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Modulation of *N*-Methyl-D-Aspartate Receptors (NMDAR), Bcl-2 and C-Fos Gene Expressions on Exposure to Individual and Mixtures of Low Concentration Metals in Zebrafish (*Danio rerio*)

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Abstract Currently, there is limited information on the toxicity of low concentration of metal mixtures in the environment. Of particular interest is the effect of low levels of metal mixtures on neurodevelopment of aquatic organisms. This study reports the neurological gene expressions after exposing zebrafish embryos to low concentration toxic heavy metals, 120 h post fertilization (hpf). Embryos were exposed to low concentration individual and mixtures of lead (Pb), mercury (Hg), arsenic (As), and cadmium (Cd). Quantitative real-time PCR was used to assess gene expressions. The findings of this study confirmed that exposure to low concentration heavy metals upregulated *N*-methyl-D-aspartate (NMDA) receptor subunits NMDAR2A (NR2A), NMDAR2B (NR2B), and NMDAR2D (NR2D) and B cell lymphoma (Bcl-2) genes. NR2A genes were significantly upregulated by 90 and 74%, respectively, on exposure to Pb + As and Pb + Cd. NR2B genes were upregulated by 85.3, 68.6, 62.7, and 62.7% on exposure to As, Pb + Hg, Pb + As, and Pb + Cd, respectively. Exposure to As, Pb + Cd, and Pb + Hg + As significantly upregulated Bcl-2 genes by 2.01-, 1.84-, and 1.80-fold, respectively. NR1A and C-fos gene expressions were not significantly different from control. Upregulation of NMDAR subunits and Bcl-2 genes in this study was largely a counter measure against insults from exposure to low concentration heavy metals. Principal component analysis confirmed the influence of low concentration individual and mixtures of Pb, Hg, As, and Cd on gene expression of NMDAR subunits and Bcl-2. These data suggest that altered expression of NMDA receptor subunits and Bcl-2 genes may explain toxicity of low concentration individual and mixtures of Pb, Hg, As, and Cd.

Heavy metals are generally ubiquitous and nonbiodegradable. Their use and release into the environment continues to increase due to anthropogenic activities, such as mining, smelting, industrial discharge, and agricultural uses (Ali et al. 2013; Wuana and Okieimen 2011). Interaction of heavy metals with living systems is associated with disease conditions, such as reduced intelligence, coordination problems, impaired development (Iqbal 2012; Wuana and Okieimen 2011), ulcers and damage to brain, kidney, and lungs (Ainza et al. 2010; Ruszkiewicz and Albrecht 2015), renal failure and chronic anemia (Awofolu 2005; Panhwar et al. 2015), and disruption of cellular processes, such as oxidative phosphorylation and ATP synthesis (Tripathi et al. 2007).

There is currently a growing interest among the scientific community on the toxicity of low concentration heavy metals on aquatic organisms. Sarkar et al. (2014) reported that low levels of arsenic trioxide (50 μg/L) increased the generation of reactive oxygen species (ROS), malondialdehyde, and conjugated dienes in the brain of zebrafish after 90 days of exposure. It was further observed in the same study that, mRNA expression of glutathione peroxidase-1 (GPx1), catalase (Cat), manganese superoxide dismutase (Mn-Sod), copper/zinc superoxide dismutase (Cu/Zn-Sod), and cyclooxygenase-1 (Cox1) were all upregulated. B-cell lymphoma 2 (Bcl-2) genes were, however,

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downregulated. On exposure of low concentrations of Cu, Zn, and Cd (0.001–1.000 mg/L) for 72 h to embryos of rare minnow (*Gobiocypris rarus*), Zhu et al. (2014) reported significant upregulation of stress and metabolism-related genes (*hsp70, cyp1a* and *mt*). It was observed, however, that development-related genes (*wnt8a, vezf1* and *mstn*) expression were downregulated. Güner (2016) observed behavioral changes in zebrafish on chronic exposure to As (10 mg/L), Cd (5 mg/L), Cr (50 mg/L), Co (10 mg/L), and Al (300 mg/L). Sonnack et al. (2015) reported neuromast damage in zebrafish on exposure to low levels of Cd (2 mg/L), Cu (0.01 mg/L), and Co (0.2 mg/L). Several other studies reported the toxicity of low concentrations of metals on aquatic organisms (Cambier et al. 2010; Kesari et al. 2012; Seriani et al. 2015; Zahran and Risha 2014).

