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^{1}H NMR-based serum metabolic profiling of *Carassius auratus gibelio* under the toxicity of Pb^{2+} and Cd^{2+}

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Abstract Lead and cadmium are the two widely recognized toxic metals, with known ecological risk to freshwater fish in contaminated ecosystems. However, their effect at environmentally realistic level in a mixture has been rarely investigated. In the present study, serum metabolic responses of Crucian carp exposed to Pb (30 μgL^{-1}), Cd (100 μgL^{-1}) and Pb+Cd (30 + 100 μgL^{-1}) for 21 days were investigated by ^{1}H NMR-based metabolomics. The metabolic responses were compared to control by multivariate techniques (PCA, PLS-DA and OPLS-DA), and metabolites that significantly contributed to the variation were identified. Metal dependent metabolic responses revealed a decline of alanine, lysine and tyrosine in Pb exposed fish, indicating changes in neurotransmitters, and amino acid metabolism, while fish exposed to Cd showed significant decrease in lysine, isoleucine, leucine, alanine and increase in 3-hydroxybutyrate, acetone, lactate, choline, inosine, guanosine and threonine. The coexposure of Pb and Cd had additive effect on metabolic profile with increase in pyruvate, guanosine and inosine. The overall metabolic changes due to Pb and Cd were characterized by disturbed energy metabolism, impaired osmotic regulation and a shift from aerobic to anaerobic respiration. Moreover, ^{1}H NMR-based metabolomics was proved to be a powerful tool in elucidating the toxic effects of environmental pollutants and underlying mechanism.

Introduction

Lead (Pb) and cadmium (Cd) are the two nonessential heavy metals that can be highly toxic even at low concentration due to their reactivity with sulfur (S) and nitrogen (N) atoms in the biomolecules and their constituents (Thapa et al. 2012). Excessive accumulation of Pb and Cd in the body can cause serious damage to different organisms at cellular and molecular level. They have the ability to bind with sulfhydryl groups thereby disrupting the structures and functions of proteins, or they can compete with some essential elements from a variety of proteins that serve as enzymes and transcription factors (Qi et al. 2015). In addition, these metals have the characteristics to induce oxidative stress and stimulate the production of reactive oxygen species (ROS) which lead to the impairment of normal redox cycle in cells (Rodriguez-Serrano et al. 2009; Schutzendubel et al. 2001). Studies have shown that both Pb and Cd can affect organs and various systems of an organism and may cause acute and chronic intoxications (Matovic et al. 2015).

The toxicity of Pb and Cd have been comprehensively investigated in various in vivo and in vitro studies,

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revealing different molecular, cellular and intracellular mechanisms to indicate the toxicological impacts of these two toxic metals (Clemow and Wilkie 2015; Hambach et al. 2013; Zaza et al. 2015). However, the degree of pathogenesis and deleterious health consequences due to Pb and Cd exposure is multifaceted, and the exact mechanisms underlying their toxicity are yet not fully understood (Matović et al. 2011). Recently, Pb and Cd exposure have been found to influence gene expression, apoptosis, DNA damage and repair (Al Bakheet et al. 2013; Banfalvi et al. 2012; Khalaf et al. 2012; Zhou et al. 2013). The knowledge pertaining to the complete understanding of these mechanisms is far from being achieved and has always remained controversial with only the oxidative stress as the most documented molecular mechanism for Pb and Cd toxicity (Çavaş 2008; Maceda-Veiga et al. 2015; Birben et al. 2012).

Beside natural occurrence of Pb and Cd in the ores and racks, their contamination in the ecosystems can greatly depend on the anthropogenic mobilization. The main sources through which they are mobilized to the environment are mining, smelting, industrial settings and agricultural activities (Sfakianakis et al. 2015). Aquatic ecosystem is the ultimate sink among all contaminated systems that constantly receive these metals from the natural and anthropogenic sources (Wang et al. 2012). The most available and reactive form of Pb and Cd in water that effect organisms are their ionic species that impart serious problem via bioaccumulation, biomagnifications through food chain and toxicity to human (Guzinski et al. 2013). Fish, being a dominant creature in aquatic environment can be better used as bio-indicator of toxic metal pollution. Besides their significance in global food supply in terms of nutritious and balance diet, fish also play crucial role in ecological balance and energy flow in aquatic ecosystems (Li et al. 2014). Fish are particularly sensitive to contaminants in water, because they show marked physiological and biochemical changes in contaminated environments, and thus, fish can serve as suitable bioindicator in environmental monitoring and quality assessment of aquatic ecosystem (Gül et al. 2004).

Metabolomics is the key "omic" science in system biology that has been widely used in toxicity assessment, disease diagnosis and safety evaluation (Laghi et al. 2014). Among various analytical techniques (GC–MS, LC–MS, NMR) used for metabolic profiling, NMR has many advantages. It is considered as unbiased, nondestructive, rich in structural information and rapid with little or no separation (Wei et al. 2015; Xu et al. 2015). Recently, metabolomics is more commonly used for biomarkers identification associated with prognosis or diagnosis of disease and toxicity assessment to improve our understanding of cellular response to toxic substances or other environmental stressors (Mamas et al. 2011). During stress or disease, cells respond to adjust themselves to intra-and/or extracellular environment in order to maintain homeostasis. One way to attain homeostasis is the modulation of biofluids in response to disease, medications and exposure to toxic substances. Such regulation is expressed in a specific metabolic pattern which results in biochemical changes that are pertinent to the disease or external stress (Dudka et al. 2014; Lindon et al. 2004).

The term environmental metabolomics refers to the study of organism environment interactions and the risk associated with the effects of environmental pollutants on physiological function and health of organism at a molecular level (Bundy et al. 2008). A number of species both from animal and plant kingdom have been subjected to the study of environmental metabolomics (Viant 2008). Recently, some of the freshwater fish including gold fish (*Carassius auratus*) have been successfully investigated for the environmental risk of organic and inorganic pollutants using ^{1}H NMR-based metabolomics approach (Jordan et al. 2012; Li et al. 2014; Lu et al. 2014; Xu et al. 2015). However, to the best of our knowledge, there is no information available on metabolomics responses of freshwater fish to toxic heavy metals, Pb and Cd. The present study was conducted at Wuhan PR. China, in 2015 to elucidate the individual and combined toxic effects of Pb and Cd at environmentally realistic concentrations using ^{1}H NMR-based metabolomics technique in Crucian carp, an ecologically important freshwater fish species.

