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Global metabolic response in the bile of pejerrey (*Odontesthes bonariensis*, Pisces) sublethally exposed to the pyrethroid cypermethrin

Pedro Carriquiriborde [a,*,1], Damián J. Marino [a,b,1], Gabriela Giachero [a], Eduardo A. Castro [b], Alicia E. Ronco [a]

[a] Centro de Investigaciones del Medio Ambiente (CIMA), Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de la Plata. Calle 47 y 115 s/n, CONICET, La Plata B1900, Bs. As., Argentina
[b] INIFTA, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de la Plata, CONICET, La Plata B1900, Argentina

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ABSTRACT

The metabolic profile of *Odontesthes bonariensis* and its global response to the insecticide cypermethrin were studied using HPLC–MS-based metabolomics. Three experiments using either juveniles or adults of *O. bonariensis* were performed by exposing fish (6, 24, or 96 h) to sublethal concentrations of cypermethrin (5 or 10 μg/L). Metabolic profiling was performed on either whole bile or aqueous and organic extracts. Chromatography was performed using a C18 column and an ACN/H_2O mobile phase. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces were used in positive and negative modes. Full scan MS data were processed using the XCMS software, log-transformed, and analyzed using either regression analysis or principal component analysis (PCA). The highest amount of information (1163 peaks) was yielded by analyzing the whole bile with the ESI(−) interface. Complementary information, useful for metabolite confirmation, was obtained from the aqueous and organic extracts and using the ESI(+) and APCI interfaces. The bile metabolic profile of *O. bonariensis* was characterized by some abundant metabolite ions corresponding with taurine conjugated bile acids, which were useful as reference peaks. A characteristic global metabolic response to cypermethrin was identified in the bile of *O. bonariensis*. A ten-fold or higher variation in abundance was observed in the whole bile of exposed fish for a small group of peaks (32), and these peaks corresponded to an even smaller number of metabolites (nineteen). Both regression analysis and PCA were useful in identifying those peaks, better explaining differences between exposed and control groups, but slight differences were suggested by each of those methods. Using unsupervised PCA scores, we were able to distinguish organisms from each treatment on the basis of the metabolic changes induced by the cypermethrin, this variability being explained mainly by only one principal component (PC3, 17.7 percent total variance). Two cypermethrin metabolites were identified as major contributors within the augmented peaks: the known glucuronide of 4′-hydroxy–cypermethrin and the sulfate of 4′-hydroxycypermethrin, not previously reported in fish bile. The HPLC–MS-based metabolomic approach demonstrated to be a powerful ecotoxicological tool for identifying biological responses to pollutants, discovering new metabolic pathways and proposing specific biomarkers using non model organisms.

1. Introduction

Cypermethrin is a pyrethroid insecticide that has been widely used in household, agricultural, forestry, and veterinary applications due to its efficacy and low toxicity to mammals. As a consequence of its cost effective control of insects, in recent years this insecticide has become one of the major biotech (BT) soybean pesticides in the South American Pampas region (Carriquiriborde et al., 2007). BT soybean is a crop that currently covers more than 90 million ha around the world, mostly in developing countries, with Brazil and Argentina in the second and third places, respectively, for plantation area in the world (James, 2009). Previous studies have demonstrated that soybean cypermethrin is able to reach water courses draining agricultural basins, with concentrations of concern for the protection of aquatic biota (Marino and Ronco, 2005).

Pyrethroids are highly toxic to some aquatic organisms, such as arthropods and fish (Haya, 1989). In particular, *Odontesthes bonariensis* is very sensitive to cypermethrin's acute lethal effects,

* Corresponding author. Fax: +54 221 4229329.
E-mail address: pcarriquiriborde@gmail.com (P. Carriquiriborde).
[1] These authors equally contributed to this work.

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presenting a 96 h LC50 value of 0.2 μg/L (Carriquiriborde and Ronco, 2006). However, it was also observed that the organic matter content of surface waters significantly reduces cypermethrin toxicity, protecting fish from lethal acute effects in the field, even when environmental concentrations exceed the LC50 values (Carriquiriborde et al., 2007). Little is known about other possible chronic effects at sublethal levels in the field. Effects on growth were observed under laboratory conditions after chronic exposure of *O. bonariensis* larvae to this insecticide over 4 weeks (Carriquiriborde et al., 2009), but no specific biological responses are currently available to evaluate exposure or effect in the field.

The primary toxic action of pyrethroids is associated with altered nerve function, principally involving neuroexcitatory effects in the brain, spinal cord, and elements of the peripheral nervous system. Particularly, Type II or CS (choreoathetosis with salivation) and pyrethroids (e.g., cypermethrin) interfere with the voltage-sensitive sodium channels in the nerve membrane, causing lower amplitude of the action potentials or total blockage of neural activity because of a marked depolarization of the membrane (Coats, 1990). Pyrethroids also act on some isoforms of the voltage-sensitive calcium channel, an effect that may contribute to the release of neurotransmitters that is associated with pyrethroid intoxication. The blockade of voltage-sensitive chloride channels is associated with the production of salivation in mammals, a hallmark of the CS intoxication syndrome, and may also contribute to enhanced excitability in the central nervous system (Soderlund et al., 2002).

In fish, responses in cardiovascular and blood chemistry variables (i.e., cough frequency, increases in arterial O_2, and pH decrease) (Bradbury et al., 1991) are correlated with central nervous system activity. Increase in anaerobic metabolism (i.e., increase in lactate dehydrogenase) (Philip et al., 1995; Singh and Agarwal, 1993) was related with the change in cardiovascular and blood chemistry variables. However, an additional nonneural site of action also seems to be an important factor in the toxicodynamics of pyrethroids in aquatic vertebrates. Inhibition of ATPase systems (Suvetha et al., 2010) and disruption of the hematological parameters (Dorucu and Girgin, 2002; Saxena and Seth, 2002) were reported.

The greater susceptibility of fish to pyrethroids compared with that of mammals and birds is caused not only by greater organ sensitivity or a site of action selectivity, but also by the mechanism of uptake and lower detoxification rates (Edwards et al., 1986). In trout, elimination rates of the *cis*- and *trans*-cypermethrin are comparable, and the principal route of excretion is the bile. Detoxification is principally the consequence of oxidation in the liver, but *trans*-cypermethrin can be also hydrolyzed in the plasma. For both isomers, 4′-hydroxy-cypermethrin, glucuronide is the major metabolite (79.6–35.5 percent) present in the bile, together with thirteen other identified and nonidentified metabolites (Edwards et al., 1987).