For some time, studies on the toxicity from metal exposures have centered mostly on individual metals. In reality, metals exist in the environment, most often as mixtures rather than as individual metals (Ge et al. 2014; Smith et al. 2012). Currently, data are limited on toxicity of low concentration metal mixtures in the environment. Of particular interest is the effect of low concentration of metal mixtures on neurodevelopment of aquatic organisms; some studies have been conducted (Chow et al. 2008; da Silva Acosta et al. 2016; Karri et al. 2016; Li et al. 2011; Powers et al. 2010). However, the effect of low levels of metal mixture exposure on neurodevelopment rarely has been investigated.

N-methyl-D-aspartate receptors (NMDARs) are important molecular devices that have vital roles in synaptic plasticity, excitatory synaptic transmission, learning, and memory functions (Li and Tsien 2009; Papadia and Hardingham 2007). They are glutamate-gated ion channel receptors that occur as heteromultimeric protein complexes, which are broadly expressed in the nervous system (Paoletti and Neyton 2007). These receptors incorporate different subunits within a collection of three subtypes: NR1, NR2 (NR2A-D), and NR3 (NR3A and NR3B).

Although their activation is necessary for cell survival, overreaction is a signal for cell death (Waxman and Lynch 2005). NMDA receptor stimulation is associated with the activation of several pathways, which mostly facilitate excitotoxicity. This results in activation of MAPKs, calcium-dependent enzymes, and mitochondrial dysfunction (Waxman and Lynch 2005). Luo et al. (2012) observed that As exposure altered expression of NMDA receptor complex and postsynaptic signaling proteins. In their study, they suggested that altered expression of NMDA receptor complex may explain As-induced toxicity. Spatial memory damage as a result of As exposure has been associated with reduced NR2A expression in hippocampus of rats by Luo et al. (2009). Toscano et al. (2002) demonstrated that low Pb levels altered the subunit composition of NMDAR complexes with subsequent effects on Ca-sensitive signaling pathways involved in CREB phosphorylation. The toxic effect of Pb also was associated with the differential effect on expression of NR1 and NR2B subunits in cortical and hippocampal neurons, respectively in rats (Lau et al. 2002).

B-cell lymphoma 2 (Bcl-2) is the founding member of the Bcl-2 family of regulator proteins that regulate cell death by either inducing or inhibiting it (Gómez-Fernández 2014). Studies show that Bcl-2 regulates the central nervous system's programmed cell death and may play a role in neurodegenerative diseases (Shacka and Roth 2005). Alterations in Bcl-2 have been linked to conditions, such as Parkinson's, Huntington's, and Alzheimer's diseases and Amyotropic lateral sclerosis (Akhtar et al. 2004; Merino and Bouillet 2009; Shacka and Roth 2005).

C-fos is a proto-oncogene, and it is part of the Fos family of transcription factors (Milde-Langosch 2005). C-fos expression can serve as a marker of neuronal activation and is usually assessed in different regions of the brain. Brain C-fos can be upregulated in zebrafish (*Danio rerio*), especially after exposure to alarm pheromone, predators, and/or novelty stress (Stewart et al. 2014). The overexpression of C-fos is linked to tumors. The expression of C-fos has been reported to modify transcription of target genes and has been related to cell proliferation and differentiation (An et al. 1993). Additionally, induction of C-fos gene is known to be associated with apoptosis (Kalra and Kumar 2004).

There is currently a paucity of information on the effect of low levels of metal mixtures at the molecular level on subunits that make up the NMDA receptor, Bcl-2, and C-fos mRNA. This study, hence, attempted to elucidate the effects on NMDA receptors, Bcl-2 and C-fos gene expressions as a result of exposure to low concentration mixtures of Pb (0.01 mg/L), Hg (0.001 mg/L), As (0.01 mg/L), and Cd (0.005 mg/L) using the zebrafish model. The concentrations of metals used in this study are the maximum permissible limits stipulated in the National Standard of the Republic of China for Municipal Water Standards (GB5749-2006). These are metal concentrations in drinking water, deemed safe for ingestion. The zebrafish model was employed in this study, because it has been found to have many advantages as a model for the study of neuronal development and the role of receptors. The zebrafish model is exceptional, because their maintenance is relatively cheap and large numbers can be produced for each experiment. They are small, thin, and their embryos can be immobilized. They also respond to many pathological factors.