Materials and methods

Chemicals and reagents

Lead nitrate and cadmium chloride of purity >99 %, nitric acid, acetic acid (conc. glyacial), sodium thiosulfate, EDTA (disodium salt of EDTA), potassium iodide crystal, magnesium sulfate, ammonium chloride and ammonium hydroxide were purchased from Sinopharm Chemical Reagents Co., Ltd (Beijing, China). The deionized water used for preparation of reagents and elemental stock solutions were passed through Millipore purification apparatus (Millipore, MA, USA) to a resistivity higher than 18.2 MΩ cm. ICP-Multi-element certified reference materials (CRM) were obtained from PerkinElmer No. N9300281, 1 Shelton, Connecticut, USA. All the chemicals were analytical grade and used without any further purification.

Experimental condition and exposure assay

Crucian carp of appropriate body weight (92 ± 4.2 g) and length (12 ± 2.6 cm) were procured from a freshwater fish

breeding base in Wuhan, China, and acclimated to the laboratory condition for 1 week in the laboratory tanks (50 cm × 30 cm × 30 cm) containing dechlorinated tap water with continuous supply of oxygen. The important water quality parameters were regularly assessed according to the standard methods of APHA (1992). The optimum condition (total hardness 156.32 ± 4.43 mgL^{-1} as CaCO$_3$, temp. 22.41 ± 2.11 °C, pH 7.6 ± 0.31, dissolved oxygen 8.26 ± 0.68 mgL^{-1}) for water quality was maintained till the end of the experiment. During acclimation, fish were fed with artificial feed once a day until a day before termination of acclimation period. Half of the aquarium water was renewed everyday to clean the residual feed and ammonia produced by fish. All the experiments were carried out according to the guidelines of Chinese Law for Animal Health Protection and Instructions for Granting Permits for Animal Experimentation for Scientific Purposes [Ethics approval No. SCXK (YU) 2005-0001]. Forty acclimated fish were randomly divided into four different groups: control ($n = 10$), Pb ($n = 10$), Cd ($n = 10$) and Cd+Pb ($n = 10$), without making any distinction between sexes. Control group was kept under similar experimental condition but without any addition of test chemical, while Pb group was exposed to 30 µgL^{-1} Pb as Pb(NO$_3$)$_2$. The Cd group was exposed to 100 µgL^{-1} Cd in the form of CdCl$_2$, whereas Cd+Pb group was exposed to a combination of the two test chemicals at the same rate. The exposure duration was 21 days for all groups. During exposure, the respective concentrations were closely monitored in the aquarium at 2 days interval by ICP-OES to maintain the desired concentration in the tanks. The exposure concentration of Cd and Pb was selected on the basis of previous studies (Khan et al. 2015; Qu et al. 2014), which suggested that exposure to these concentrations might significantly inhibit the activity of antioxidant enzymes and induce a pro-oxidant condition in the various tissues of freshwater fish. Moreover, these concentrations are somewhat related to the contamination levels of rivers and lakes in China (An et al. 2010; Bing et al. 2013; Li et al. 2013; Wang et al. 2012; Yang et al. 2009; Zhou et al. 2007).

Sample collection and preparation for ^{1}H NMR

After 21 days of exposure, blood samples were obtained from the caudal vein of each fish in all groups and allowed to clot for 30 min. The serum was then obtained by centrifugation at 3500 rpm for 5 min. All serum was snap-frozen with liquid nitrogen immediately after collection and stored at −80 °C prior to NMR analysis. For sample preparation, 200 µL of each serum was mixed with 400 µL of phosphate buffer (45 mM, pH 7.40, 50 % D$_2$O) followed by centrifugation at $16{,}099 \times g$, 4 °C for 10 min. The supernatant (550 µL) from each sample was then transferred into a 5 mm NMR tube and employed directly for NMR analysis.

^{1}H NMR serum analysis

^{1}H NMR spectra of serum were acquired at 298 K on an Agilent DD2-600 MHz spectrometer (599.83 MHz for proton frequency) equipped with an Agilent inverse cryogenic probe. A water-pre-saturated Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (recycle delay-90°-(τ-180°-τ)n-acquisition) was employed to attenuate NMR signals from macromolecules. For all experiments, 128 transients were collected with 32 k data points and a spectral width of 16 ppm. A 90° pulse length was adjusted to about 10 µs, and recycle delay (RD) was set to 2 s. For all spectra, an exponential window function was applied with a line-broadening factor of 1 Hz prior to Fourier transformation.

Pre-processing of ^{1}H NMR data and peak assignment

All ^{1}H NMR spectra of serum were phased and baseline-corrected manually using MestReNova (V7.0, Mestrelab Research SL). The serum spectra were referenced to the anomeric proton signal of α-glucose (δ5.23). The spectral regions of δ 0.6–9.0 were then integrated into bins with the width of 0.002 ppm using AMIX software package (V3.8.3, Bruker Biospin). The spectral regions contained residual water signals (δ 4.60–5.20) and ethanol signals (δ 1.155–1.199, δ 3.63–3.689) which were discarded prior to data normalization. All spectra were mean-centered, and the integral values of each spectrum were probability quotient normalized to account for different dilutions of samples. NMR signals were assigned by querying publicly accessible metabolomics databases such as Human Metabolome Database (HMDB; http://www.hmdb.ca) (Wishart et al. 2013) and Madison-Qingdao Metabolomics Consortium Database (MMCD; http://mmcd.nmrfam.wisc.edu/) (Cui et al. 2008).

Statistical analysis

Multivariate statistical data analyses including Principal Component Analysis (PCA), Projection to Latent Structure with Discriminant Analysis (PLS-DA) and Orthogonal Projection to Latent Structure with Discriminant Analysis (OPLS-DA) were performed with the SIMCA-P+package (V.12, Umetrics, Sweden). First, the unsupervised pattern recognition method, PCA was used to differentiate the similarities and differences in the serum metabolic profiles of control and exposed groups (Wold et al. 1987). Based on PCA, metabolites differentiating the control group from each of the treated group were identified and integrated.