Despite the knowledge on the toxicology of pyrethroids, no specific biomarkers have been identified previously to evaluate the exposure and/or affects of these insecticides. Biomarkers were defined as early warning signals of pollution stress observed at the below-individual level and at concentrations lower than those causing irreversible damage (Van Gestel and Van Brummelen, 1996). Emerging "omics" technologies have offered new possibilities in the identification of the modes of action of chemicals, and exhibit opportunities for the discovery of new biomarkers (Ankley et al., 2006; Miracle and Ankley, 2005; Viant et al., 2003). In particular, metabolomics, metabonomics, or metabolic profiling implies the comprehensive assessment of the global metabolic composition and dynamic of a biological system and its response to disease and genetic and environmental perturbations. Metabolomics is at the bottom of the "omics cascade" and is the closest to phenotype (Dettmer et al., 2007). Two major analytical platforms are currently available for metabolomic studies: nuclear magnetic resonance spectroscopy and mass spectrometry (MS) (Dettmer et al., 2007).

Despite the promise of metabolomics, no previous studies have used this approach to evaluate the adverse effects of pyrethroids on fish. Therefore, the aims of the present study are as follows: (i) to evaluate alternative methodological conditions for assessing the metabolic profiling of the bile in the South American fish *O. bonariensis* (Valenciennes, 1835) using the HPLC–MS platform, (ii) to study under laboratory conditions the global metabolic response in the bile of organisms exposed to waterborne sublethal concentrations of cypermethrin, and (iii) to identify cypermethrin metabolites and compare them with the established metabolic pathway for this insecticide.

2. Materials and methods

2.1. Chemicals

Acetonitrile LiChrosolv, dichloromethane UniSolv, ethanol absolute LiChrosolv, formic acid Suprapur, and hydrochloride acid Ultrapur were purchased from Merck (Darmstadt, Germany). Diethyl ether Ultra Resi-Analyzed and ammonium acetate, HPLC grade, were from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ). Aqueous solutions were prepared in NANOpure water (Barnstead Thermolyne D4751 NANOpure water system). Cypermethrin active ingredient (91 percent purity, technical grade) was provided by Gleba Argentina S.A.

2.2. Test organisms

Pejerrey (*O. bonariensis*, Aterinopsidae) is a medium size (maximum length, 500 mm) freshwater teleost characteristic of the fish found in the southern sector of the Rio de la Plata Basin (South America) (Bonetto and Castello, 1985) and is particularly abundant in shallow lakes of the Pampas region, the core soybean area of Argentina. Pejerrey is a close relative of the North American inland silverside (*Menidia beryllina*) and is very sensitive to pollutants, showing levels of tolerance similar to those reported for salmonids (Carriquiriborde and Ronco, 2002, 2006). Because of the quality of the flesh and its attractiveness as a game fish, pejerrey is one of the most studied South American fish, and its aquaculture has been encouraged in several parts the world (Somoza et al., 2008).

Experimental fish were obtained from aquaculture station of the Ministerio de Asuntos Agrarios of the Buenos Aires Province, Argentina. Fish were transported and acclimated to laboratory conditions 2 weeks previous to the experiments.

2.3. Experiments

Three experiments were conducted for assessing cypermethrin metabolites and the metabolic profile of the bile of exposed fish:

Experiment one. One and a half year old pejerrey juveniles (2.94 ± 0.13 g; 66.08 ± 1.85 mm, $n=10$) were exposed for 24 h to 5 μg/l cypermethrin. A control group (3.60 ± 0.37 g; 71.95 ± 2.41 mm; $n=10$) was kept in clean water.

Experiment two. Oneand a half year old pejerrey juveniles (7.14 ± 0.63 g; 93.45 ± 3.74 mm; $n=6$) were exposed for 6 h to 10 μg/l cypermethrin. A control group (5.64 ± 0.57 g; 88.90 ± 6.88 mm; $n=6$) was also included.

Experiment three. Four year old pejerrey adults (195.1 ± 2.81 g; 263.3 ± 1.92 mm; $n=3$) were exposed for 96 h to 5 μg/l cypermethrin, with a control group (205.0 ± 2.36 g; 262.5 ± 3.5 mm; $n=3$) maintained in clean water.

Experimental concentrations of cypermethrin were established considering the sensitivity of *O. bonariensis* to the insecticide as observed in previous studies (Carriquiriborde et al., 2009, 2007; Carriquiriborde and Ronco, 2006) and the range of environmental concentrations reported for surface waters in the Pampas region (Marino and Ronco, 2005).

All experiments were conducted in 50 L glass aquaria using La Plata tap water dechlorinated and filtered through activated carbon (Ca^{2+}, 0.85 mM; Mg^{2+}, 0.90 mM; Na^{+}, 3.13 mM; K^{+}, 0.67 mM; Cl^{-}, 0.11 mM; hardness, 180 mg/L as $CaCO_3$; pH 7.8). Aquarium temperature was maintained at 21 °C and the photoperiod was set to 16:8 (light:dark). Fish were starved for 24 h before initiation of the experiments and were not fed during the trials. Cypermethrin was added, dissolved in ethanol. Ethanol was also added to the control groups, and the final concentration of dissolvent was the same in all the treatments and was always

lower than 0.1 percent. Fish were killed in ice water, the gallbladders dissected, and the bile carefully withdrawn using a 1 ml syringe. In experiments 1 and 2, due to the small volume, the bile of two fish from the same treatment was pooled.

Experiments 1 and 2 were useful for refining the methodology and the exposure conditions. To be concise, only data from experiment 3 are presented in Section 3.

2.4. Bile processing

Different pretreatments of bile samples were tested.

Ethylic extraction of unmodified bile: Bile samples (100 μl) were extracted directly (3 × 400 μl) with diethyl ether. The ethylic extracts were dried under a stream of N_2 and the residue taken up in 100 μl of mobile phase. Both, the ethylic and the complementary aqueous extracts were filtered through a 0.22 μm filter and injected (20 μl) into the HPLC–MS system for analysis.