Materials and Methods

Test Chemicals

Analytical grade lead acetate [$Pb(CH_3COO)_2$], cadmium chloride ($CdCl_2$), mercury chloride ($HgCl_2$), and sodium arsenite ($NaAsO_2$) were purchased from Sinopharm Chemical Reagent Co. Ltd. Stock solutions of 1000 mg/L were prepared using water purified by several processes, including reverse osmosis. Low concentrations of Pb (0.01 mg/L), Hg (0.001 mg/L), Cd (0.005 mg/L, and As (0.01 mg/L) were prepared through serial dilutions. PrimeScript RT reagent kits were purchased from TaKaRa (Dalian, China). SsoFast™ EvaGreen® Supermix kit and iQ™ 5 Multicolor Real-Time PCR Detection System were bought from Bio-Rad, USA.

Fish Care and Maintenance

Zebrafish (AB strain) for the study were bought from the National Zebrafish Resources of China (Shanghai, China). The brood stock had been maintained in our laboratory for more than 1 year. Zebrafish were cultured at a temperature of 28 ± 0.5 °C in dechlorinated water using a flow-through system, in a photoperiod consisting of a 14-h light/10-h dark cycle each day. Feeding of fish was done twice a day, with brine shrimps (*Artemia nauplii*), and embryos were examined under a stereomicroscope after 120 hpf. Unfertilized eggs and those with cleavage irregularities or injuries were disposed off.

Chemical Exposure

Normally developed zebrafish embryos that have attained the blastula stage were selected for the study. They were categorized into ten groups and exposed to low concentrations of individual and mixtures of Pb, Hg, Cd, and As (Table 1). Approximately 120 zebrafish embryos were randomly distributed into a six-celled microplate as a group and exposed to test solutions (5 mL per cell) until a 120-hpf period. Each cell contained 20 embryos, with the control group exposed to dechlorinated tap water. By 120 hpf, most zebrafish embryos develop into free-swimming larvae and most had completely developed organs, such as heart, brain, and liver (Amsterdam et al. 2004; Chan et al. 2009). Exposure to all groups were done in triplicates, and all embryos were incubated at 28 ± 0.5 °C in a 14-h light/10-h dark cycle each day. Test solutions were completely (100%) changed every 24 h. At the end of 120 hpf, larvae were randomly sampled and immediately frozen in liquid nitrogen and stored at −80 °C for subsequent gene expression analysis.

Quantitative Real-Time PCR Assay

Isolation of total RNA, synthesis of first-strand cDNA and qRT-PCR were done as previously described in Xu et al. (2013). Briefly, 20 zebrafish larvae per sample were homogenized and used for total RNA isolation using the RNAprep pure Tissue kit (TIANGEN-Biotech, Shanghai, China), following the manufacturer's instructions. DNA contamination was removed with the help of RNase-free DNase I (Promega, Madison, WI). The quantity of RNA was measured using an UV spectrophotometer at 260 nm. The purity and quality of RNA was assessed by determining the 260/280 nm ratios and by 1% agarose gel electrophoresis.

cDNA was synthesized for each sample from 100 ng total RNA using the iScript™ cDNA Synthesis kit (Bio-Rad, USA) following the manufacturer's protocol. Quantitative real-time PCR was done using the SsoFast™ EvaGreen® Supermix kit (Bio-Rad, USA) and an iQ™ 5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). The reaction mix was made up of 10 µl of SsoFast™ EvaGreen® Supermix (2×), 0.5 µM of each primer, and 1 µl of cDNA. The PCR amplification protocol was as

Table 1 Experimental design for low-dose (mg/L) individual and mixtures of heavy metals administered to embryos in groups

Group	Dose
Control	0
Lead (Pb)	0.01
Mercury (Hg)	0.001
Arsenic (As)	0.01
Cadmium (Cd)	0.005
Lead + mercury	0.01 (Pb) + 0.001 (Hg)
Lead + arsenic	0.01 (Pb) + 0.01 (As)
Lead + cadmium	0.01 (Pb) + 0.005 (Cd)
Lead + mercury + arsenic	0.01 (Pb) + 0.001 (Hg) + 0.01 (As)
Lead + mercury + cadmium	0.01 (Pb) + 0.001 (Hg) + 0.005 (Cd)
Lead + mercury + cadmium + arsenic	0.01 (Pb) + 0.001 (Hg) + 0.005 (Cd) +0.01 (As)

follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and the fluorescent signal was measured at the extension step. Melt curve analyses was performed to validate the specificity of the PCR amplicons. A Ct-based relative quantification with efficiency correction normalizing to ribosomal protein L13A (*rpl 13a*) was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Duplicate RT-qPCR reactions were done on triplicate samples for each tested gene. The primer sequence of genes is shown in Table 2.

