The in vitro and in vivo Effect of *Clinoptilolite* on Decreasing of Copper Ion and DNA Damage of *Anodonta Cygnea*

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Abstract

The ability of natural zeolite (Clinoptilolite) to remove copper from aqueous ecosystem was studied in real and laboratory conditions. Fresh water mussels (Anodonta Cygnea) of different sizes were Exposed to copper (150, 350, 450 µgl^{-1}) for 10 days. Copper exposure induced DNA damage in the haemolymph cells of Anodonta of all sizes. In connection with real samples, the amount of damage and even much higher than the first (150 µgl^{-1}) and second (450 µgl^{-1}) treatments was observed. Presence of zeolite in the aquatic environment removed Cu^{2+} and specially reduced DNA damage in all samples. In vitro results showed a significant reduction of copper metal. Decreasing of other heavy metals Concentration is also observed in vivo. Damage to DNA and to other biomolecules by copper, mean that the availability of Cu^{2+} ions in vivo must be carefully controlled. analysis of variance and other tests were performed for determination of optimum quantities and exposure time of adsorbent in vivo and in vitro.

Keywords*: Anodonta Cygnea, Cu^{2+}, Clinoptilolite, DNA damage.*

Introduction

Numerous aquatic systems may receive contaminants present in waste water releases as a Consequence of elevated concentrations of metals present in many types of waste water [1]. Thousands of chemical compounds have been released into aquatic ecosystems and can cause hazardous effects in marine and freshwater organisms. These substances (heavy metals, oil Products, chlorinated pesticides, halogenated aromatic hydrocarbons) have the ability to accumulate in water organisms [2].

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Invertebrates present over 90% of the species in aquatic communities and have a particularly important role in the ecosystem function [3]. Mollusks have been widely used as indicator organisms, because they are ubiquitous. Furthermore, they have highly conserved control and regulatory pathways that are often homologous to vertebrate systems, and are extremely sensitive to anthropogenic inputs [4]. Comparatively high bioaccumulation factors for organic pollutants, relatively low metabolic detoxification rates and a sessile filter-feeding life style allow using the bivalves as sensitive organisms in biomonitoring studies [5]. Freshwater mussels store toxicants in their tissues and pseudofaeces and may play a key role in maintaining water quality [6,7]. Dixon et al Different methods have been developed for detection of both double- and single-strand breaks of DNA, DNA-adducts, micronuclei formation, and chromosome aberrations. The assessment of cytogenetic damage has been presented as a very important assay in identification of pollution effects in marine environments. Double strand breaks in DNA duplex are thought to be biologically significant sources of cell lethality, because appear to be less readily repaired by DNA repair mechanisms [8]. The Comet assay is widely influenced by laboratory procedures suggesting that standard protocols are required for both fish and mussel cells. Recently, Pavlica et al have used comet assay on mussel. The comet assay offers considerable advantages over other cytogenetic methods for DNA damage detection, because the cells studied do not need to be mitotically active [9].

It is known that copper is an essential trace metal for living organisms and it is present in all natural waters and sediments. This metal plays a crucial role in many biological enzyme systems that catalyze oxidation/ reduction reactions and have molecular oxygen as a co-substrate. However, if copper is present at relatively high concentrations in the environment, toxicity to aquatic organisms can occur [10]. Copper is a common pollutant of the aquatic environment and it induces cellular reactions in mussel by reacting with the structural and enzymic components of cell membranes, DNA and organelles [11]. Among various available treatment processes for the removal of heavy metals such as precipitation, phytoextraction , ultra filtration and reverse osmosis, adsorption is considered to be a cost effective method provided that low cost adsorbents such as zeolites and clays [12].