Ethylic extraction with acidification and aqueous dilution of the bile: Bile samples (100 μl) were diluted in NANOpure water (20 vol.), adjusted to pH 2 with 0.1 M HCl, and extracted (3 ×) with diethyl ether (2 vol.). The ethylic extracts were dried under a stream of N_2 and the residue taken up in 100 μl of mobile phase (Edwards et al., 1987). Both the ethylic and the aqueous extracts were filtered through a 0.22 μm filter and injected (20 μl) into the HPLC–MS system for analysis.

Whole bile analysis: Bile samples (100 μl) were diluted in mobile phase, filtered through a 0.22 μm filter, and injected (20 μl) into the HPLC–MS system.

2.5. LC–MS instrument and operational conditions

The LC–MS equipment consisted of a binary-pump Agilent 1100 LC system (Agilent Technologies Inc, USA) equipped with and a diode array detector (DAD) in tandem with an MSD VL quadrupole (Agilent Technologies, USA) with an electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interface. A Rheodyne 7725*i* injector with a 20 μl loop was used.

The chromatographic separations were performed on a C-18 Gemini column (Phenomenex, Inc., USA) (150 × 4 mm; 3 μm pore size). The column was maintained at room temperature (22 ± 1 °C). A number of alternative mobile phases and run conditions were evaluated, combining different ratios of acetonitrile to water (from 20:80 to 80:20), with either formic acid (0.1 percent) or ammonium acetate (10 mM) as additives. Elution was performed at a flow rate of 0.5 ml/min.

Bile extracts were analyzed by HPLC–MS using the APCI and ESI sources either in positive or negative mode. In both cases, nitrogen was used as nebulizer gas at a flow rate of 7 L/min, a nebulizer pressure of 40 psi, and a drying gas temperature of 330 °C. The capillary voltage was set at 3000 V. The APCI conditions were set at the following values: gas flow, 1 ml/min; vaporizer temperature, 330 °C; corona needle current, 4 and 15 μA in positive and negative modes, respectively. Mass spectral data were acquired in full-SCAN mode in the *m/z* range 100–1000.

2.6. Data analysis

Data acquisition was performed using LC/MSD Agilent ChemStation Rev. A.10.02 (1757). The mass spectral data were first processed using the XCMS software for peak identification and matching (Smith et al., 2006). The output peak intensities of each sample were log–transformed and then subjected to both regression analysis and the unsupervised multivariate method of principal component analysis (PCA). Both analysis were performed using a commercial statistical software pack.

Regression analysis evaluated the relationship between the mean peaks intensity in controls with respect to exposed fish. This method allowed us to distinguish those peaks that fall above or below the prediction interval with a significance value of α=95 percent by means of the residual analysis. Residuals were calculated as the observed value minus the predicted value (Y_i–$\overline{Y}$). The prediction interval is the area in which you expect 95 percent of all data points to fall. It was calculated using the following equation:

$$\hat{\alpha}+\hat{\beta}X_0 \pm t_{\alpha/2,n-2}S_{YX_0},$$

where $t_{\alpha/2,n-2}$ is Student's t distribution with significance level α and degrees of freedom n–2, and S_{YX_0} is the standard error of the residues, calculated as follows:

$$S_{YX_0}=\hat{S}_R\sqrt{1+\frac{1}{n}+\left(\frac{X-\hat{X}}{S_x\sqrt{n}}\right)^2}$$

The unsupervised multivariate method PCA was chosen to identify those peaks (variables) that better explain the variations of the global metabolic response to cypermethrin exposure, avoiding an "a priori" classification of the individuals from each experimental group. The number of principal components (PCs) was established using the "variance explained criteria," keeping a number of PCs that accounts for the 80 percent of the total variation. The "component loadings" are the correlation coefficients between the variables and factors, the proportions of variance in the variables, explained by a factor. The "component scores" are defined as the values obtained from the projection of the case on the principal components.

Metabolite identification of relevant peaks was performed by applying a combination of different mass spectrometric methods such as (i) structural elucidation from logical fragmentation; (ii) prediction of the isotopic abundance, the latest using the ISOFORM (NIST Formula and Isotopic Pattern Generator) software version 1.02; (iii) searching for mass spectra of putative compounds in open access databases (e.g., Human Metabolome Database, METLIN, MassBank, Lipid Bank); and (vi) comparison with results from previous chromatography and mass spectrometry studies.

3. Results

3.1. Chromatographic and spectrometry conditions influencing bile profiling

From the different tested chromatographic conditions, satisfactory results were obtained by applying a linear gradient elution with acetonitrile aqueous solution of ammonium acetate (in negative mode) or aqueous formic acid (in positive mode), starting at 35 percent acetonitrile and rising to 85 percent in 15 min. The number of extracted peaks (ions) obtained from the whole bile of *O. bonariensis* using ESI(+), ESI(−), and APCI(+) is shown in Fig. 1. The number of ions produced by the ESI source was more than the double that produced by APCI. In addition, the ESI in the negative mode yielded 15.5 percent more ions than in the positive mode.

In Fig. 2, the total numbers of extracted peaks from the bile are presented in relation to the sample extraction method. The number of peaks showing a variation of intensity in exposed fish higher than 10-fold up or down, with respect to controls, is also shown in the graph. The number of total ions obtained by injecting the whole bile was almost the same as that obtained by adding those from the aqueous and organic extracts. In addition, the numbers of total ions were similar in the complementary aqueous and organic extracts. The presence of some metabolites (i.e., cypermethrin metabolites) in the aqueous or organic extract was dependent on the previous dilution and acidification (or lack thereof) of the aqueous phase (data not shown).

