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Heavy Metals-Induced Expression of ABCB10 Gene in Zebrafish *Danio rerio.*

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ABSTRACT

ABCB10 is a mitochondrial ATP-binding cassette (ABC) transporter. It is involved in multi-drug resistance and it has a potential role in the regulation of oxidative stress induced by toxic substances, such as heavy metals. Little is known about the role of ABCB10 gene in metal detoxification in fishes generally and Zebrafish particularly. Also the effect of *Tubifex* worms on the gene expression has not been discussed before, despite the importance of these worms in metals transfer and bioavailability. Therefore, the impact of contaminated sediment with Cu and Cu on ABCB10 gene expression was investigated in this study. In addition, the effect of *Tubifex* worms on the expression of Zebrafish ABCB10 gene was also studied. Zebrafish was exposed for 7 days to contaminated sediment with 315 mg/kg and 84.8 mg/kg of Cu and Cd, respectively. Relative gene expression was recorded in Zebrafish different organs (brain, gills, muscles and digestive tract), in 4 experimental groups in presence and absence of *Tubifex* worms.

The highest level of ABCB10 expression was recorded in digestive tract samples in all tested groups followed by muscles then gills, while brain samples recorded the least induction level for ABCB10 expression. *Tubifex* worms showed an effective influence on Zebrafish ABCB10 expression with higher up-regulation level compared with those recorded for Cu and Cd contamination.

Keywords: ABCB10, heavy metals, gene expression, *Tubifex* worms, Zebrafish.

INTRODUCTION

ABCB10 is a multi-drug resistance protein, it is also called Mitochondrial ATP-binding cassette, sub-family B (MDR/TAP), member 10 (Thiriet, 2012). This protein encoded by ABCB10 gene which is localized in the mitochondrial inner membrane.

The ABCB10 gene is conserved in Chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, Zebrafish, fruit fly, mosquito and *C.elegans*. It is highly expressed during erythroid differentiation and hemoglobin synthesis (Liesa *et al*., 2012).

ABCB10 gene may regulate oxidative stress by its transport activities. Also it has a potential therapeutic role against diseases involved in increasing mitochondrial ROS production and oxidative stress (Liesa *et al*., 2012).Therefore, it participates in mitochondrial antioxidant response in eukaryotic cells (Hyde *et al*., 2012).

Heavy metal is a serious source of contamination threatening all aquatic systems specially lakes. Due to lake's closed system, toxic materials can rapidly accumulate in water, sediment and aquatic organisms (Gurcu *et al*., 2010).

One of the most heavily polluted lakes in Egypt is Lake Manzala (Abdel-Gawad & El-Sayed, 1998). It is continuously receiving high amounts of wastewater from 5 governorates Port Said, Cairo, Sharqia, Daqahlia and Qualiobia (Mageed, 2007). Lake Manzala's sediment and water have exceeded the maximum permissible limits for worldwide reference (Saeed & Shaker, 2008).

Heavy metals contamination has a genotoxic effects on the aquatic organisms (Myers *et al*., 2008; Valko *et al*., 2006), by activating the production of reactive oxygen species (ROS), leading to oxidative stress and DNA damage (Farombi *et al*., 2007), that could be transferred to the next generations (Bolognesi & Hayashi, 2011). As a defense mechanism against heavy metal toxicity, aquatic organisms activate the cellular detoxification mechanisms. Different studies have illustrated the role of some genes in multi-xenobiotic resistance process to extrude toxins outside the cell. Such genes are ABC transporters (Aleo *et al.*, 2005; Kingtong *et al.*, 2007; Morgan *et al*., 2007).

Zebrafish, *Danio rerio* is one of the perfect vertebrate models for toxicology, pharmacology, developmental biology, and genetics studies. Its importance as a model organism is due to its small size, short life cycle, rapid growth and its completed genome sequence (http://www.ensembl.org/Danio_rerio) (Broughton *et al*., 2001; Gilbert, 2006).

Danio rerio has been extensively used in toxicological studies to investigate the mutagenic effects of water pollution on the integrity of DNA (Gonzalez *et al*., 2006; Scholz *et al.,* 2008; Popovic *et al*., 2010).