Zeolites are microporous materials, which are widely used in industry as sorbents, ion exchangers, and catalysts. These materials have well-defined pore structures with molecular dimensions that are suitable for selectively adsorbing various organic molecules. The adsorption properties of zeolites determine their usefulness as catalysts and sorbents. As certain guest molecules are adsorbed into

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certain zeolites, phase changes can occur in the framework [13]. The structure of zeolites of consists of the three dimensional frameworks of SiO_4 and AlO_4 tetrahedra. Replacement of Si^{4+} by Al^{3+} produces negative charge in the lattice. The net negative charge in balanced by the exchangeable cations (Na, K, or Ca). These cations are exchangeable with certain cations in solutions, such as lead, cadmium, zinc, manganese and copper [14]. Selective absorptivity, Cation-Exchange Capacity (CEC), low price and abundance of natural zeolites in environment are very important factors in selection of suitable adsorbent. That is why we used Clinoptilolite in this research [15]. Blanchard et al. reported that wastewater containing heavy ions such as Pb^{2+}, Zn^{2+}, Cu^{2+} besides NH_4^+ ions can be treated effectively by filtration on clionoptilolite columns. The order of efficiency was $Pb^{2+}> NH_4^+ > Cd^{2+}$, Cu^{2+}, $Sr^{2+}> Co^{2+}$ [16].

Experimental

Animals and treatments

Mussel (Anodonta Cygnea) of 9-11.5 cm in length were collected from Anzali wetland in north of Iran. 9 animals were used in each exposure in 8 tanks. Three different concentrations of $CuSO_4$ as treatment were chosen: 150, 350 and 450 $\mu g l^{-1}$ (with and without zeolite simultaneously). Two samples were used as real sample (Anzali wetland). In order words different concentrations of copper were tested in the three treatments in vitro. Simultaneous determination of copper ions concentration of water sample was studied in ecosystems (Anzali Wetland) and tested as the real sample to be compared in vitro and in vivo. The same experiment was repeated at the same conditions with Clinoptilolite. The exposure period was 10 days.

Zeolite process

50 ml solution was collected from 8 tanks (4 tanks with zeolite, 4 tanks without zeolite) everyday then zeolitic samples were filtered and all samples kept in refrigerator. Finally, The Samples obtained during the 10 days after filtration procedure were transferred to the induction coupled plasma (ICP) for measuring the adsorption rate and subsequent analysis.

Obtained results are presented in Table 1. The equilibrium isotherm in this study was analyzed using the Langmuir, model. This isotherm is useful for estimating the total amount of adsorbent needed to absorb the required amount of absorbent from the solution [17].

Table 1. ICP Analysis results.

450 ppb		350 ppb		150 ppb		Real sample		Day
With Zeolite	Without Zeolite	With Zeolite	Without Zeolite	With Zeolite	Without Zeolite	With Zeolite	Without Zeolite	
60.25	95.06	59.93	79.11	54.11	59.92	74.39	79.11	1
83.49	110	80	111.08	57.1	67.09	92.02	107.11	2
91.38	117.68	91.38	114.35	90.22	111.57	105.66	138.77	3
119.09	152.79	117.39	124.16	111.71	125.78	125.43	163.22	4
134.77	173.31	119.48	141.81	116.63	129.38	131.24	170.27	5
143.11	175.09	133.83	171.54	117.39	168.66	145.26	193.15	6
151.82	195.94	151.84	164.06	141.6	149.91	152.26	207.52	7
173.15	210.47	156.65	192.25	153.83	183.12	165.7	232.42	8
201.8	211.81	166.77	193.48	166.77	176.56	170.83	262.28	9
207.22	256.01	192.43	242.83	192.12	353.73	182.25	273.32	10

Comet assay

Anodonta 's haemolymph was collected for comet assay test at the end of each experiment. DNA Changes were studied in the absence and presence of zeolites. Comet assay was conducted as described by Singh et al and Taban with slight modifications.[18,19] Comet assay test was used to evaluate percentage of DNA damage and the cytogenetic changes after Cu^{2+} exposure (Tables 2-3). For each animal 100 cells per slide (500 cells per sample) were visually scored at random and given an empirical score depending on the degree of damage they exhibited. DNA damage was classified in four classes, according to the tail length of the comet: 0, undamaged; 1, minimum damage; 2, medium damage; 3, maximum damage, according to Kobayashi et al [20]. The damage categories were allocated according to the approximate percentage of DNA in tail as described by Wilson et al [21].