3.2. Bile metabolic profile in O. bonariensis

An extracted ion chromatogram showing the characteristic metabolic profile of *O. bonariensis* bile is shown in Fig. 3. The

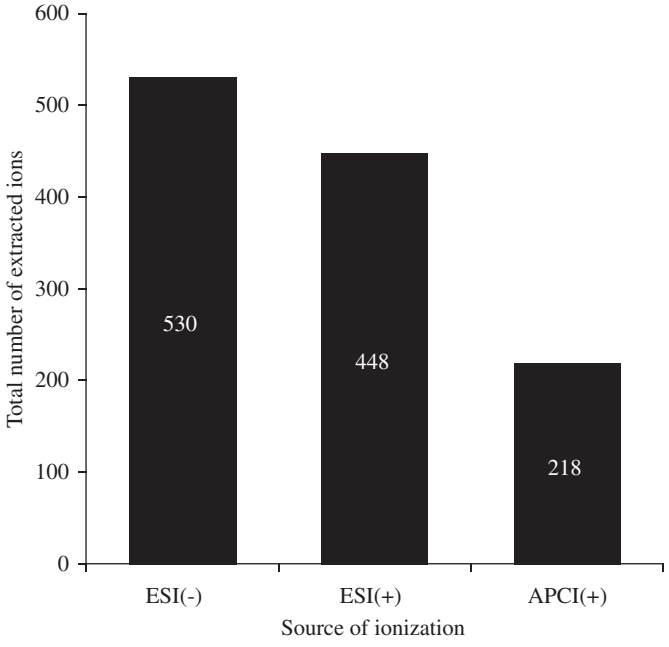


Fig. 1. Relationship between the ionization interface and the total number of ions in the organic extract of *O. bonariensis* bile. (+), Positive mode; (−), negative mode.

representative mass spectrum of a characteristic bile acid, taurocholic acid (TCA), found in the bile of the *O. bonariensis* is shown in Fig. 4. The fragments at *m/z* 80, 107, and 124 were useful for the identification of these metabolites, since those ions are typical of taurine-conjugated bile acids. In addition, dimers of these compounds (i.e., *m/z* 1029.1 for TCA) were also observed. Other abundant taurine-conjugated acids were a C27 bile acid, putative taurotrihydroxycoprostanic acid (TTHCA) or isomers, and taurodehoxycholic acid (TDCA). No glycine-conjugated bile acids (characteristic fragment at *m/z* 74) were found. Bilirubin was identified using the APCI(+) source as the $[M+H]^+$ ion [*m/z* at 585 and 586; retention time (RT) 582 s]. Relative isotopic abundances were confirmed by the isotope calculator. Corroboration was also obtained from the DAD spectrum (maximum abs, 450 nm). In addition, using the ESI(−) source, a fragment of the molecule (product of the loss of a pyrrolic ring) was found as the base peak (*m/z* at 450) together with the $[M-H]^-$ ion (*m/z* at 583) at RT 586 s.

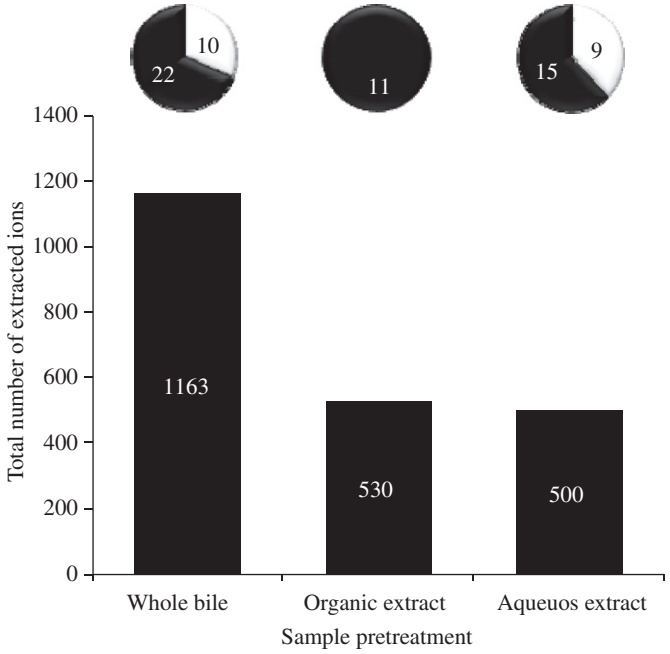


Fig. 2. Relationship between sample pretreatment and the total number of ions in the bile of *O. bonariensis* (bar chart) obtained with the ESI interface in the negative mode. Inserted pie charts show the respective numbers of 10-fold upregulated (black) or downregulated (white) ions in exposed organisms with respect to controls.

3.3. Response in O. bonariensis bile metabolic profile to cypermethrin exposure

A clear response was observed in the bile metabolic profile of the fish exposed to cypermethrin compared to controls.

The shift in the abundance of some characteristic ions was clearly visualized in a correlation plot of the log of the mean values of exposed with respect to control fish (Fig. 5A). Since XMS software processes raw spectra data, and with no blank subtraction, those levels of abundance below 10^3 should be considered background levels. In addition, those extracted ions presenting abundance values three times above or below the standard error of the residues were identified by the regression analysis (Fig. 5B). The major responding ions were labeled in the graph indicating the mass (*M*) and RT.

The PCA was also able to identify those ions contributing to the shift in the metabolic profile of the bile in response to the exposure to cypermethrin. Of the total variation, 81.6 percent was explained by the first three PCs (PC1, 37.1 percent; PC2, 26.7 percent; PC3, 17.7 percent). The differences among exposed and control groups were mainly accounted for by PC3, with an eigenvalue of 205.6. The component loading together with component scores for PC3 and PC1 are shown in a join plot (Fig. 6). The "component loadings" indicate the contribution of the variables (peaks) to the components. The major variables (absolute values of factor loadings greater than 0.85) contributing to PC3 are listed in the same graph. The "component scores" were useful to identify cases (fish) that cluster together (more similar spectra)