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From the integrated data, relative intensity of each of the metabolites was then calculated. Further, a supervised pattern recognition method, PLS-DA, was performed to maximize the separation of control and treated groups based on metabolites identified from PCA with rigorous permutation test (200 permutations) (Gu et al. 2011; Solanky et al. 2003). For clearer separation of the treated groups and improved predictive quality, OPLS-DA was also run for the data (Bylesjö et al. 2006). The validity of the models against over fitting and predictive ability was explained by the parameters R^2Y and Q^2Y, respectively. A low or negative value for Q^2Y indicates no statistically significant difference between the control and exposed groups. To make sure that discrimination in the OPLS-DA model was not due to data over fitting, a validation of the model was performed by rigorous permutation test (100 times) (Brindle et al. 2002; Llorach et al. 2010). The established models were validated by the observed value of P (≤ 0.05) at 95 % confidence level. Different responses were identified and visualized by S-plots and color-coded loadings plots.

Results and discussion

^{1}H NMR spectroscopy of fish serum

The representative ^{1}H NMR spectra of serum obtained from control and metal exposed (Pb, Cd and Pb+Cd) groups are shown in Fig. 1. A number of metabolites classes were identified including amino acids, energy storage compounds, citric acid cycle intermediates and unsaturated fatty acids. However, the ^{1}H NMR spectra mainly comprised of amino acids, and unsaturated fatty acids were found to be 10 to 100 fold greater in intensity as compared to other metabolites. Although the obtained spectra for different groups seems to be similar in overall appearance and composition, yet marked differences in the spectra can be observed by visual inspection due to the inherent quantitative nature of ^{1}H NMR technique as differences in the ^{1}H NMR signals intensities are directly related to the concentration of metabolites containing the protons.
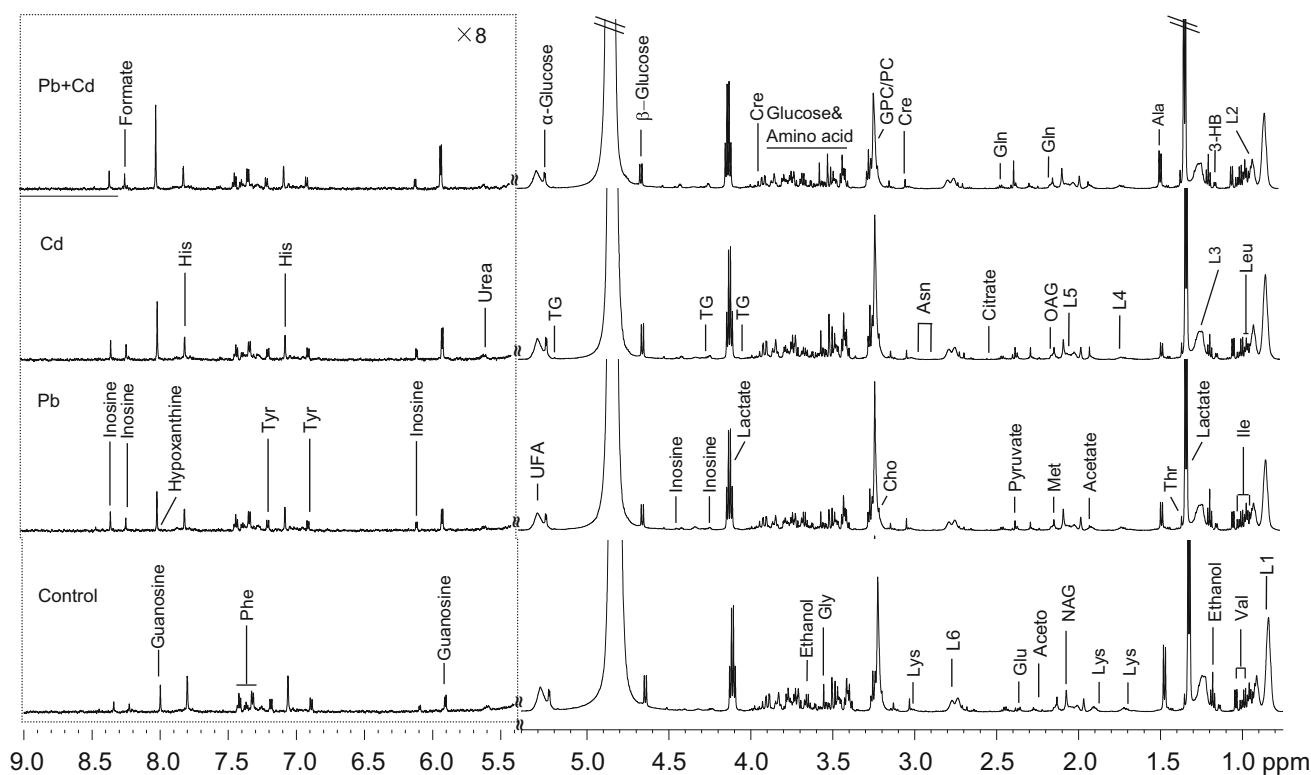