Another macrobenthic organism is used as a bioindicator to sediment contamination , *Tubifex tubifex* worm is a conveyor belt feeder that has a great role in metal transfer and bioavailability between sediment and water column during its feeding process (Anschutz *et al*., 2012; Ciutat & Boudou, 2003). Moreover, it is used as a favourable food for Zebrafish.

Few previous studies investigated the role of ABCB10 gene in cellular detoxification and toxins secretion in humans (Zhang *et al.*, 2000), but no recorded reference about the role of Zebrafish ABCB10 in heavy metals detoxificatoion. In addition, it is not previously studied the impact of *Tubife*x worms on the gene expression of ABCB10 gene in Zebrafish.

Therefore, the aim of this work was to investigate the impact of contaminated sediment (with relevant concentrations of Cu and Cd found in Lake Manzala) on Zebrafish ABCB10 expression. The effect of *Tubifex* worms on Zebrafish ABCB10 gene expression was also investigated.

MATERIALS AND METHODS

Zebrafish *Danio rerio* maintenance

Adult males *D. rerio* were purchased from a local pet farm in Bordeaux-France, and transferred to the acclimation tank at 21 ° C. Fishes were fed twice a day (50mg diet /fish/day) for acclimation period of 30 days.

Sludge worm *Tubifex tubifex* maintenance

Sixty batches of *Tubifex* worms, 200 worms each were purchased from natural pond (SARL GREBIL père & fils, Paris - France). Worms were acclimated on clean sediment from Garonne River, Bordeaux-France at 20º C for 15 days.

Heavy metals treatment

Sediment from Garonne River, Bordeaux-France was sampled and sieved. Mixture of Copper (Copper standard, Cu-Titrisol, Merck) and Cadmium (Cadmium standard, $CdCl_2$-Titrisol, Merck) stock solutions were added and homogenized with the sediment to have a final concentration of 315 mg/kg and 84.8 mg/kg of Cu and Cd

respectively. Sediment was stored at 4ºC for 7 days before the beginning of the experimental protocol.

Experimental design

Experimental units (EUs) were divided into 4 different experimental conditions (3 units each). Two control groups; group C (uncontaminated sediment + fish –worms), and group D (uncontaminated sediment + fish+ worms), and two contaminated groups; group F (contaminated sediment +fish – worms), and group G (contaminated sediment + fish+ worms).

Four Zebrafishes were distributed in each unit, while worms were distributed as 56000 worms/m^2 in D and G conditions only. All experimental unites were arranged in a thermostatic chamber in Arcachon marine station, Arcachon-France. Water temperature was set at 21º C (optimum temperature for Zebrafish and *Tubifex* worms) (Schaefer & Ryan, 2006), with source of aeration and 12 hr photoperiod of florescent light for 7 days of exposure.

Total RNA Isolation

After 7 days of exposure, 5 fish samples from each condition were dissected to collect skeletal muscles, digestive tract, gills and brain samples. All tissue samples were stored in RNA later solution (Qiagene) at -80 ºC.

Total RNAs were isolated from tissues samples using absolutely RNA miniprep kit (Agilant), then first strand of cDNA was synthesized using Affinity Script cDNA synthesis kit (Agilent).

Primers design

Primers for Zebrafish ABCB10 gene (XM_001343182) and the housekeeping gene β-Actin gene (NM_131031) were designed according to their sequence in the GenBank.

Real-Time PCR

RT-PCR was performed in real-time PCR device (STRATAGENE) in a total volume of 25 µl; 1 µl cDNA template, 2 µl primer, 12.5 µl Brilliant SYBER green master mix, and 9.5 µl deionized water. The PCR thermal profile was as follows: one cycle for 10 min at 95ºC, 40 cycles: 30 s at 95ºC, 30s at 55 ºC and 30s at 72 ºC.

C_ts for PCR reactions were collected and relative quantification for gene expression was calculated according to the equation: $2^{-(\Delta C_t)}$, $\Delta C_t = C_t$ (Housekeeping gene) – C_t (Target gene).

