM mixture on CAT enzyme.

4. DISCUSSION

4.1 Inhibition of growth rate

With significant growth in both urban and industry development, the use of heavy metal has raised serious environmental problems in water and damage marine life [24,25]. Therefore, assessment of the heavy metals toxicity upon wild microalgae from polluted sites is of exciting importance in ecotoxicology studies, particularly because such wild species are naturally exposed to highly pollution, and consequently transmission transmit heavy metals to the food chain. The most commonly used organisms for toxicity tests are the micro-green algae and diatoms, which used as the most standard form, measuring any change in growth rate, so it can survive as integrated environmental monitoring factor [7]. Growth inhibition of microorganism’s due to increasing heavy metals concentration in water has been studied in the last two decades [26]. The toxicity of heavy metals depends on both the concentration of heavy metal and the microalgal species, as well as the period of exposure. It is clear from the cited results that, except within low concentration, the inhibition rate of the studied alga (S. obliquus) was increased with increase of the heavy metals (Cu, Cd, Zn and HM mix) concentrations as well as longer exposure period. Concerning exposure of S. obliquus to different Cd concentrations show that, the growth inhibition was increase gradually and the strong inhibition followed the exposure to the highest levels of Cd, which recorded up to 75.8% within 100 µg L-1 after 96h of exposure. There a scientist found that, growth of Scenedesmus obliquus was affected by Cd concentrations more than 1 µg L-1 which directly correlated with the extent of inhibition [10]. Other scientist reported that, growth Tetraselmis chuii was markedly affected by 60% inhibition when exposed to 50.0 µg L-1 of soluble Cd, which agree with the sited results in this study [28]. The decrease in growth rate in Scenedesmus quadricauda after addition of Cd to its attribution on the respiratory process [29]. On the other hand, the zero effect of the lowest Cd concentration may be deriving from the fact, Cu and Zn have a significant effect and that of the HM mixture present in the same side appeared as a dotted line. In contrast to the above, Cd negatively affected the growth, both copper and zinc tended to relate with each other in minor subgroup. The most noticeable result in (Figure 4) is the high dissimilarity between the effect of the two heavy metals and the activity of the two enzymes (CAT and GR) with especial highlight on their dissimilarity between the effect of each heavy metal alone and the mixture of them on the activity of GR enzyme, which related by low similarity with effect of both Cd and HM mixture on CAT enzyme.

4.2 Effects of metals on the photosynthesis

The present experiment did not study the mechanism of copper toxicity, but pointed out that, copper may affect the permeability of the cell and then disrupting both enzyme activity and cell division, hence reducing the cell growth [36].
All the three tested heavy metals and its mix, inhibited the growth of S. obliquus, and the effects were both dose-dependent and time-dependent, the toxic order was HM mix > Cd > Cu > Zn. Unlike the effect on the growth, the impacts on the photosynthesis were more complicated. A researcher also reported that, growth and photosynthesis are independent processes unrelated to each other [37]. Thus, it is necessary to take both growth and photosynthesis into account when estimating the ecological risk of a toxicant, especially under sub-lethal concentrations. Where both zinc and copper enhance the production of chla and b within the low concentration during the entire period. The result which agree with that reported that, both copper and zinc acts as a micronutrient favouring some physiological activities within low concentrations and then supporting the algal growth [38].

On the whole in spite of some exception (increase of chla and b within low concentration of zinc and copper), the higher concentration of Cu and Zn beside Cd and HM mixture (by all concentrations) reduce carotenoid, chla and b. The acute inhibition of photosynthesis related to the role of high concentration of heavy metals which both interrupt the physiological properties of the cell and destruct the chloroplast [39]. In fact, it is well known that Cd2+ disorganizes chloroplast causing the damage of photosynthetic pigments [40]. High concentrations copper is highly toxic to the algae, affecting both photosynthetic activity is mostly influenced by the amount present in the water [48]. Also, it could be used in enzyme synthesis. Stauber and Florence, and also Wildt reported that, the possible mode of zinc and cadmium toxicity are related to the cell membrane, where it may interrupt the uptake of calcium which is necessary for the Ca-ATPase activity during cell division [41,42].

4.3 CAT activity

Generally, concerning enzymatic activity, low concentrations of heavy metals have stimulated CTA activity, while the response is reflected in the case of high concentration (100 μm L1). This phenomenon can be explained that, small amounts of heavy metals (spatially Zn and Cu) could be used in enzyme synthesis. Stauber and Florence, and also Wildt reported that, the possible mode of zinc and cadmium toxicity are related to the cell membrane, where it may interrupt the uptake of calcium which is necessary for the Ca-ATPase activity during cell division [41,42].

4.4 GR activity

Anent GR activity, the recorded results showed that, except HM mixture, the heavy metals support its activity within all concentrations during the entire period of experiment. Previous studies suggested that heavy metals can induce oxidative stress by generating reactive oxygen species (ROS) in aquatic organisms. Indeed, ROS production by exposure to Cd, Cu, and Zn, mainly superoxide and peroxides, was detected using fluorophores [43-45]. However, a research reported that, the mechanisms by which heavy metals induces antioxidant responses and to what extent different plant species may share a common defence mechanism are not yet fully understood [46].

4.5 Up Take of heavy metals

Microalgae considered as an efficient organism in heavy metal removal from the aquatic environment. They can eliminate metal ions from water in short time by biosorption in uncomplicated systems, without any problems of toxicity. Different microorganisms, have different ability to uptake the same metal, and also, the same microorganisms may be more or less damaged by different metals [47]. Scenedesmus sp has the ability to uptake and accumulate heavy metal in their cells, and known as one of the most efficient microalgae in this process. The data illustrated in table (4) performed that, accumulation of cobalt, zinc and copper by Scenedesmus obliquus increased with increase of the heavy metals (Cu, Cd and Zn) concentrations as well as longer exposure period. Where, the uptake of any element from the surrounding media is mostly influenced by the amount present in the water [48]. Also, it can be seen that the tested alga (Scenedesmus obliquus) accumulated a considerable amount of cadmium more than other that of copper and zinc. However, no significant difference was observed between copper and zinc. Metal accumulation by Scenedesmus was shown to be in an order of Cd > Cu > Zn. This noticeable high range of uptake in case of Cd (0.09 at 5 μg L-1 after 24h to 4.24 mg/g at 100 μg L-1 after 96h) may due to the fact that, cadmium has no known function in cell metabolism at all, so it is solely up taken by adsorption. A research also reported that, Cd toxicity leads to severe disturbances in physiological processes, such as nitrogen fixation, photosynthetic activity and growth [49].

The internally accumulation of cadmium ion in microalgae occurred in two phases of uptake process [50-53]. The first phase is a rapid physicochemical adsorption of cadmium ion onto cell wall binding sites, which followed by period of steady intracellular uptake phase (energy dependent phase).

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