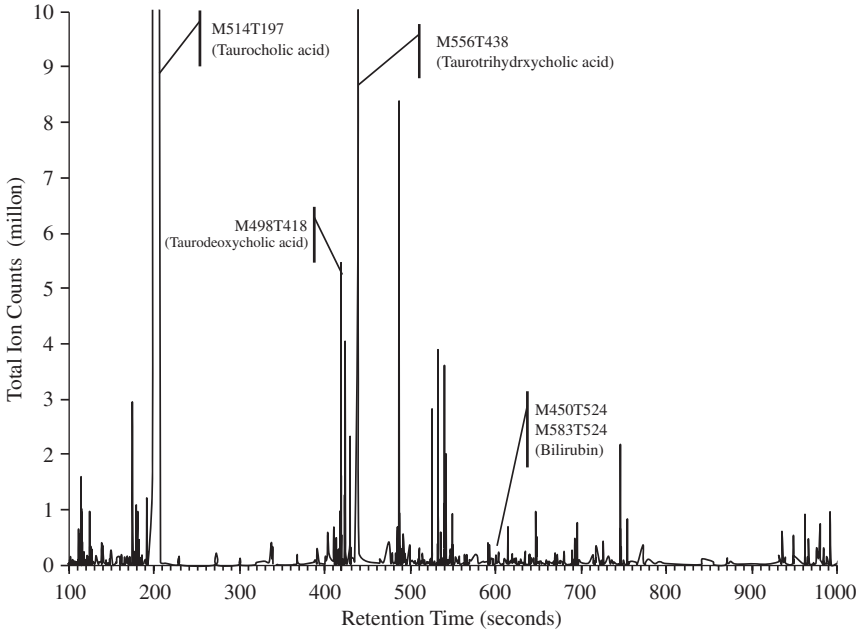


Fig. 3. Representative extracted ion chromatogram obtained from the whole bile of control fish showing some major bile components. Mass spectra data were obtained by LC–MS and ESI(−), and were later processed using XCMS software. The mayor bile components are labeled. *M*, *m/z*; *T*, retention time.

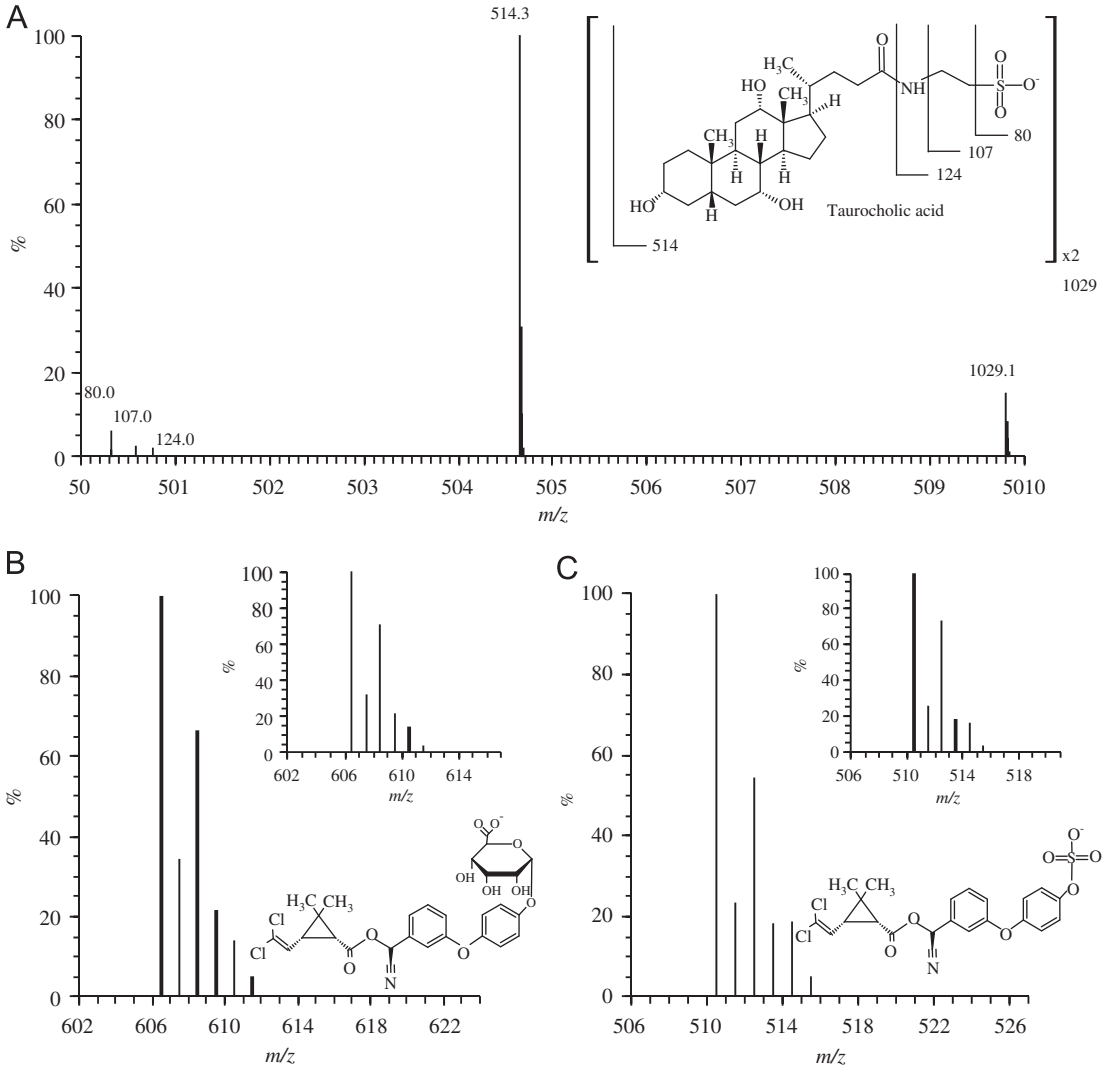


Fig. 4. Mass spectra of a representative bile acid (A) and the metabolites of the insecticide detected in the bile of exposed *O. bonariensis*, glucuronide of 4′-hydrohy-cypermethrin (B), and sulfate of 4′-hydroxy–cypermethrin (C). Mass spectra were obtained using an ESI interface in negative mode (150 eV) and a single quadrupole (Agilent G1956A VL MSD). The chemical structure of the molecular ions and the fragment assignments are shown in the graph. The isotopic distributions predicted by the ISOFORM software for the cypermethrin metabolites are inserted in the respective panels.

from cases that do not. The score plot shows that PC3 clearly segregates control (positive scores) and exposed (negative scores) fish.

Both analyses showed that the intensity of characteristic peaks was up or down in response to insecticide exposure. However, some of the relevant ions proposed from each analysis are not exactly the same. The list of the most important ions obtained by the regression analysis and principal component analysis are shown in Figs. 5B and 6.