In addition, the differential gene expression was calculated as follows:

D/C= differential gene expression for group D against C $=2^{-\Delta Ct\,(D)}/2^{-\Delta Ct\,(C)}$,

F/C= differential gene expression for group F against C $=2^{-\Delta Ct\,(F)}/2^{-\Delta Ct\,(C)}$,

G/C= differential gene expression for group G against C $=2^{-\Delta Ct\,(G)}/2^{-\Delta Ct\,(C)}$,

F/D= differential gene expression for group F against D $=2^{-\Delta Ct\,(F)}/2^{-\Delta Ct\,(D)}$,

G/D= differential gene expression for group G against D $=2^{-\Delta Ct\,(G)}/2^{-\Delta Ct\,(D)}$,

G/F= differential gene expression for group G against F $=2^{-\Delta Ct\,(G)}/2^{-\Delta Ct\,(F)}$.

Statistical analysis

The deferential gene expression was tested for significance using non parametric Mann-Whitney test ($P<0.05$), after applying the Shapiro-Wilk normality test (1% risk) using SigmaStat 3.5 program.

RESULTS

Relative expression of Zebrafish ABCB10 gene

After 7 days of exposure to contaminated sediment with Cu and Cd, the relative gene expression of ABCB10 gene to β-actin was evaluated in Zebrafish different organs in presence and absence of *Tubifex* worms.

Among the 4 different tissues, the highest level of Zebrafish ABCB10 gene expression was recorded in digestive tract samples in all tested groups. Samples from control group D recorded the highest induction in the gene expression compared to samples from all other groups, while gill samples showed very low level of gene expression in all treatments.

Generally, *D. rerio* different organs almost recorded same trend in gene expression levels in the 4 experimental groups, as digestive tract samples showed the highest level of gene expression followed by muscles samples then gills and brain samples (Fig.1).

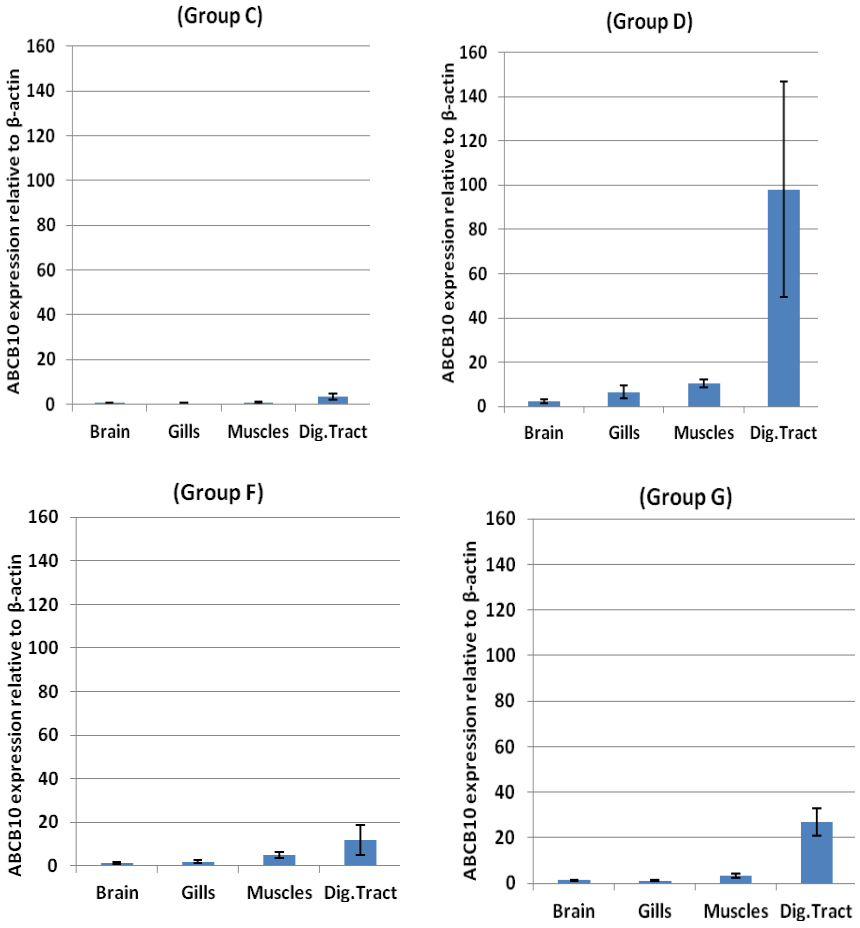

Fig. 1: Relative expression of ABCB10 gene in Zebrafish *Danio rerio* Zebrafish ABCB10 relative expression to β-actin in group C (Uncontaminated sediment +fish -worms), group D (Uncontaminated sediment +fish +worms), group F (Contaminated sediment +fish -worms), and group G (Contaminated sediment +fish +worms)