Table 2. mean and the Percentage of DNA damage in each Tank (without zeolite).

Tanks	Real sample	150 ppb	350 ppb	450 ppb
(mean±S.D.) of DNA Damage	199.344.70±	178.661.71±	190.16 15.40 ±	255.66 19.62 ±
percentage of DNA damage	66.43 %	62.55%	63.38%	85.22%

Table3. mean and the Percentage of DNA damage in each Tank (with zeolite).

Tanks	Real sample	150 ppb	350 ppb	450 ppb
(mean±S.D.) of DNA Damage	52.520.54±	65.8318.59±	77.525.68±	83.16 30.48 ±
percentage of DNA damage	17.5%	21.94%	25.83%	27.72%

The mean number of damaged nucleotides (classes 1–3) was calculated per specimen exposed to 8 tanks for exposure period. The main Hydrochemical parameters such as dissolved oxygen, pH and temperature were examined during 10 days (Tables 4 and 5).

Table 4. Physical and chemical parameters of water (without zeolite).

Day 5			Day 4			Day 3			Day 2			Exposure Parameters
Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Tanks
5.60	8.31	20.9	5.78	8.26	19.8	5.78	8.28	19.2	6.31	8.18	19.1	**Real sample**
5.7	8.66	21.8	5.64	7.44	21.7	6.2	8.34	19.3	6.45	7.79	19.3	**Sample 1**
5.67	8.46	19.8	6.32	8.1	19.8	5.89	8.42	19.1	5.90	7.96	19.1	**Sample 2**
5.41	8.43	20.1	5.90	8.15	19.5	5.81	8.61	19.1	6.20	8.11	18.90	**Sample 3**
Day 9			Day 8			Day 7			Day 6			Exposure Parameters
Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Tanks
5.85	8.17	22.4	6.13	8.21	21.1	6.02	8.61	21.5	6.20	8.30	21.3	**Real sample**
5.79	8.72	21.3	6.12	7.79	20.7	6.09	8.26	21.5	6.44	7.70	21.2	**Sample 1**
6.23	8.70	21.8	5.96	8.16	20.8	5.76	8.47	21.4	6.32	7.85	20.9	**Sample 2**
5.57	8.59	22	5.91	8.27	21.1	5.78	874	21.9	6.16	8.41	21	**Sample 3**

Table 5. Physical and chemical parameters of water (with Zeolite).

Day 5			Day 4			Day 3			Day 2			Exposure Parameters
Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Tanks
5.85	8.17	22.4	5.42	8.17	21.7	6.25	8.23	20.7	6.30	8.15	20.5	**Real sample**
5.79	8.72	21.3	5.89	8.44	20.8	6.55	8.41	18.8	6.42	8.43	19.9	**Sample 1**
6.23	8.70	21.8	5.56	8.39	21.4	6.58	8.43	20.7	6.53	8.33	20.5	**Sample 2**
5.57	8.59	22	5.56	8.37	20.7	6.58	8.32	19.4	6.60	8.31	20.4	**Sample 3**
Day 9			Day 8			Day 7			Day 6			Exposure Parameters
Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Oxygen (mg/L)	pH	T (ºC)	Tanks
5.82	8.28	21.4	4.30	8.21	21.9	5.73	8.62	23.5	5.91	8.34	22.6	**Real sample**
6.12	8.03	23.7	5.23	8.56	21.1	5.25	8.95	22.1	6.28	8.62	20.9	**Sample 1**
6.00	8.25	22.01	6.22	8.58	21.6	5.24	9.04	22.3	6.29	8.67	22.0	**Sample 2**
5.76	8.34	21.5	4.55	8.49	21.9	5.29	8.97	22.8	6.18	8.56	21.30	**Sample 3**

Statistical analysis

All statistical analyses were carried out using SPSS. One-way analysis of variance (ANOVA) with Tukey ($P \leq 0.05$) was performed to compare the mean differences between real samples and treatments. ICP data also analyzed using Excel and Curve Expert program packages.