Fig. 1 Typical 600 MHz ^{1}H NMR spectra of serum from Control, Pb, Cd and Pb+Cd exposed fish. The region of δ 5.45–9.0 (in the *dashed box*) was vertically expanded 8 times compared with the region of δ 0.5–5.45 for the purpose of clarity. Keys: *Ala* Alanine, *Asn* asparagine, *Arg* arginine, *Cho* choline, *Cre* creatine, *Gln* glutamine, *Glu* glutamate, *Gly* glycine, *GPC* glycerolphosphocholine, *3-HB* 3-Hydroxybutyrate, *His* histidine, *Ile* isoleucine, *Leu* leucine, *Lys* lysine, *NAG* N-acetyl glycoprotein signals, *OAG* O-acetyl glycoprotein signals, *PC* phophocholine, *Phe* phenylalanine, *Thr* threonine, *Tyr* tyrosine, *Val* valine, *TG* triglyceride, *Met* methionine, *Aceto* acetoacetate, *Lipid1(L1)* R-C$\underline{H}_3$(ω-6), *Lipid2(L2)* CH$_3$(CH$_2$-C$\underline{H}$=CH)$_n$(ω-3), *Lipid3(L3)* R-C$\underline{H}_2$(ω-6), *Lipid4(L4)* C$\underline{H}_2$CH$_2$C=C, *Lipid5(L5)* C$\underline{H}_2$C=C, *Lipid6(L6)* C=CC$\underline{H}_2$C=C, *UFA* unsaturated fatty acid
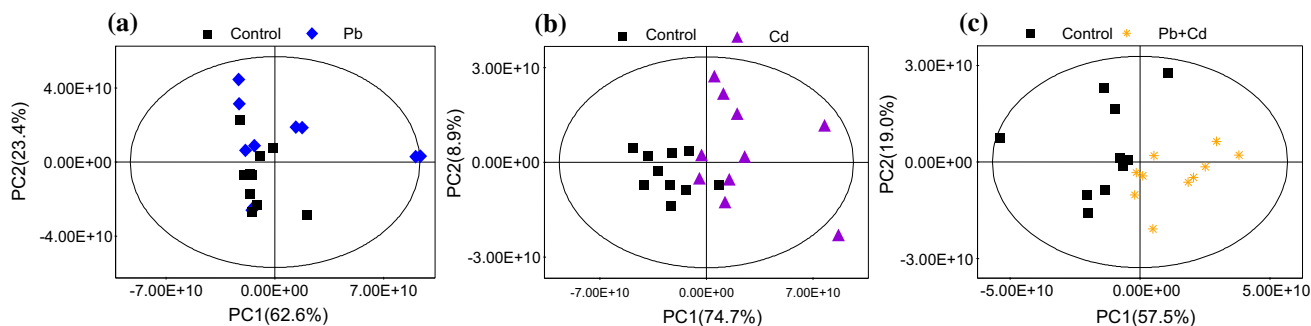
Fig. 2 Principle component analysis (PCA) sore plots obtained from ^{1}H NMR-based metabolomics data of Crucian carp's serum in the spectral region of δ 0.6–9.0 representing significant separation between control and Pb (**a**), Cd (**b**) and Pb+Cd (**c**) exposed groups at 30, 100 and 30+100 µg L^{-1} for 21 days, respectively

Pb- and Cd-induced altered metabolic profile by PCA

^{1}H NMR spectral data sets obtained from control and exposed groups were first analyzed by PCA for reduction of dimensionality and detection of outliers. The distinct separation of each exposed group from control can be seen in respective PC score plots as given in Fig. 2, except the Pb versus control group, where no obvious separation was observed between serum metabolic profiles of the two groups ($P < 0.05$). The corresponding PC loadings plots pointed out which metabolites were important in the separation of the two groups and the direction of their changes. The explained variation (R^2) along X-axis between the control and exposed groups were 86.0, 83.6 and 76.4 % with predictability of the PCA model ($Q^2 > 0.5$) 0.78, 0.70 and 0.63 for Pb versus control, Cd versus control and Pb+Cd versus control group, respectively.

Pb- and Cd-induced altered metabolic profile by PLS-DA and OPLS-DA

The ^{1}H NMR spectral data from control and exposed fish serum were also subjected to PLS-DA and OPLS-DA modeling to differentiate serum metabolic pattern between control and exposed groups. Results from the PLS-DA model revealed significant ($P < 0.05$) demarcation between the control, Cd and Pb+Cd exposed groups as shown in Fig. 3. There was no obvious separation observed for serum metabolic profile of Pb versus control group (Fig. 3a), whereas the predictability of PLS-DA model as explained by Q^2 (proportion of variance in the data predictable by the model) was 0.6 and the explained variation was $R^2X = 30.6$ % and $R^2Y = 91.5$ % which showed good predictability of the model. The serum metabolic profile of Cd and Pb+Cd groups versus control showed clear separation with good predictability and high value parameters R^2X, R^2Y and Q^2 24.9, 98.7, 0.816 % and 28.3, 98.3, 0.851 %, respectively. The Q^2 intercept for Pb versus control, Cd versus control and Pb+Cd versus control were 0.157, 0.0504 and 0.158, respectively, was <0.5 which further confirmed the validity of the PLS-DA model to predict the metabolomics data.

The OPLS-DA analysis was carried out using the ^{1}H NMR data as X matrix and class information as Y matrix with unit variance scaling. The score plots and loadings plots generated from MATLAB (Mathworks, Natick, MA) following back-transformation, which shows color-coded signals with correlation coefficients to reveal significantly altered metabolites of Pb versus control, Cd versus control and Pb+Cd versus control group are presented in Fig. 4. The score plots from OPLS-DA revealed clear separation between control and exposed groups with high predictive and modeling parameters R^2X and Q^2 30.6, 0.58 for Pb versus control, 24.9, 0.84 for Cd versus control and 28.3, 0.83 for Pb+Cd versus control group, respectively.

According to the color-coded correlation coefficient plots in Fig. 4, several metabolites were changed due to Pb, Cd and Pb+Cd exposure in the serum of Crucian carp. Unlike Cd and Pb+Cd exposure, Pb caused significant decrease in few but important metabolites like alanine, lysine and tyrosine as shown in Table 1. Fish serum from Cd exposed group revealed significant decrease in the level of lysine, isoleucine, leucine and alanine, but increase in the level of 3-hydroxybutyrate, acetone, lactate, choline, inosine, guanosine and threonine as depicted in Table 2. The co-exposure of Pb and Cd caused the most significant variation in the serum metabolic profile of fish by enhancing the level of pyruvate and inosine, but declining the level of alanine, lysine, glutamine, asparagine, R-C$\underline{H}_3$(ω-6), CH$_3$(CH$_2$C$\underline{H}$=CH)$_n$(ω-3), R-C$\underline{H}_2$(ω-6), unsaturated fatty acids and O-acetyl glycoprotein as shown in Table 3.

