Subcellular energy balance of *Odontesthes bonariensis* exposed to a glyphosate-based herbicide

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ABSTRACT

Water pollution by agrochemicals is currently one of the most critical problems for the conservation of aquatic ecosystems. Glyphosate [N-(phosphonomethyl) glycine); PMG] is the main broad-spectrum post emergence herbicide used for the control of a wide range of pests in soybean crops. Adenylate energy charge (AEC) reflects the energy balance of the cells, a measure of the energy available from the adenylate pool: adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP). Background adenylates, phosphagens and the AEC index of two year old *Odontesthes bonariensis* were determined in some tissues by HPLC, and the impact on subcellular energy balance of sublethal glyphosate-based herbicide exposure was analyzed. The doses used were 0 (control tank), 1 or 10 mg PMG L^{-1}, trials were carried out during 15 days. AEC values in brain, liver and muscle from control fish were 0.37 ± 0.02, 0.49 ± 0.05 and 0.56 ± 0.03, respectively (means $\pm$ SEM). While brain ATP concentrations were undetectable (hence low values of AEC), the muscle tissue showed the highest concentrations of the more energetic molecules: 0.18 μmole ATP g^{-1} and 8 μmole phosphocreatine g^{-1} (PCr g^{-1}). In the brain, no significant changes were detected in exposed fish compared to controls. Instead, in both the liver and muscle of animals exposed to the highest concentration of the herbicide, significant changes in the AEC (reduction of 26% and 15%, $p < 0.05$) with respect to the control group were determined. Chronic exposure (15 days) of *Odontesthes bonariensis* to 1 and 10 mg L^{-1} of formulated glyphosate did not affect brain AEC. However, the highest concentration of the herbicide produced a significant decrease in liver and muscle AEC manifesting adverse sublethal effects on the energy metabolism. These results suggest the usefulness of AEC as a biomarker of fish glyphosate exposure.

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1. Introduction

Water pollution by agrochemicals is currently one of the most serious problems for the conservation of aquatic ecosystems. In Argentina, transgenic soybean production has continued growing over the last decade. The cropped area increased from approximately 9 million ha at the end of the 1990's up to 20 million ha in 2012/13 (MAGyP, 2014). This led to a parallel increase in the pesticide application rate (herbicides plus insecticides, fungicides, acaricides, among others) from 127.5 thousand tons in 1999 to more than 280 thousand tons in 2013 (CASAFE, 2014).

In particular, glyphosate [N-(phosphonomethyl) glycine; PMG] is the main broad-spectrum herbicide used for the control of a wide range of weeds in soybean crops. The application of glyphosate formulations commonly used in Argentine agriculture increased from 70 thousand tons to more than 180 thousand tons between 1999 and 2013 (CASAFE, 2014). The physical properties of PMG, such as the high solubility in water explain its fast and easy distribution in aquatic compartments of ecosystems, and strong binding capacity to soil organic matter explain the permanence and retention of PMG in the environment (WHO 1994). Once applied to the crop, part of the herbicide may remain adsorbed to soil particles until the degradation by microorganisms. Also, the herbicide can be mobilized by the influence of a number of factors such as the wind, rain or irrigation that increase infiltration and surface run-off (Perez et al., 2011; Menéndez–Helman et al., 2013).

Thus, the organic compound can reach the nearer aquatic environments and adversely affect the biota.

USA, Brazil and Argentina are the countries with large areas under genetically modified crops with concomitant increase of glyphosate use. In adition, a marked global increases of the average rate of glyphosate application per Ha is associated with the appearance of a growing number of tolerant or resistant weeds. Application rates up to 5.6 kg active ingredient/Ha were reported by Giesy et al. (2000). For this reason, it is not surprising that higher levels of glyphosate are determined in water bodies in these countries. In this regard, some authors have reported high levels of glyphosate and its metabolite aminomethylphosphonic acid (AMPA) in sediment, soil and water, particularly in the surroundings of cultivation areas (Edwards et al., 1980; WHO, 1994; Peruzzo et al., 2008, Ronco, 2010), as well as in plants and soybeans (Arregui et al., 2004; Lorenzatti et al., 2004).