Data were verified for normality and homogeneity using the Kolmogorov–Smirnov and Levene's test, respectively. Data are shown as mean ± standard error (SEM). Differences among groups were determined using one-way analysis of variance (ANOVA), followed by Duncan's test, using SPSS 16.0 (SPSS Inc., Chicago, IL). A p value <0.05 was considered statistically significant. Multivariate analysis was used to ascertain associations among NMDA receptor subunits, Bcl-2 and c-fos genes exposed to individual and mixtures of metals. In this, the principal component analysis was used to compare molecular responses resulting from exposure to individual and mixtures of metals.

Results and Discussion

In this study, the effect of low concentration individual and mixtures of Pb, Hg, As, and Cd on NMDA receptor subunits (NR1A, NR2A, NR2B, and NR2D), B-cell lymphoma 2 (Bcl-2), and C-fos genes were assessed. Low concentrations of the metals did not significantly change mRNA levels of NR1A compared with control. However, within the groups, exposure to Pb, Hg, and Cd individually, slightly inhibited NR1A gene expression by 33.0, 24.5, and 46.1%, respectively (Fig. 1a). Several studies report on the inhibition of mRNA levels of NMDA receptor subunits, such as NR1A, on exposure to individual toxic metals (Borges et al. 2007; Fan et al. 2010; Marchetti and Gavazzo 2005; Nihei and Guilarte 2001; Vidal et al. 2007). For instance, Pb is thought to inhibit NR1A expression in rats by reducing intracellular Ca^{2+} levels, which have the ability to alter intracellular signaling pathways. This can disrupt the activation of transcription factors that regulate NR1A gene expression (Fan et al. 2010). It also could be due to Pb induced perturbations in the sp1 transcriptional regulation of the NR1A gene (Lahiri et al. 2007).

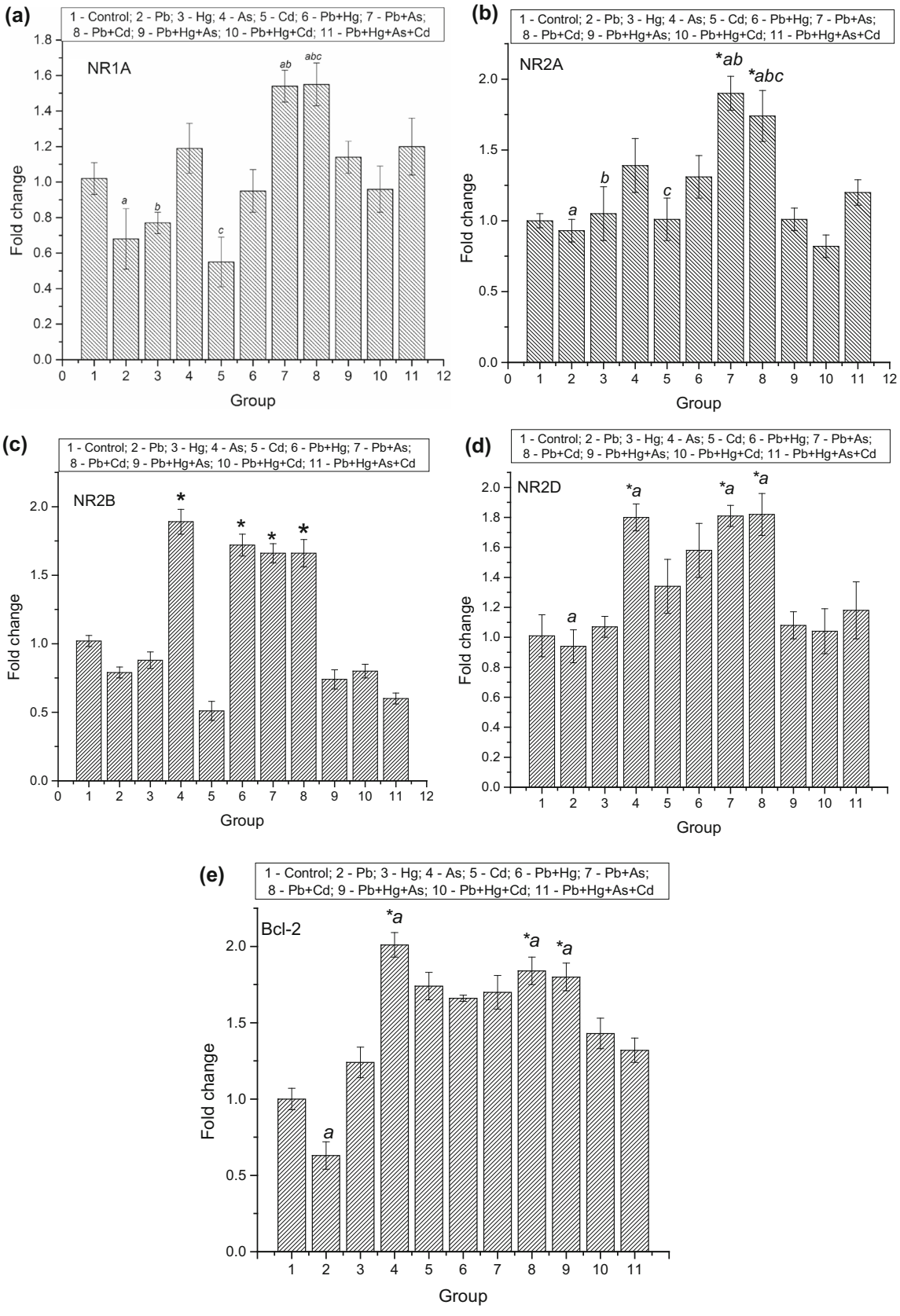
Marginal increases in NR1A gene expressions, resulted from exposure to Pb + As (1.54-fold), Pb + Cd (1.55-fold), and Pb + Hg + As + Cd (1.20-fold) (Fig. 1a). Among the groups, there were significant differences in NR1A gene expression on exposure, especially to Pb and Pb + As. Co-exposure of Pb and As slightly upregulated NR1A mRNA gene expression compared with the individual metals (Fig. 1a). Pb and Pb + Cd similarly showed a significant difference in NR1A expression and synergism. Co-exposure of Pb and Cd also showed increased NR1A expression in zebrafish embryo compared with Pb + Cd mixture.