Transcriptional response of Zebrafish ABCB10 gene to Cu and Cd contamination

Impact of Cu and Cd contamination on ABCB10 gene expression was evaluated by comparing the relative gene expression of ABCB10 gene in contaminated group F against control group C, and contaminated group G against control group D. Significant up-regulation by 6.4-times was recorded in muscles samples of the contaminated group F compared to C group. In addition to up-regulation in the gene expression by 2.2-, 4.1- and 3.4-times in brain, gills and digestive tract samples respectively.

The presence of *Tubifex* in both control group D and the contaminated group G had a significant effect on Zebrafish ABCB10 expression. *Tubifex* worms caused down-regulation in gene expression in the 4 tested organs of the contaminated group G compared to D group samples (Table1and Fig. 2).

Table 1: The differential expression of ABCB10 gene in Zebrafish *Danio rerio*

Tissue	D/C	F/C	G/C	F/D	G/D	G/F
Brain	3.8	2.2	1.9*	0.6	0.5	0.9
Gills	14.3	4.1	2.5	0.3*	0.2*	0.6
Muscles	13.1	6.4*	4.2*	0.5	0.3*	0.7
Digestive Tract	28.4*	3.4	7.7	0.1	0.3	2.3
*Statistically Significant difference ($p<0.05$)						

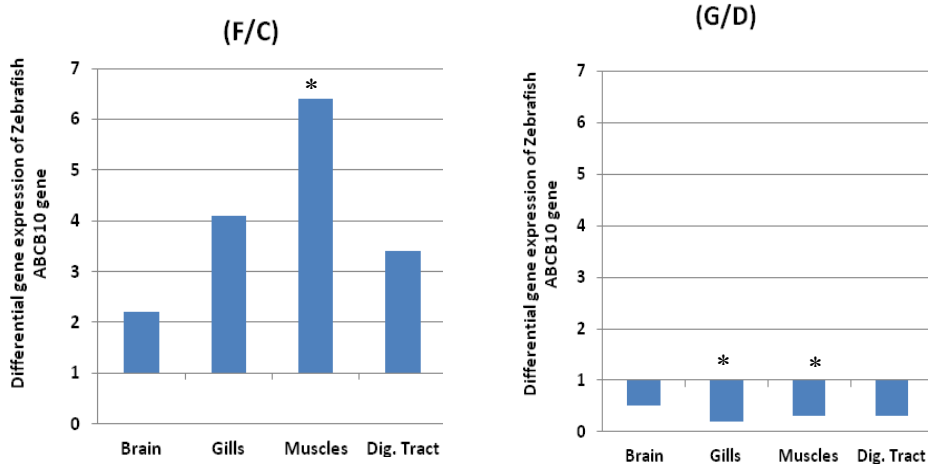

Fig. 2: Differential gene expression of Zebrafish ABCB10 in relation to heavy metals contamination *Statistically Significant difference ($p<0.05$)

Transcriptional response of Zebrafish ABCB10 gene to *Tubifex* worms

Zebrafish ABCB10 expression was highly up-regulated in presence of *Tubifex* worms in control group D compared to control group C; brain, gills, muscles and digestive tract samples recorded 3.8-, 14.3-, 13.1- and 28.4-times up-regulation respectively.

The presence of worms in contaminated environment showed down-regulation in Zebrafish ABCB10 gene in brain, gills, and muscles samples of G group compared to F group. In addition to 2.3-times up-regulation in the gene expression in digestive tract samples of G group compared to F group samples (Table 1and Fig. 3).