The toxic effect of Pb and Cd in terms of carcinogenesis, histopathology and genotoxicity together with several other toxicological aspects has been extensively studied but their effects caused at metabolite level still remain almost unknown. In the present study, ^{1}H NMR-based
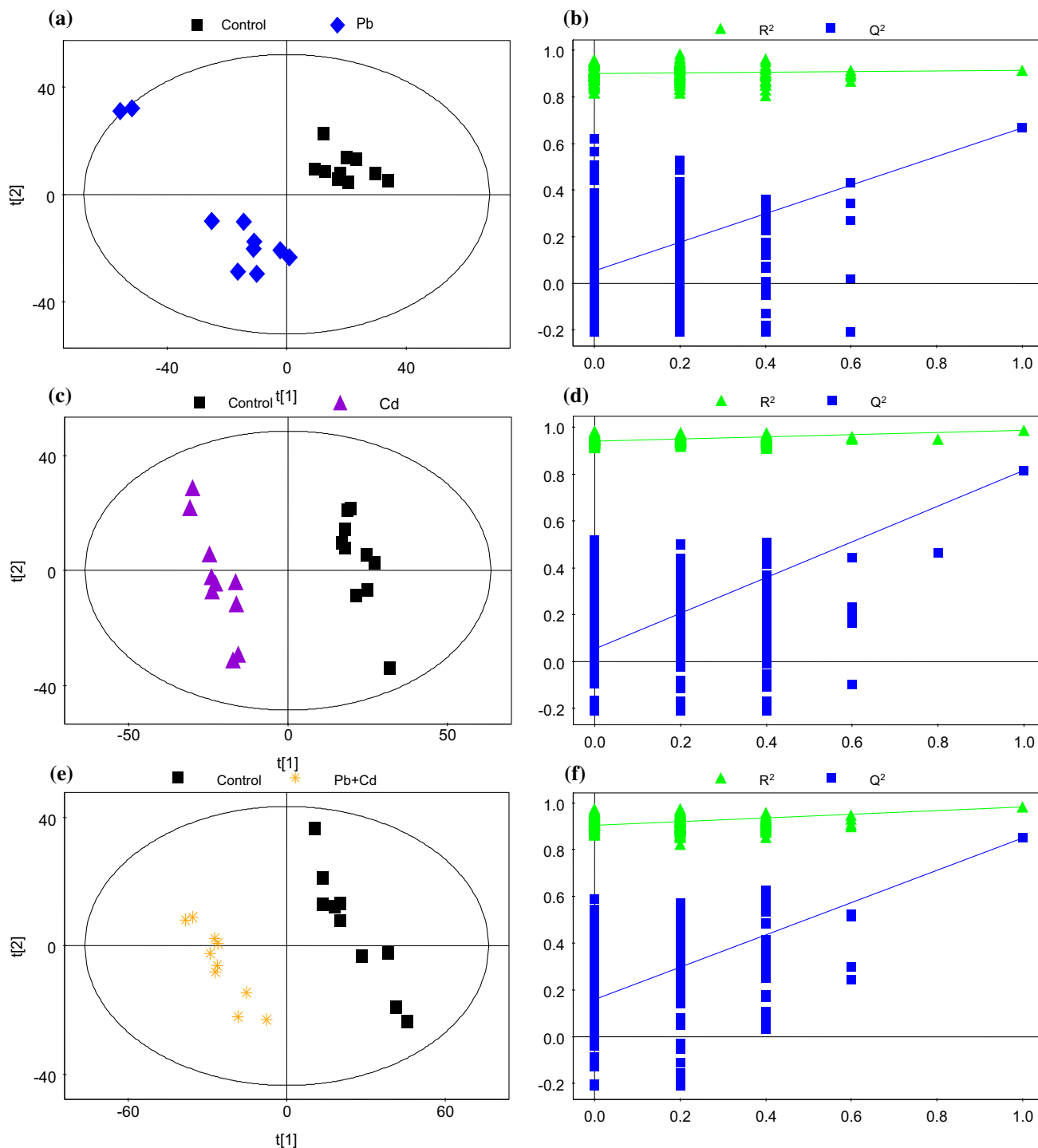
Fig. 3 Based on ^{1}H NMR metabolomics data, the score plots of PLS-DA model (*on left*) showing separation between the serum metabolites of control verses Pb (**a**), Cd (**c**) and Pb + Cd (**e**) exposed Crucian carps at 30, 100 and 30+100 µgL^{-1} for 21 days, respectively. The

validation plot (*on right*) of 100 permutation test with Q^2-intercept 0.0, 0.0173 (**b**), 0.0, 0.0504 (**d**) and 0.0, 0.158 (**f**) were <0.5, indicating a valid model

metabolomics technique was used to explore changes in the serum metabolic profile of control and exposed (Pb, Cd, Pb+Cd) Crucian carps and to identify the altered metabolites as potential biomarkers of exposure. The data