mRNA gene expression levels of NR2A were significantly upregulated by 1.90- and 1.74-fold on administration of Pb + As and Pb + Cd, respectively (Fig. 1b). Co-exposure of Pb and As in Pb + As showed synergistic interactions. A similar observation was made in that of Pb and Cd. Synergistic metal interactions in Pb + As and Pb + Cd largely suggests that mRNA expression of NR2A of individual metals (Pb, As, and Cd) were lower than when co-administered (Fig. 1b).

Fig. 1 Expression of **a** NR1A, **b** NR2A, **c** NR2B, **d** NR2D, and **e** Bcl-2 after exposure to individual and mixtures of low concentration Pb, Hg, As, and Cd. The results are mean ± SEM. of triplicate samples. *Significance at $p < 0.05$ compared with control. [a,b,c]Like *symbols* signify that gene expression resulting from exposure to individual metal was significantly different from corresponding mixture ($p < 0.05$)

Table 2 Primer sequence for quantitative reverse transcription polymerase chain reaction used in the study

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	GenBank Accession #	Coupling Eff. (%)	Reference
NR1A	TGGCCGATTCAAGGTGAACA	CCATGCCTAGGATACGTGCAGA	NM 017010.1	98.4	Fan et al. (2010)
NR2A	TCGATACCGGCAGAACTCCAC	CATCCGCAGACAGGCATCA	NM 012573.3	98.4	Fan et al. (2010)
NR2B	CGCCTAGAGGTTTGGCGTCTAC	GAACGAGCTTTGCTGCCTGA	NM 012574.1	98.4	Fan et al. (2010)
NR2D	TAGTGTCAGTGCGCAGATCC	ACCATGAACCAGACGTAGCC	L31612	98.4	Santillo et al. (2014)
Bcl-2	GATTGTGGCCTTCTTTGAGTT	AGTTCCACAAAG GCATCCCA	–	98.4	Pan et al. (2011)
C-fos	ATCCGAAGGGAAAGGAATAA	TCTGGGAAGCCCAGGTCAT	–	98.4	Pan et al. (2011)
rpl13A	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	NM_212784	98.4	Tang et al. (2007)