Results and discussion

Heavy metal pollution in rivers and its impact on aquatic ecosystems is a dynamic process. Mussels are ideal indicators of heavy metal contamination in aquatic systems [22].

The environmental impact of these heavy metals affects not only to the species biodiversity whose natural environment results seriously damaged but also to human populations whose marine economic resources are decreased Many recent studies on sea environment organisms and comet assay as effect biomarker [23-25]. Most of these works showed that gill cells result very suitable for comet assay evaluation, since they

filter great quantities of contaminant agents present in water. Moreover, heavy metal exposure has been previously determined in mussels by means of comet assay [26-27]. In addition, their low biodegradation in mussels may contribute to this fact during certain periods of time [28].

Taban et al reported a significantly higher % DNA in the comet tail of cells from sea urchins and mussels compared to untreated control cells (mean values of 24% and 14% DNA in the comet tail in sea urchins coelomocytes and mussels haemocytes, respectively Mussels have been effectively used as bioindicator organisms in other marine. Two different mineral were used in order to examine the removal of Cu^{2+} from aqueous solutions.

Marinos et al. reported Natural zeolite (clinoptilolite) and vermiculite (clay) were supplied by S&B Industrial minerals S.A and I.G.M.E. (Institute of Geology & Mineral Exploration).

It is necessary using Clinoptilolite because of bioaccumulation and biomagnifications Cu^{2+} cations in anodont's tissue and it's increasing in environment and the synergic effects with other heavy metals on living organisms. Mussels exposed to $CuSO_4$ (450 µgl^{-1} and real samples without zeolite) showed more damaged cells with a significant increase in the number of damaged nucleotides in relation to their respective treatments (Figure 1). Mussel DNA damage in zeolite containing samples show a significant decrease in zeolite free ones (Figure 2).
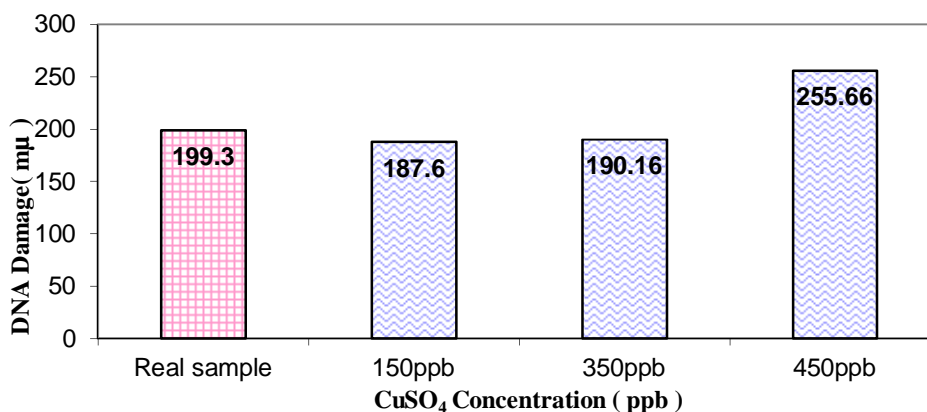