obtained from ^{1}H NMR spectra were analyzed by multivariate statistical techniques including PCA, PLS-DA and OPLS-DA for pattern recognition and metabolite identification. PCA is an unsupervised exploratory pattern
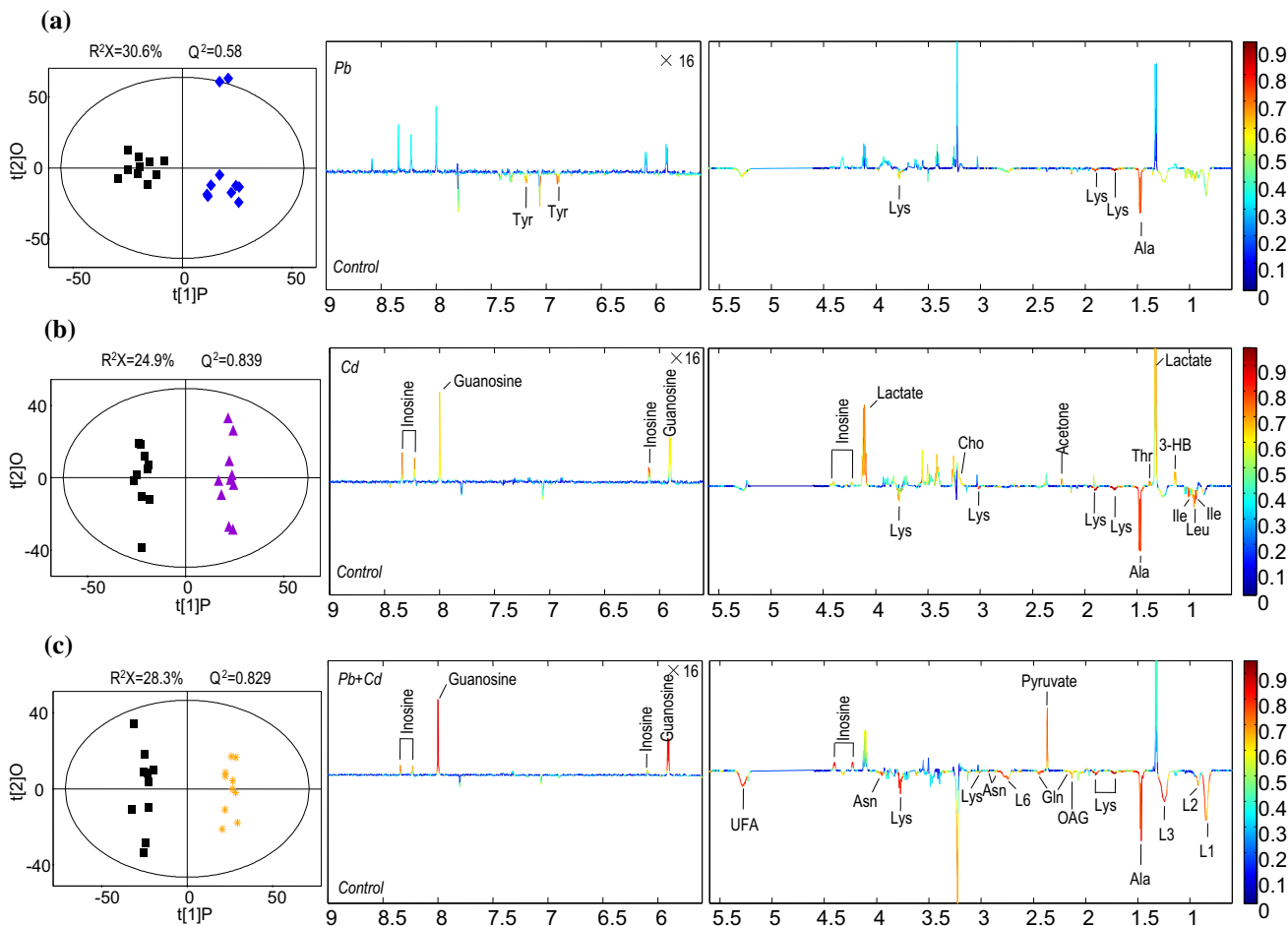
Fig. 4 Cross-validated OPLS-DA score plots (*left*) and corresponding color-coded correlation coefficient plots (*right*) derived from ^{1}H NMR spectra of control verses Pb (**a**), Cd (**b**) and Pb + Cd (**c**) exposed Crucian carps at 30, 100 and 30+100 μgL^{-1} for 21 days, respectively. The *color-coded* map indicates significance of metabolite variations between control and exposed group. *Red color* shows important discriminatory metabolites, and *blue color* indicates no discrimination at $P < 0.05$

Table 1 Significantly changed metabolites with corresponding correlation coefficients between Control and Pb^{2+} exposed group

No.	Metabolite	Chemical shift	Correlation coefficient
1	Alanine	1.48(d), 3.77(q)	−0.806
2	Lysine	1.49(m), 1.72(m), 1.91(m), 3.02(t), 3.77(t)	−0.794
3	Tyrosine	6.90(d), 7.19(d)	−0.799

The correlation coefficient of $|r| > 0.602$ was used as the cutoff value for the statistical significance based on the discrimination significance at the level of $P < 0.05$ and df (degree of freedom) $= 9$

d Doublet, *t* triplet, *q* quadruple, *m* multiplet

[a] Correlation coefficients, positive and negative signs indicate positive and negative correlation in the concentrations, respectively

[b] Multiplicity: *d* doublet, *t* triplet, *q* quadruple

recognition tool irresponsive to the status of each sample and serve to reduce dimensionality, identify outliers and indicate similarity and difference between multiple NMR spectral data sets. On the other hand, PLS-DA is a supervised pattern recognition method more commonly employed to classify multiple classes by probing variables at *X* matrix which are correlated with class variables at *Y* matrix. In PLS-DA model, the *X* matrix is the measured matrix (NMR data) and *Y* matrix consists of dummy variables which indicate the class for each treatment (Zhang et al. 2011). Compare to PLS-DA model, the OPLS-DA model analyzes data with orthogonal filtration of *X* matrix on a vector *Y*. It splits variance in the *X* matrix into predictive variance and orthogonal variance. The structure

Table 2 Significantly changed metabolites with corresponding correlation coefficients between Control and Cd^{2+} exposed group

No.	Metabolite	Chemical shift	Correlation coefficient
1	Isoleucine	0.93(t), 1.00(d), 1.99(m), 1.26(m), 1.47(m), 3.68(d)	−0.792
2	Leucine	0.96(d), 0.97(d), 1.72(m), 3.73(t)	−0.741
3	Alanine	1.48(d),3.77(q)	−0.839
4	Threonine	1.38(d), 3.58(d), 4.24(m)	0.764
5	Lysine	1.49(m), 1.72(m), 1.91(m), 3.02(t), 3.77(t)	−0.973
6	3-Hydroxybutyrate	1.14(d), 2.32(m), 2.42(m), 4.16(m)	0.701
7	Acetone	2.22(s)	0.735
8	Lactate	1.33(d), 4.12(q)	0.715
9	Choline	3.19(s), 3.53(t), 4.07(t)	0.656
10	Inosine	8.34(s), 8.23(s),6.09(d),4.45(m),4.30(m)	0.783
11	Guanosine	8.00(s), 5.90(d)	0.703