(a)
1 - Control; 2 - Pb; 3 - Hg; 4 - As; 5 - Cd; 6 - Pb+Hg; 7 - Pb+As;
8 - Pb+Cd; 9 - Pb+Hg+As; 10 - Pb+Hg+Cd; 11 - Pb+Hg+As+Cd
NR1A
Fold change
Group
(b)
1 - Control; 2 - Pb; 3 - Hg; 4 - As; 5 - Cd; 6 - Pb+Hg; 7 - Pb+As;
8 - Pb+Cd; 9 - Pb+Hg+As; 10 - Pb+Hg+Cd; 11 - Pb+Hg+As+Cd
NR2A
*ab
*abc
Fold change
Group
(c)
1 - Control; 2 - Pb; 3 - Hg; 4 - As; 5 - Cd; 6 - Pb+Hg; 7 - Pb+As;
8 - Pb+Cd; 9 - Pb+Hg+As; 10 - Pb+Hg+Cd; 11 - Pb+Hg+As+Cd
NR2B
Fold change
Group
(d)
1 - Control; 2 - Pb; 3 - Hg; 4 - As; 5 - Cd; 6 - Pb+Hg; 7 - Pb+As;
8 - Pb+Cd; 9 - Pb+Hg+As; 10 - Pb+Hg+Cd; 11 - Pb+Hg+As+Cd
NR2D
Fold change
Group
(e)
1 - Control; 2 - Pb; 3 - Hg; 4 - As; 5 - Cd; 6 - Pb+Hg; 7 - Pb+As;
8 - Pb+Cd; 9 - Pb+Hg+As; 10 - Pb+Hg+Cd; 11 - Pb+Hg+As+Cd
Bcl-2
Fold change
Group

Studies show that exposure to As (68 mg/L), however, only decreased NR2A protein expression in rats (Luo et al. 2009, 2012), implying that NR2A gene expressions are dose-dependent. Additionally, exposure to only Pb in this study reduced mRNA levels of NR2A (0.93-fold). This is concordant with studies by Guilarte and McGlothan (1998), where developmental Pb exposure produced reductions in NR2A genes expressions in hippocampal regions of rats. Co-exposure of Pb and As was found to affect the central monaminergic system of adult mice (Mejía et al. 1997). In the same study, Pb + As provoked a 38% decrease of norepinephrine in the hippocampus and a 90% increase of serotonin in the frontal cortex (Mejía et al. 1997).

Exposure of zebrafish embryo to low-dose As significantly upregulated mRNA levels of NR2B by 1.89-fold (Fig. 1c). However, inorganic As exposure to cortex and hippocampus of male and female pups downregulated NR2B subunits in a study by Ramos-Chávez et al. (2015). Further increases were observed when embryos were exposed to binary mixtures, such as Pb + Hg (1.72-fold), Pb + As (1.66-fold), and Pb + Cd (1.66-fold) in the study. It was observed that, although not significantly different ($p < 0.05$) from control, the ternary and quaternary mixtures slightly inhibited mRNA levels of NR2B. NR2B subunits modulate the functional and pharmacological properties of the NMDA receptor (Mony et al. 2009). It has been implicated in modulating the synaptic functions in activities, such as learning, memory processing, and feeding behaviors (Mehta et al. 2013). Modulation of NR2B genes may be due to the effect of oxidative stress in the developing embryo of the zebrafish (Scimemi et al. 2009).

Messenger-RNA levels of NR2D were significantly upregulated after administration of As (1.80-fold), Pb + As (1.80-fold), and Pb + Cd (1.82-fold). There was generally no significant change in mRNA levels after exposure to ternary and quaternary mixtures (Fig. 1d). The presence of Pb did not have much effect on As in Pb + As mixture, because NR2D levels did not change. Interaction in Pb + Cd was observed to be antagonistic, as NR2D levels decreased after Pb + Cd exposure, compared with individual metals (Pb and Cd).

During the study, Bcl-2 gene transcripts were significantly upregulated on exposure to As (2.01-fold), Pb + Cd (1.84-fold), and Pb + Hg + As (1.80-fold). Among the groups, there were significant differences between Pb and Pb + Cd-exposed groups. It was observed that co-exposure of Pb and Cd exhibited antagonistic interactions. Although Pb and Cd are non-redox metals, their toxicity to living systems are due to the generation of reactive species and/or depletion of antioxidant defense system, which results in oxidative stress (Matović et al. 2015). Similar antagonistic interactions were observed in exposure to Pb + Hg + As, as Bcl-2 gene expression of mixture was lower compared with individual metals (Fig. 1e). Overexpression of Bcl-2 genes from exposure to these groups, resulted as a counter measure, to insults from exposure to low concentration metals (Cherbonnel-Lasserre and Dosanjh 1997). Increased levels of Bcl-2 however, are associated with neurodegenerative diseases, including Parkinson's disease in humans (Marshall et al. 1997). Studies also showed that Bcl-2 is pro-oxidant in *E Coli*., as it appears to influence levels of reactive oxygen intermediates that induce endogenous cellular antioxidants (Steinman 1995).