Among the group of ions showing an increase in abundance, a cluster of ions (*m/z* 606, 608, 607, 609) with the same RT (565 s) was clearly distinguished by both analyses. This cluster of ions was identified as the glucuronide of 4′-hydrohy-cypermethin. The molecular mass together with the characteristic isotopic signature of the chlorine and ^{13}C together with specific fragmentation products allowed us to elucidate the structure. This metabolite was also confirmed in the positive mode and using the APCI source. In addition, this metabolite was readily extracted in the ethylic phase. A second cluster of ions (*m/z* 510, 512) at RT 682 s was successfully identified as sulfate of 4′-hydroxy–cypermethrin, using the same approach. No other common ions were distinguished by both analyses within this group. Two temporal clusters at RTs 566 s and 537/8 s were found for the ions *m/z* 963–610 and *m/z* 981–982, but independently by each of the methods.

Within the group of ions that showed a decrease in abundance in response to cypermethrin exposure, only one ion (*m/z* 249; RT 930 s) was common in both analysis. All of the others ions were found only in one of the two applied methods. Other important ions are listed in Figs. 5 and 6. The identification of these metabolites was not the aim of the present study and will require further analysis using more sophisticated equipment and confirmation with standards.

4. Discussion

4.1. Methodological approaches and metabolomic information

Metabolomic studies applying LC–MS are relatively recent, and still no standardized protocols are available. In consequence, in the present study, different methodological approaches were evaluated with the aim of defining strategies toward assessing characteristic metabolic profile responses in the bile of

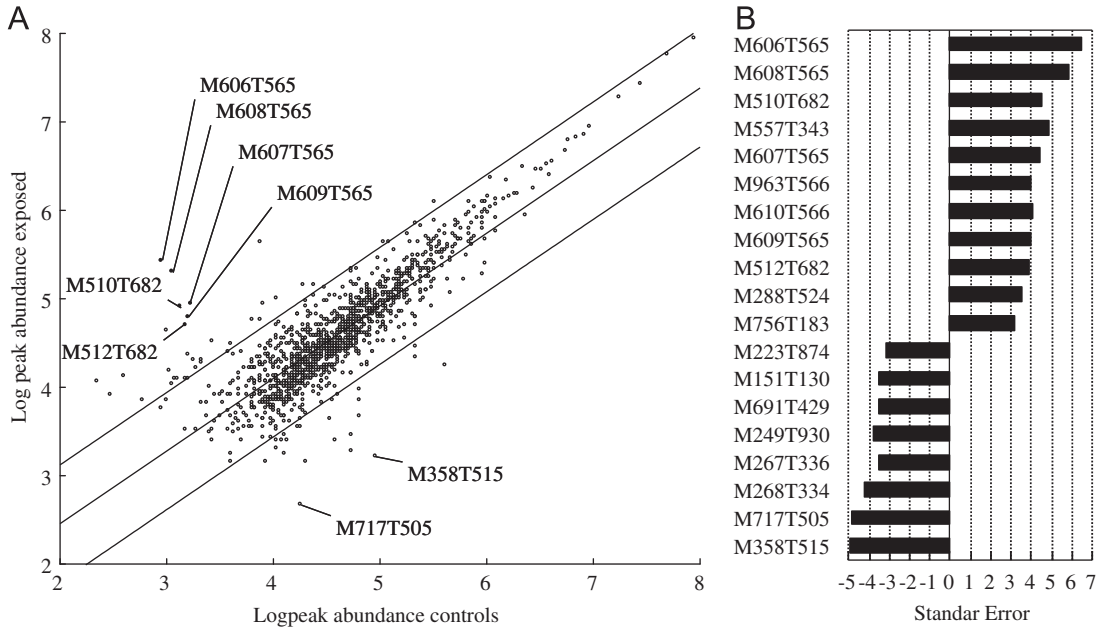
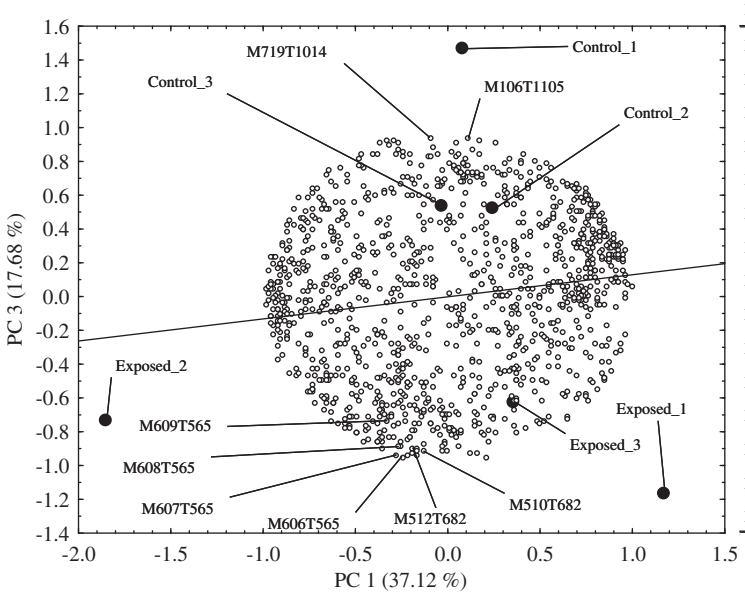


Fig. 5. Regression analysis of the log of the mean peak values obtained in control and exposed groups. The scatter plot (A) shows the extracted peaks and the regression line (continuous line), the prediction bands (dashed lines), and the labels (mass and time) of the main peaks above (cypermethrin metabolites) and below (unidentified metabolites) the prediction limits. In addition, the bar graph (B) shows the output of the residual analysis, listing those peaks with residue values three times greater than the standard error of the residuals.



Peak	PC 3	Peak	PC 3
M492T526	-0.948	M505T600	0.853
M606T565	**-0.946**	M292T420	0.865
M982T537	-0.939	M813T418	0.869
M607T565	**-0.937**	M198T74	0.874
M963T410	-0.933	M249T930	0.880
M594T154	-0.923	M302T991	0.896
M938T746	-0.920	M129T99	0.906
M399T826	-0.920	M212T101	0.907
M510T682	**-0.914**	M342T424	0.914
M478T490	-0.911	M925T755	0.916
M557T343	-0.908	M301T991	0.920
M574T338	-0.901	M251T987	0.921
M981T538	-0.900	M495T793	0.931
M512T682	**-0.893**	M106T1105	0.936
M608T565	-0.888	M719T1014	0.941

Fig. 6. Results of PCA of all the peaks extracted from exposed and control fish. The factor scores (filled dots) and factor loadings (empty dots) are shown together in the graph, and the loading values of the 30 peaks that contributed more to the PC 3 are shown in the inserted table. Cypermethrin metabolites and those extremely depressed in exposed fish are labeled in the plot. Cypermethrin metabolites are also shown in bold in the table.