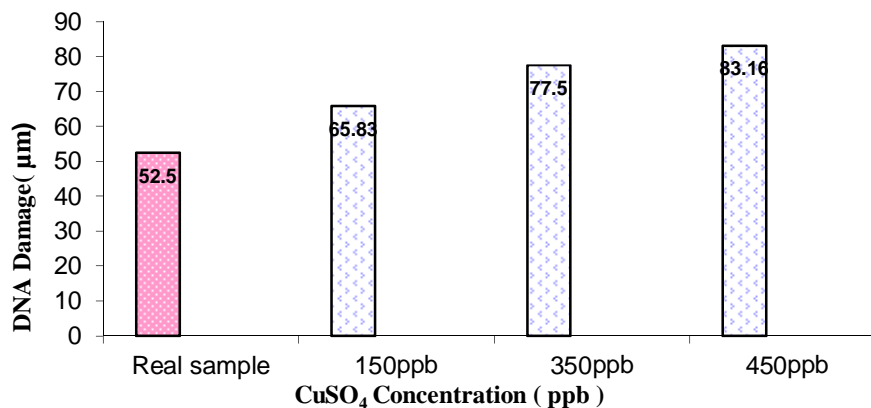**Figure1**. statistically significant difference in zeolite-free treatments (ANOVA with Tukey test).

Figure 2. Statistically significant difference in zeolite treatments (ANOVA with Tukey test).

Results of the comet assay expressed as the percentage of damaged haemocytes of *Anodonta* *cygnea* after exposure to CuSO$_4$ (Without and with zeolite) are presented in Figure 3.
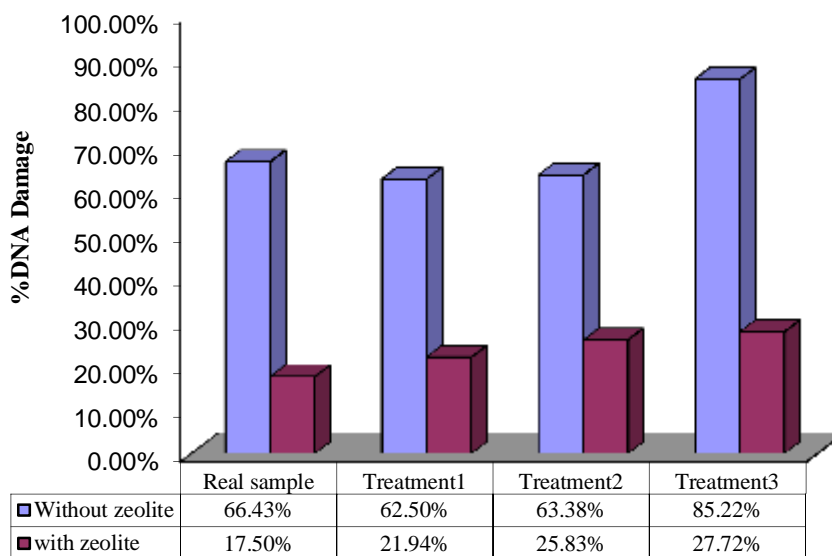

	Real sample	Treatment1	Treatment2	Treatment3
■Without zeolite	66.43%	62.50%	63.38%	85.22%
■with zeolite	17.50%	21.94%	25.83%	27.72%

Figure 3. Comparing the percentage of damaged cells.

The results showed that the variation of them was almost negligible during the experiment period. The amount of adsorption at equilibrium, q$_e$ (mol/g), was calculated by:

$$q_e = \frac{(C_0 - C_e)V}{W} \qquad (1)$$

Where C$_0$ and C$_e$ (mol/l) are the initial and equilibrium liquid-phase concentrations of dye, respectively. V (L) is the volume of the solution and W (g) is the mass of sorbent used. The equilibrium isotherms in this study were analyzed using the Langmuir isotherm. The Langmuir isotherm theory assumes monolayer coverage of adsorbate over a homogenous adsorbent surface [29]. A basic assumption is that sorption takes place at specific homogenous sites within the adsorbent. Once an adsorbate molecule occupies a site, no further adsorption can take place at that site.

The Langmuir adsorption isotherm has been successfully used to explain the adsorption of heavy metals from aqueous solution. The Langmuir isotherm is:

$$\frac{1}{q_e} = \frac{1}{K_L q_{max}} \frac{1}{C_e} + \frac{1}{q_{max}} \qquad (2)$$

Where:

q$_e$: the amount adsorbed (mol/l).