Exposure of individual and mixtures of metals did not significantly change c-fos gene expression during the study. However, among the groups, there were significant differences in c-fos gene expressions between Pb and Pb + As-exposed groups. Similar observations were made between Pb and Pb + Cd-exposed groups.

Intergene Correlations

Pearson's rank correlations of genes exposed to individual (*i*) and mixtures (*m*) of metals are presented in Table 3. There were very strong significant correlations ($r > 0.75$) between NR1A*i* - NR2A*i* ($r = 0.934$, $p < 0.01$), NR1A*i*–NR2B*i* ($r = 0.996$, $p < 0.01$), NR1A*i*–c-fos*m* ($r = 0.825$, $p < 0.01$), NR1A*m*–NR2A*i* ($r = 0.840$, $p < 0.05$), NR2A*i*–NR2B*i* ($r = 0.938$, $p < 0.01$), NR2A*i*–NR2Di ($r = 0.924$, $p < 0.01$), NR2A*i*–c-fos*m* ($r = 0.951$, $p < 0.01$), NR2A*m*–NR2B*m* ($r = 0.838$, $p < 0.05$), Bcl-2*i* - Bcl-2 *m* ($r = 0.974$, $p < 0.01$), Bcl-2*i* - c-fos*i* ($r = 0.977$, $p < 0.01$), Bcl-2*i*–c-fos*m* ($r = 0.862$, $p < 0.05$), Bcl-2*i*–c-fos*i* ($r = 0.977$, $p < 0.01$), and Bcl-2 *m*–c-fos*i* ($r = 0.974$, $p < 0.01$). The strong relationship between the genes, after exposure to individual and mixtures of low concentration metals, indicates a strong physiological relationship. It also suggests that their expressions are influenced in a similar way.

Multivariate Statistical Analysis

In this study, principal component analysis was performed to identify possible relationships and evaluate its extent among NMDA receptor subunits, Bcl-2 and c-fos genes, after exposure to metals. Principal component analysis results based on the correlation matrix of receptor subunits, Bcl-2 and c-fos genes, are presented in Table 4. They include loadings with Varimax rotation and eigen values Boldfaced values are loadings that represent the importance of variables for the component, correlation coefficients ≥0.70 were considered highly correlated.

In all, three components with eigen values >1 were extracted, which accounted for 99% of total variance. The first component (PC1), which generally explained the

Table 3 Pearson's rank correlation for associations among NMDA receptor subunits, Bcl-2, and c-fos genes after administration of low concentration individual and mixtures of heavy metals

	NR1A*i*	NR1A*m*	NR2A*i*	NR2A*m*	NR2B*i*	NR2B*m*	NR2D*i*	NR2D*m*	Bcl-2*i*	Bcl-2*m*	C-fos*i*	C-fos*m*
NR1A*i*	1	0.694	0.934**	0.673	0.996**	0.564	0.744	0.735	0.496	0.500	0.329	0.825*
NR1A*m*		1	0.738*	0.840*	0.640	0.436	0.555	0.711	0.605	0.462	0.437	0.639
NR2A*i*			1	0.510	0.938**	0.238	0.924**	0.488	0.772	0.770	0.643	0.951**
NR2A*m*				1	0.609	0.838*	0.165	0.965**	0.084	0.590	−0.124	0.535
NR2B*i*					1	0.532	0.770	0.689	0.507	0.530	0.351	0.812*
NR2B*m*						1	−0.129	0.930**	−0.414	0.600	−0.587	0.402
NR2D*i*							1	0.118	0.914**	0.948**	0.859*	0.892*
NR2D*m*								1	−0.095	0.556	−0.302	0.464
Bcl-2*i*									1	0.974**	0.977**	0.862*
Bcl-2*m*										1	0.934**	0.659
C-fos*i*											1	0.736
C-fos*m*												1

* Correlation significant at 0.05 level (2-tailed)

** Correlation significant at 0.01 level (2-tailed)

Table 4 Rotated component matrix of NMDA receptor subunits, Bcl-2 and c-fos, resulting from exposure to individual and mixtures of heavy metals