O. bonariensis to identify exposure and responses to environmental pollutants.

The obtained results showed that when an LC–MS platform is used, the amount and type of information obtained from a given sample markedly depends on the type of ionization interface. In the fish bile, a greater amount of ions was obtained using the ESI interface in negative mode. Similar results were obtained in the bile of rats and dogs using UPLC–ESI–MS (Plumb et al., 2009). Another study with human serum also obtained a higher amount of ions using the ESI rather than the APCI source, but in this case the numbers of ions in positive and negative modes were comparable (Nordstrom et al., 2008). This indicates that the amount of information obtained in the positive or negative mode will not only depend on the ionization interface, but also on the nature of samples. But the amount of information was not the only matter of consideration, since complementary information was also obtained using ESI(+) and APCI interfaces. The contributional significance of analyzing this complementary information in metabolomic studies has been discussed previously by Nordstrom et al. (2008).

The pretreatment of the samples was also an important factor regarding the amount and nature of the obtained information. The most informative, and practical, procedure was the direct injection of the diluted whole bile. However, useful information for metabolite identification was obtained using different extract

procedures (aqueous and organic, with or without previous acidification). For example, the glucuronide of 4′-hydroxy-cypermethrin was found in the whole bile, in the nonacidified aqueous extract, and in the organic extract that was previously diluted and acidified, but not in the organic extract without previous acidification. On the other hand, the sulfate of 4′-hydroxy-cypermethrin was found in the whole bile and the aqueous extract, but not in the organic extract. This could explain why this metabolite was not reported by Edward et al. (1987) in the bile of trout, where only the organic extract was analyzed. The partition behavior was in agreement with the polarity of the elucidated metabolite.

4.2. Characterization of O. bonariensis bile metabolic profile

The major components of the bile are the bile acids and salts, cholesterol, free fatty acids, lecithin (phospholipids), bile pigments, and small amounts of other metabolic end products, bicarbonate ions, and trace metals (Guyton and Hall, 2006). Although, the aim of the present study was not to do an exhaustive description of the bile components, the identification of some major compounds was useful as a reference to check the correct peak filtering, identification, and matching among samples. Among the total number of peaks detected in the whole bile, those clusters presenting peaks of higher intensity corresponded with mass spectra of bile acids, particularly those conjugated with taurine. This group of bile acids were easily identified when looking for the fragments corresponding to the taurine moiety (Griffiths and Sjövall, 2010). In addition, the formation of dimmers in the spraying chamber was indicative of the high abundance of these compounds. The primary bile acid, TCA, was the major component of the *O. bonariensis* bile. Another relevant peak (but less intense) was the primary bile acid, TDCA. The observed relative abundance of these two bile acids was in good correspondence with that reported for other teleosts (Yeh and Hwang, 2001). Also in concordance with Yeh and Hwang (2001), no glycine-conjugated bile acids were found in the bile of *O. bonariensis*, since no fragments of the glycine moiety were found (Griffiths and Sjövall, 2010). Although not reported in the previous study, another important component of the *O. bonariensis* bile was a taurine-conjugated C27 bile acid, putative TTHCA (3α,7α,12α-trihydroxy-5β-cholestan-26-oyl acid taurine), or isomers of this compound. TTHCA is a precursor of the cholic acid found in the bile of humans with peroxisomal 3-oxoacyl–coenzyme A thiolase deficiency (Clayton et al., 1990). Two different taurine-conjugated C27 bile acids (3α,7α,12α-trihydroxy-5β–cholestan-27-oyl acid taurine and 1β,3α,7α-trihydroxy-5β-cholestan-27-oyl taurine), isomers of the TTHCA, were also reported in the bile of the tinamou *Rhynchotus rufescens* (Hagey et al., 2009). Other relevant bile components like bilirubin were also detected.

4.3. Global metabolic response in the bile of O. bonariensis exposed to cypermethrin

Fish bile was historically used in ecotoxicology to assess exposure to pollutants by means of a variety of targeted analyses for metabolite identification (Beyer et al., 2010; Gibson et al., 2005). However, the "omic" approach offered opportunities for the discovery of new biomarkers (Ankley et al., 2006; Miracle and Ankley, 2005; Viant et al., 2003). In the present study, a characteristic response at the global metabolic profile level was identified in the bile of those organisms sublethally exposed to cypermethrin for 96 h with respect to a control group. This response was characterized by a shift in the abundance of specific bile components. Only ten and nine clusters of ions (peaks with the same retention time) were up- and down regulated, respectively, in response to the insecticide exposure. Two of the upregulated clusters were identified as metabolite products of the detoxification pathway of the cypermethrin. The other clusters presumably corresponded to endogen metabolites. No major bile components were identified within the group of peak clusters responding to the exposure. This indicates that cypermethrin is affecting specific metabolic pathways with little impact on general biliary metabolism. Furthermore, the identification of these specific metabolites in complementary studies will help us to gain insight into the biological mechanisms underlying the observed response.

Both regression analysis and PCA were useful to identify those ions that better explain the variations observed among the treated and control groups. The lists of the major ions characterizing the metabolic response in *O. bonariensis* bile to cypermethrin exposure are shown in Figs. 5B and 6. A clear visualization of the relevant ions was obtained using regression analysis. On the other hand, differences among experimental groups were better identified by PCA, which was able to summarize the insecticide effect in only one variable (PC3). Log transformation of the peak intensities obtained from the XCMS analysis was a prerequisite to obtain biologically sound results from both methods. This is explained as a consequence of the great differences in the concentrations among bile metabolites. Metabolites of cypermethrin were certainly identified as major contributing ions using both approaches. However, some differences depending on the analytical method were observed regarding those ions corresponding with endogenous metabolites. These results would indicate that metabolic fingerprints not only depend on method of sample extraction and instrumental setup, but also on the methodology used for the analysis of data. Consequently, standardized methodologies will be necessary to use this approach to assess global metabolic responses as biomarkers.