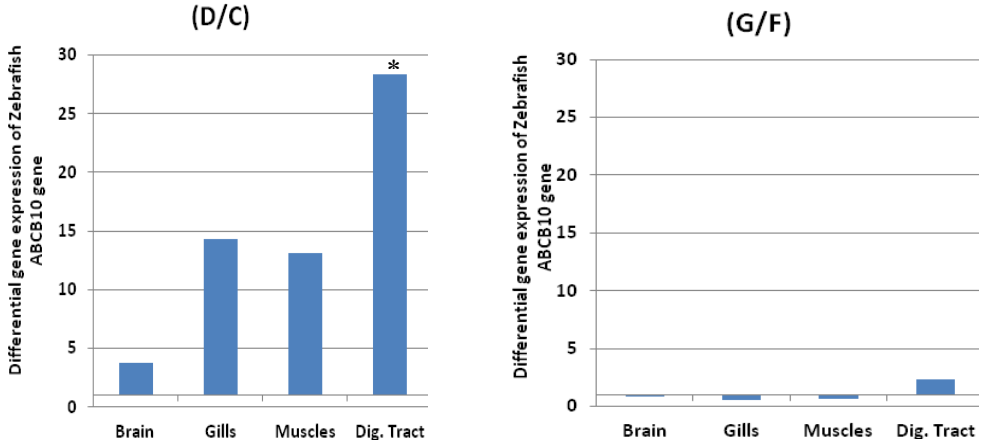

Fig. 3: Differential gene expression of Zebrafish ABCB10 in relation to *Tubifex* worms *Statistically Significant difference ($p<0.05$)

Transcriptional response of Zebrafish ABCB10 gene to both Cu and Cd contamination and *Tubifex* worms

To evaluate the combined effect Cu and Cd contamination and *Tubifex* worms on Zebrafish ABCB10 expression, the differential gene expression was calculated in G group against C group. Significant up-regulation was recorded in the gene expression in brain and muscles samples in G group compared to C group, also gills and digestive tracts have recorded 7.7- and 2.5-times up-regulation respectively (Fig.4).

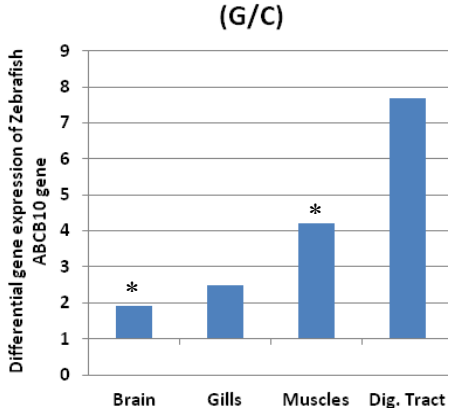

Fig. 4: Differential gene expression of Zebrafish ABCB10 in relation to both heavy metals contamination and *Tubifex* worms
*Statistically Significant difference ($p<0.05$)

DISCUSSION

ABCB10 gene expression was used in this study to invistigate Zebrafish defense mechanism against polymetallic contamination with Cu and Cd. *Tubifex* worms were used for its effective role in metal transfer and bioavailabilty between sediment-water column as bioturbator organism (Ciutat & Boudou, 2003). Therefore, its influence on Zebrafish ABCB10 expression was also investigated in this study.

ABCB10 is a mitochondrial ATP-binding cassette transporter, sub family B (MDR/TAP) member 10. It is involved in multidrug resistance, heme biosynthesis in liver and spleen (Nilsson et al., 2009), heme transport (Lee *et al*., 2012), and it is highly expressed in erythrocytes (Chambers *et al*., 2007).

Results of relative gene expression showed high induction in gene expression in digestive tract samples in all the experimental groups, with the highest expression level in the control group D (+fish+worms), followed by samples from group G, then samples from group F and group C.

ABCB10, as a member of ABCB transporters which are located in blood-brain barrier, liver, mitochondria and participates in peptides and bile transports (Annilo *et al*., 2006), and it has a serious role in cellular detoxification through lipophilic anion extrusion (Bellamy, 1996) . It was clear from the differential gene expression results that ABCB10 gene was up-regulated in 4 tested organs as a result of metallic contamination with Cu and Cd, as it has a serious role in heavy metals detoxification (Bellamy, 1996).

The comparison of relative gene expression in tissue samples in the contaminated group G (+fish+worms) against control group D (+fish+worms) showed significant down-regulation in gene expression in all tissues samples,

this is due to the high level of gene expression in control group D in all the tested organs.