Parameter	Component		
	PC1	PC2	PC3
Bcl-2*i*	**0.995**	−0.029	−0.100
Bcl-2*m*	**0.988**	−0.104	0.114
C-fos*i*	**0.969**	−0.238	−0.071
NR2D*i*	**0.950**	0.183	0.254
C-fos*m*	**0.871**	0.482	−0.094
NR2A*i*	**0.813**	0.544	0.207
NR2D*m*	−0.067	**0.998**	−0.002
NR2A*m*	0.079	**0.942**	−0.326
NR2B*m*	−0.368	**0.904**	0.216
NR1A*i*	0.552	**0.775**	0.307
NR2B*i*	0.569	**0.730**	0.379
NR1A*m*	0.581	0.667	−0.467
Control	0.098	0.027	**0.995**
Eigen value	6.347	4.909	1.744
% of variance	48.82	37.76	12.41
Cummulative (%)	48.82	86.59	99.00

i = individual, *m* = mixture

majority of total variance (48.82%), had high loadings on Bcl-2*i*, Bcl-2 *m*, c-fos*i*, c-fos*m*, NR2D*i*, and NR2Ai.

The second component (PC2) explained 37.76% of total variance, with high loadings on NR2D*m*, NR2B*m*, NR2A*m*, NR1A*i*, and NR2B*i*. PC3 had high loading on the control and explained 12.41% of total variance (Table 4).

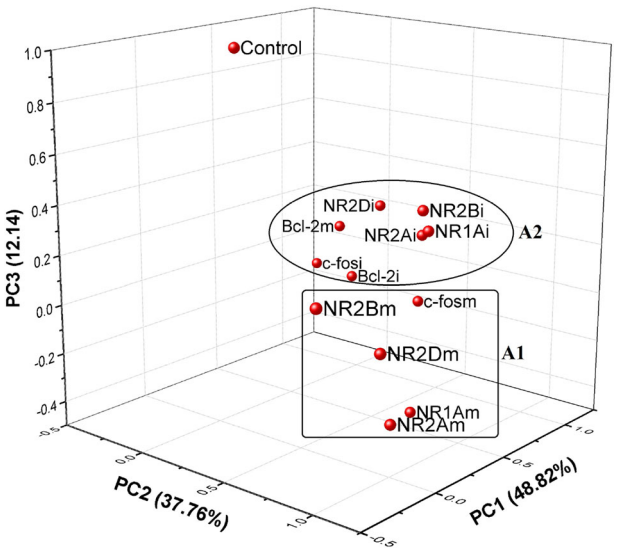


Fig. 2 Principal component analysis of NMDA receptor subunits, Bcl-2 and c-fos, gene expression on exposure to low-dose individual and mixtures of toxic metals (Pb, Hg, As, and Cd). The total variance explained by the components was 99.0%. Clusters containing groupings resulting from exposure to mixtures and individual metals are shown in A1and A2, respectively

In all, neurological gene expressions were divided into two main groups, according to their correlation between metal administrations (score) and their expressions (loadings) (Fig. 2). The first group (A1) was on the positive side of PC1 and PC2 but on the negative side of PC3 (Fig. 2). This depicted the influence due to exposure mixtures of heavy metals on NMDA receptor subunits (NR1A, NR2A, NR2B, and NR2D) and c-fos genes.

The second group (A2) was on the positive side of PC1 and the negative and/or positive sides of both PC2 and PC3 (Fig. 2). This demonstrated the expressions resulting from the influence exerted from exposure of individual metals (Fig. 2). This confirms that both individual and mixtures of heavy metals influenced expressions of NMDA receptor subunits, Bcl-2 and c-fos, after exposure to low concentration Pb, Hg, As, and Cd.

Conclusions

There is limited information on the toxicity of low concentration metal mixtures in the environment. Of particular interest is the effect of low concentration of metal mixtures on neurodevelopment of aquatic organisms. The findings of this study show that exposure of low concentration individual and mixtures of metals modulate through the NMDAR signaling pathway and Bcl-2 gene expressions in zebrafish embryo. The study confirmed that *N*-methyl-D-aspartate (NMDA) receptor subunits NMDAR2A (NR2A), NMDAR2B (NR2B), and NMDAR2D (NR2D) and B-cell lymphoma (Bcl-2) genes were upregulated on exposure to low-concentration Pb, Hg, Cd, and As. Specifically, exposure to As was observed to increase NR2B, NR2D, and Bcl-2 gene expressions. Binary mixtures, such as Pb + As, Pb + Cd, and Pb + Hg, were found to increase significantly NR2A, NR2B, NR2D, and Bcl-2 genes during the study. Principal component analysis confirmed the influence of low concentration individual and mixtures of Pb, Hg, As, and Cd on gene expression of NMDAR subunits and Bcl-2.

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