The correlation coefficient of $|r| > 0.602$ was used as the cutoff value for the statistical significance based on the discrimination significance at the level of $P < 0.05$ and df (degree of freedom) $= 9$

d Doublet, t triplet, q quadruple, m multiplet

[a] Correlation coefficients, positive and negative signs indicate positive and negative correlation in the concentrations, respectively

[b] Multiplicity: s singlet, d doublet, t triplet, q quadruple, m multiplet

Table 3 Significantly changed metabolites with corresponding correlation coefficients between Control and $Pb^{2+}+Cd^{2+}$ exposed group

No.	Metabolite	Chemical shift	Correlation coefficient
1.	Alanine	1.48(d),3.77(q)	−0.895
2.	Lysine	1.49(m), 1.72(m), 1.91(m), 3.02(t), 3.77(t)	−0.943
3.	Glutamine	2.15(m), 2.43(m), 3.78(m)	−0.759
4.	Asparagine	2.84(dd), 2.94(dd), 3.95(m)	−0.824
5.	Pyruvate	2.37(s)	0.737
6.	L1:R-CH_3(ω-6)	0.84(m)	−0.696
7.	L2: $CH_3(CH_2CH=CH)_n$(ω-3)	0.92(m)	−0.788
8.	L3: R-$\underline{CH}_2$(ω-6)	1.24(m)	−0.83
9.	L6: C=CCH_2C=C	2.73(m)	−0.737
10.	unsaturated fatty acid	5.30(m)	−0.814
11.	O-acetyl glycoprotein	2.13(s)	−0.727
12.	Inosine	8.34(s), 8.23(s), 6.09(d), 4.45(m), 4.30(m)	0.757
13.	Guanosine	8.00(s), 5.90(d)	0.858

The correlation coefficient of $|r| > 0.602$ was used as the cutoff value for the statistical significance based on the discrimination significance at the level of $P < 0.05$ and df (degree of freedom) $= 9$

d Doublet, t triplet, q quadruple, m multiplet

[a] Correlation coefficients, positive and negative signs indicate positive and negative correlation in the concentrations, respectively

[b] Multiplicity: s singlet, d doublet, t triplet, dd doublet of doublets, q quadruple, m multiplet

noise of the data such as age, genetic background and physical activity is filtered and only described by orthogonal components. The predictive component, i.e., the first component, only shows the variation of scientific interests. Thus, the interpretability of the resulting model is increased (Fonville et al. 2010). In our study, both unsupervised and supervised pattern recognition methods showed distinct separation between the control and exposed groups with good predictability and validation of the model.

Regarding the toxicity of Pb, Cd and their co-exposure at environmental level, significant changes in several metabolites were observed from the color-coded correlation coefficient plots (Fig. 4). Fish exposure to 30 μgL^{-1} Pb caused significant reduction in alanine, lysine and tyrosine, while the rest of the metabolite did not show any change. Alanine is an aliphatic amino acid and plays important role in glucose-alanine cycle between different tissues and liver. It was observed that alanine level decreased in *Ruditapes philippinarum* due to Cu exposure
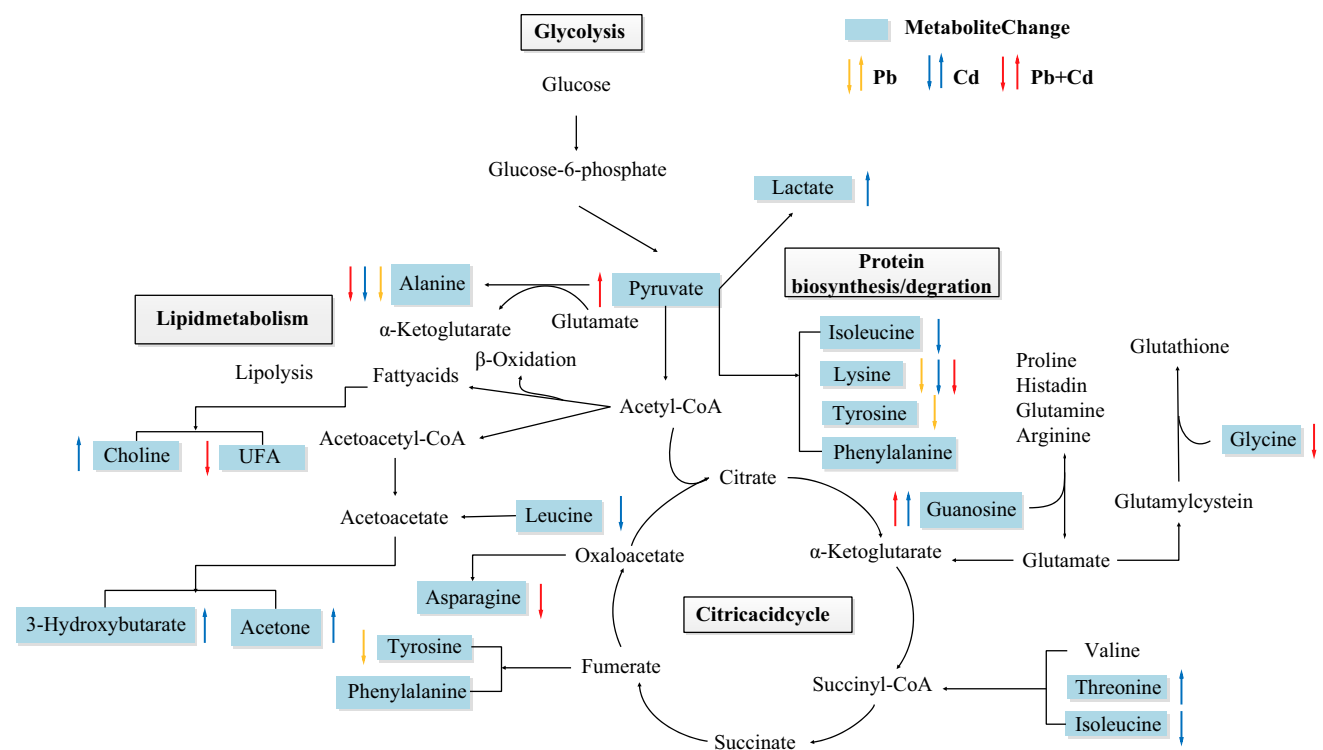
Fig. 5 Scheme representing the perturbed metabolic pathways of Crucian carp exposed to environmental concentration of Pb, Cd and Pb+Cd for 21 days as detected by ^{1}H NMR analysis. Citric acid cycle was the junction pathway of energy metabolism, lipid metabolism and amino acids metabolism. *Arrows* ("↑↓") in *different colors* representing significant increase or decrease in metabolites due to Pb (*yellow*), Cd (*blue*) and Pb+Cd (*red*) intoxication in fish as compared to their respective control group. (For interpretation of the references to color in this figure legend, the reader is referred to the webversion of the article)