It was surprising to notice that *Tubifex* worms were more effective on Zebrafish ABCB10 gene expression than Cu and Cd contamination. The significant high level of ABCB10 gene expression in presence of *Tubifex* in uncontaminated sediment in group D compared to group C could be explained due to the stimulatory effect of these worms on fishes attraction and exploration behaviour (Saglio *et al*., 1990). More exploration behaviour may cause more consumption of *Tubifex* as food, and more activity in the digestive tract to store many substances and to produce bile in the liver, then more expression of ABCB10 gene to extrude bile and to participate in blood detoxification (Field *et al*., 2003).

While the effect of *Tubifex* worms was significantly decreased in contaminated sediment, heavy metals contamination of the sediment might have caused inhibition in worms activities (Arrate *et al*., 2004) , less consumption of *Tubifex* as food, which could have less effect on the exploration and attraction activities of Zebrafish with less expression for ABCB10 gene.

The final results confirmed that there are two main organs that are associated in dissolved metal uptake inside aquatic animal's body, gills and digestive tract, while muscles are the final storage site for metals (Amiard *et al*., 1989; Lagadic *et al.,* 1997). Therefore, it was very clear that digestive tract recorded the highest expression level followed by muscles then gills which act as a short time storage organ for heavy metals (Amiard *et al*., 1989; Lagadic *et al.*, 1997). The least affected organ with heavy metals contamination in the presence of *Tubifex* worms was brain.

The combined effect of Cu and Cd contamination and *Tubifex* worms was evaluated by comparing the relative gene expression of group G samples with the gene expression of group C samples. Results showed up-regulation in the gene expression in all tested organs with significant increase in brain and muscles samples in group G compared to group C. That increase in gene expression was less than the effect of the metallic contamination or *Tubifex* worms alone. It might be due to the fact that *Tubifex* can be additional source of contamination because of its high ability to bioaccumulate different metals such as Cd in its body (Arrate *et al*., 2004; Ciutat & Boudou, 2003). Subsequently, it acts as a source of contaminated diet for Zebrafish leading to serious levels of Reactive Oxygen Species (ROS), causing oxidative stress that might cause DNA damage, inhibition in cells control on apoptotic death and by the end all the functions inside the cells could be affected (Lee & Shacter, 1999; Turpaev, 2002).

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ARABIC SUMMARY

التعبير الجيني لجين ABCB10 الناتج عن الحث بالمعادن الثقيلة في اسماك الزيبرا *Danio rerio*

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يعتبر جين ABCB10 من مجموعة ناقلات ABC وله دور مهم في مقاومة الأدوية المتعددة إلى جانب دوره المحتمل في مواجهة الضغط التأكسدى الناتج عن التعرض للمواد السامة كالمعادن الثقيلة. إلا انه حتى الآن لا يوجد معلومات كافية عن دور جين ABCB10 في إزالة سمية المعادن الثقيلة في اسماك الزيبرا. وكذلك لم يتم دراسة تأثير ديدان *Tubifex* على التعبير الجيني لهذا الجين بالرغم من دورها الهام في نقل ووفرة المعادن بين عمود الماء و الرواسب. لذلك في هذه الدراسة تم دراسة تأثير الرواسب الملوثة بالنحاس و الكادميوم على التعبير الجيني لجين ABCB10، كما تم دراسة تأثير وجود ديدان *Tubifex* على التعبير الجيني لهذا الجين.

تم تعريض اسماك الزيبرا لرواسب ملوثه بالنحاس و الكادميوم لمدة 7 أيام وذلك في وجود وعدم وجود ديدان *Tubifex*.

تم تسجيل نسبة التعبير الجيني لجين ABCB10 في 4 انسجه مختلفة (المخ و الخياشيم و العضلات و القناة الهضمية) لأسماك الزيبرا.

سجلت النتائج أعلى مستوى من التعبير الجيني لجين ABCB10 في عينات الجهاز الهضمي في جميع المعاملات التي تم اختبارها يليها العضلات ثم الخياشيم، في حين سجلت عينات المخ اقل مستوى للتعبير الجيني لجينABCB10.

وكما أظهرت ديدان *Tubifex* تأثير فعال جدا على التعبير الجيني لجين ABCB10 في اسماك الزيبرا ، وذلك بمستوى أعلى من المسجل لتأثير التلوث بالنحاس والكادميوم .