C$_e$: the equilibrium concentration of the adsorbate (mol/l).

q$_{max}$ and K$_L$: the Langmuir constants related to the maximum adsorption capacity and energy of adsorption, respectively.

When 1/qe was plotted against 1/Ce, a straight line with the slope of 1/K$_L$q$_{max}$ was obtained. The correlation coefficient R^2 of 0.99 indicated that the adsorption data of Cu^{2+} on the clinoptilolite was well fitted to the Langmuir isotherm. The Langmuir constants were calculated from Eq.

(2). Samples obtained during the 10 days after filtration procedures were transferred to the induction coupled plasma (ICP) for measuring the adsorption rate and subsequent analysis. In vitro results showed a significant reduction of copper metal. Decreasing of other heavy metals Concentration is also observed in vivo.

Based on the obtained results, we can conclude that adsorption of copper is increase with time and the variation of concentration goes to the less values (Figure 4). Using the Longmuir adsorption isotherm the equilibrium constant of the process can be obtained.
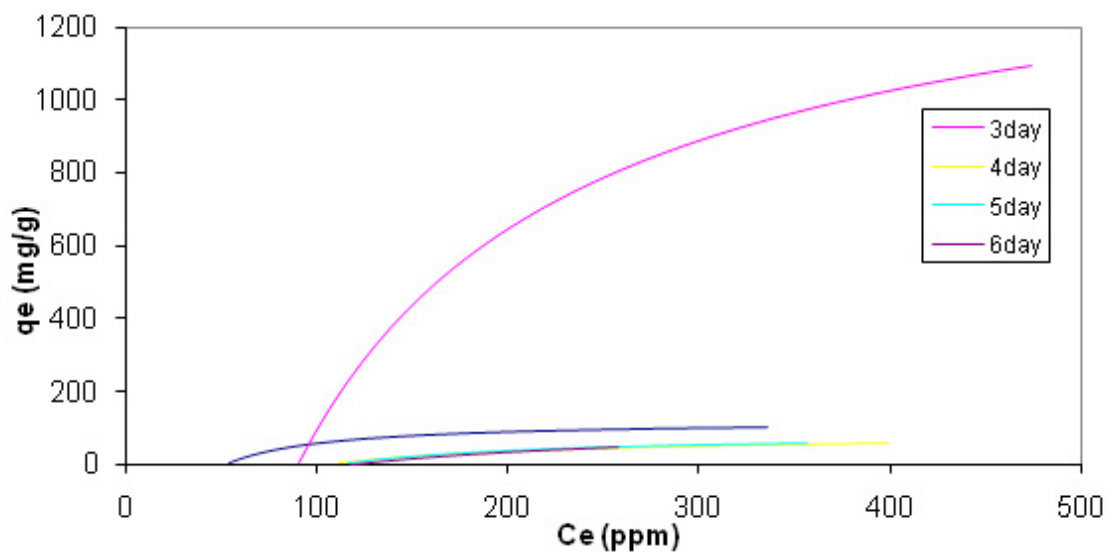

Figure 4. The relation between contact time and adsorbent.

Conclusion

Various concentrations of copper caused DNA damage. Zeolite absorbent could significantly reduce the amount of DNA damage. The natural zeolite clinoptilolite was chosen zeolite because of high surface area, selective adsorption property and Cation- Exchange Capacity (CEC). Whenever concentration of copper increases without the presence of zeolite in vitro the amount of DNA damage gets higher values. In connection with real samples, the amount of damage and even much higher than the first and second treatments was

observed. The Langmuir theory is chosen as the most appropriate isotherms. That the using the degree of solidarity and steady isotherms realized adsorptive capacity to adsorbed metal ions. Statistical and analytical calculations determined the optimal amount of zeolite for reducing pollution and making the number of days exposed by zeolite.

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