4.4. Identified cypermethrin metabolites in the context of the known detoxification pathways

The toxicokinetics and toxicodynamics of pyrethroids in fish and other vertebrates were comprehensively studied in the 1980s (Bradbury and Coats, 1989a,1989b; Bradbury et al., 1986; Coats and Bradbury, 1989; Coats et al., 1989; Edwards and Millburn, 1985). In particular, a detailed description of the detoxification pathway of *cis*-and *trans*-cypermethrin was reported for trout (Edwards et al., 1987). Both isomers, *cis* and *trans*, were equally toxic for trout and were primarily eliminated through the bile. The major metabolite was the glucuronide of 4′-hydroxy–cypermetrhin together with dichlorovinyldimetyl-cyclopropanecarboxylic acid and its glucuronide, 3-(4-hydroxyphenoxy)benzoic acid(4′-hydroxy-3BPA), and its ester and ether glucuronides, 3-phenoxybenzoyl glucuronide and 4′-hydroxy-3BPA sulfate.

When results obtained in the present study are integrated with the detoxification scheme proposed by Edwards et al. (1987), it is possible to observe that cypermethrin is also mainly metabolized to 4′-hydroxy-cypermetrhin (Fig. 7). Nevertheless, none of the other minor metabolites were detected in the bile of exposed *O. bonariensis*. This could be because the concentrations of minor metabolites were too low to be detected by the MS scan mode. On the other hand, with the global approach used in this study we were able to detect the sulfate of 4′-cypermethrin as an important product of the detoxifying metabolism of the pyrethroid in *O. bonariensis*. This metabolite was not reported by Edwards et al. (1987). Such difference in the results could either indicate differences in the metabolic pathway among fish species or be a methodological consequence, since the sulfate of 4′-cypermethrin was found only in the whole bile and aqueous extract and not in the ethylic extract, which was the assessed in the study by Edwards et al. (1987).

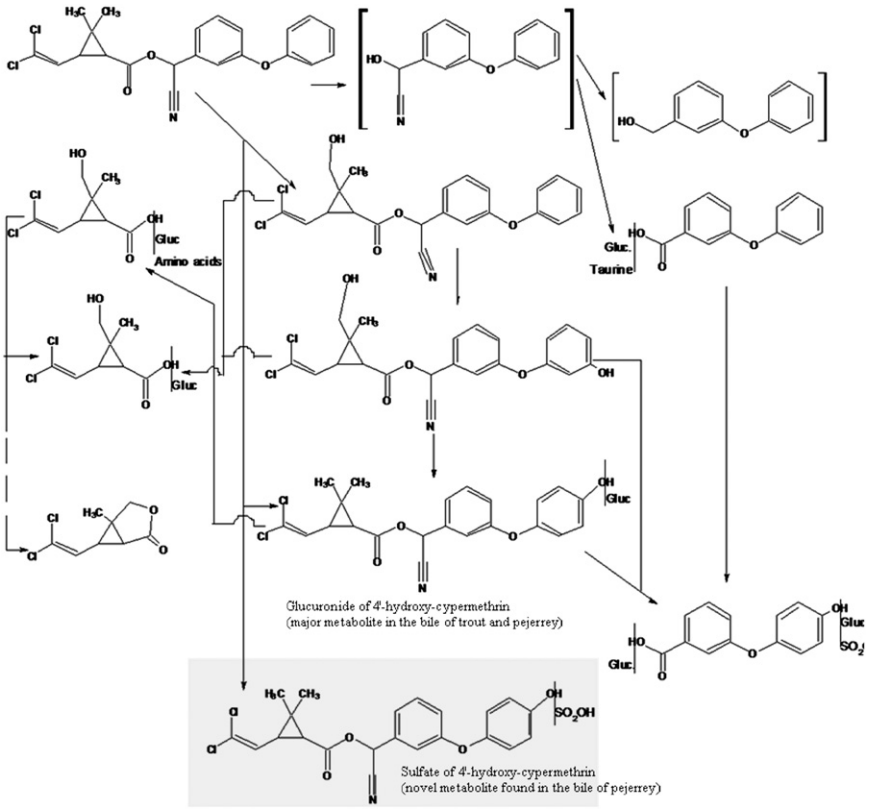


Fig. 7. Cypermethrin metabolites found in pejerrey bile integrated with the detoxifying pathway scheme proposed by Edwards et al. (1987) according to the metabolites found in the bile of trout using labeled *cis*-and *trans*-cypermethrin standards.

The high toxicity of pyrethroids to fish compared with other vertebrates, particularly mammals, is mainly attributed to the capacity of the enzymatic ester hydrolysis (Bradbury and Coats, 1989a). The minor relevance of the ester hydrolysis detoxification pathway observed in the liver of *O. bonariensis* was consistent with the high sensitivity shown by this species to the soybean insecticide (Carriquiriborde and Ronco, 2006).

5. Conclusions

The metabolic profile of *O. bonariensis* and its response to the insecticide cypermethrin were studied for the first time using HPLC–MS-based metabolomics.

The metabolic profile was dependent on the setup of the instrument, the sample pretreatment, and the statistical method used. Hence, it will be important to establish standardized procedures for the metabolomic analysis.

The taurine-conjugated bile acids were the more conspicuous metabolites of *O. bonariensis* bile. Identification of major bile components demonstrated to be helpful, as these were used as reference peaks to verify that mass spectra data were processed properly.

A global metabolic response to the sublethal exposure to cypermethrin was characterized by the up–and downregulation of a specific group of ions related to a reduced number of metabolites. The mass and retention time, together with the direction of the change in the responding ions, were determined. Subsequent identification of these metabolites will help us to understand the subjacent mechanism driving potential sublethal/chronic effects. In addition, the characterized global metabolic response stands out as a promising biomarker to assess sublethal exposure and response to the insecticide in feral fish and under field conditions.

Among the ten-fold upregulated ions, we identified the metabolites of cypermethrin: the glucuronide of 4′-hydroxy-cypermetrhin and the sulfate of 4′-hydroxy-cypermetrhin, the only two metabolites of the insecticide detected in the bile of *O. bonariensis*. In particular, the sulfate of 4′-hydroxy-cypermetrhin was reported here for the first time in fish bile.

Finally, HPLC–MS-based metabolomics demonstrated to be a powerful ecotoxicological tool for identifying biological responses to pollutants, discovering new metabolic pathways, and proposing specific biomarkers using non-model organisms.

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