because alanine is involved in anaerobic metabolism (Zhang et al. 2011). Both lysine and tyrosine are essential amino acids and a part of the intermediatiory metabolism (Fig. 5). Lysine undergoes to produce acetyl-Co A, an intermediatiory molecule via transamination with α-ketoglutarate, while tyrosine is a precursor to neurotransmitters and hormones (McCoole et al. 2012).

Significant decrease in lysine, isoleucine, leucine, alanine and increase in 3-hydroxybutyrate, acetone, lactate, choline, inosine, guanosine and threonine were observed in the serum of fish exposed to 100 μgL^{-1} Cd. Isoleucine is glucogenic as well as ketogenic amino acid which can be converted to succinyl Co A to enter into (tricarboxylic acid) TCA cycle or oxaloacetate for gluconeogenesis (Campos-Ferraz et al. 2013). Whereas leucine stimulates the synthesis of protein through activation of intracellular cell signaling pathways especially those which are used by insulin (Lynch et al. 2003). Lactate, acetone and 3-hydroxybutyrate are the intermediatiory metabolites that normally occur in the blood and play essential role in energy metabolism (Pavlides et al. 2010). However, increase in these metabolites indicated a switch of the organism to anaerobic respiration that may be due to hypoxia (Li et al. 2014; Xu et al. 2015). Choline is one of the components in

phospholipids and plays crucial role in maintaining the structural integrity of membranes (Li et al. 2014). The increased level of choline in fish serum might be due to damage caused by Cd-induced oxidative deterioration of structural membranes in various tissues. An increase in inosine, a metabolite of energy rich molecule ATP, was also noticed in fish exposed to butachlor due to mitochondrial membrane damage by oxidative stress (Xu et al. 2015).

Similar to the altered serum metabolic profiles of fish exposed to Pb and Cd, the co-exposure of Pb and Cd (30+100 μgL^{-1}) resulted in significant increase in pyruvate and inosine, while the level of several other metabolites such as alanine, lysine, glutamine, asparagine, some unsaturated fatty acids and *O*-acetyl glycoprotein was decreased. Pyruvate is a product of glucose oxidation in aerobic respiration. The increase in pyuruvate and acetate was also indicated in amyotrophic lateral sclerosis (ALS), an invariably fatal neurological disorder to meet the excessive requirement of ATP (Kumar et al. 2010). Although the metabolic response for each metal was different in our study, there are still similarities in some of the metabolites which responded in the same manner to Pb, Cd and Pb+Cd exposure. The amino acids alanine, lysine,

tyrosine, isoleucine, leucine, glutamine and asparagine are the intermediatory compounds in TCA cycle (Fig. 5) (Kokushi et al. 2012). The decrease in these amino acids may be due to lesser production under stress or being depleted in mitigating oxidative stress posed by metal toxicity (Nagato et al. 2013). Depletion of alanine in particular was not only observed for Pb, Cd and Pb+Cd exposure in our study but also indicated for several other metals (Cu, Li, Ar, Ni) in *Mytilus galloprovicialis* (Jones et al. 2012). In our study, increase in pyruvate and decrease of alanine might suggest an increase in energy metabolism because alanine can be converted to pyruvate in TCA cycle (Ekman et al. 2007; Southam et al. 2008). It was observed that organism utilize greater proportion of their energy as coping mechanism against toxins which resulted in growth impairment and compromise of other physiological activities (Nagato et al. 2013; Spann et al. 2011). On the other hand, some amino acids like lysine, leucine, isoleucine and tyrosine under specific metabolic condition can be converted to ketone bodies, that's why in our study acetone was found higher in Pb+Cd exposed fish serum (Fasulo et al. 2012). In addition to the disturbance in energy metabolism, toxic metals exposure may affect osmoregulatory status of the fish as previous studies shown that free amino acids in the blood are also required to maintain osmoregulation in aquatic organisms (Nagato et al. 2013; Shinji et al. 2012). Interestingly, it was observed that the total number of 3 metabolites significantly altered during Pb exposure, but in Cd and Pb+Cd exposure, 11 and 13 metabolites were significantly changed. This further showed that the effect of Pb and Cd on fish metabolic profile was metal specific, concentration dependant and synergistic for Pb and Cd exposure in combination.

Conclusion

This study investigated the serum metabolic responses of Crucian carp to environmental Pb and Cd by ^{1}H NMR-based metabolomics approach. The PCA, PLS-DA and OPLS-DA analyses of ^{1}H NMR spectral data sets revealed significant changes in the serum metabolic profiles of control and exposed fish. A different metabolic response was observed for each metal and its co-exposure with distinct similarities in some of metabolic biomarkers (alanine, lysine). Based on metal specific metabolic biomarkers response, changes in neurotransmitters and amino acid metabolism were induced by Pb, while impaired energy metabolism and disturbed osmotic regulation with a shift from aerobic to anaerobic respiration were observed due to Pb and Cd exposure, whereas the co-exposure of Pb and Cd in a mixture had an additive effect on metabolic disturbance. Moreover, Crucian carp was found to be a

preferable bioindicator for heavy metal pollution in freshwater ecosystem and ^{1}H NMR-based metabolomics as remarkable tool to elucidate the effect of environmental pollutants and underlying mechanism for the discovery of novel metabolic biomarkers.

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