In general, PMG does not exhibit a significant acute lethal toxicity to freshwater fish (LC50-96 h > 100 mg L^{-1}) (Folmar et al., 1979; Neskovic et al., 1996; Carriquiriborde, 2010; Menéndez–Helman et al., 2013). However, several authors have noted that formulations containing the herbicide active together with co-adjuvants such as POEA exhibit a significant increase in toxicity to diverse aquatic organisms (algae, bacteria, protozoans, molluscs, crustaceans, amphibians and fish) (Giesy et al., 2000; Tsui and Chu, 2003; Pérez et al., 2011; Menéndez–Helman et al., 2013; Wagner et al., 2013). A number of sublethal effects of the glyphosate-based herbicides have also been reported in fish: evasion of harmful concentrations (Hildebrand et al., 1982), histopathological changes (Domitrovic, 1997; Szarek et al., 2000; Jiraungkoorskul et al., 2003), metabolic changes associated with oxidative stress (Cattaneo et al., 2011; Glusczak et al., 2007; Langiano and Martínez, 2008), neurotoxicity (Glusczak et al., 2006; 2007; Menéndez–Helman et al., 2012), genotoxicity (Cavas and Könen, 2007; Kier and Kiekland, 2013), malformations (Kelly et al., 2010), hematologic changes (Alvarez et al., 2012), alterations of hormonal profiles (Soso et al., 2007), decrease in cell-mediated immune response (El–Gendy et al., 1998), among others.

The silverside (*Odontesthes bonariensis*; Cuvier and Valenciennes, 1835) belongs to the Atherinidae family and is considered native to lagoons in Buenos Aires Province, Argentina, and probably in Southern Brazil (Dyer, 2006). This teleost presents socioeconomic relevance for commercial and sport fishing, and additionally it is a promising species for aquaculture (Somoza et al., 2008). In a number of lethal toxicity studies, using silversides as test organism, relatively low LC50-96h values for several heavy metals and organic compounds have been estimated, confirming the sensitivity of this species to environmental pollutants (Carriquiriborde and Ronco, 2006).

Environmental stress triggers events associated with the physiological regulation mechanisms and induces changes from the cellular to the population level. Under these scenarios, aquatic organisms increase their energy requirements to trigger physiological compensation mechanisms (homeostasis), releasing catecholamines, glucocorticoids and/or corticotrophins, and alter the energy metabolism of tissues (Mazeaud and Mazeaud, 1981; Boonstra, 2013). The ATP is the "energy currency" of cells in all organisms. ATP stock can be regenerated immediately from two enzymatic systems catalyzed by adenylate kinase (AK) and creatine kinase (CK). Both systems maintain energy homeostasis in cells (De la Fuente et al., 2014). Therefore the analysis of the energy balance of tissue requires the knowledge of adenylate and phosphagen levels.

The adenylate energy charge (AEC) reflects the energy balance of the cells, a measure of the available energy amount from the high energy adenylate pool (Atkinson, 1968). AEC has been proposed as the main factor that regulates the flow of energy of metabolic pathways (catabolic and anabolic processes) in cells and as an indicator of the energy state of the organisms (Le Gal et al., 1997). Moreover, the use of AEC has been suggested as a tool for assessing the responses of aquatic organisms to environmental stress conditions (Le Gal et al. 1997). However, its study as a biomarker in biomonitoring and toxicity studies has not been exhaustively explored. The aim of this study was to determine basal levels of adenylates, phosphagens and the AEC index in the brain, muscle and liver of *Odontesthes bonariensis*, and the impact on subcellular energy balance after exposure to sublethal glyphosate-based herbicide.

2. Materials and Methods

2.1. Animals. Experimental design

Two year old fish were selected from the stock born and reared in the Aquaculture facilities of INTECH (Chascomús, Argentina). Three tanks, containing 120 L of groundwater (salinity: 15 g L^{-1}), continuously aerated, were located outdoors; four males and two females were placed in each tank. After acclimatization of 48 h, fish were transferred to another container while commercial formulated glyphosate was added to the tanks, after that fish were placed back into the tanks. The glyphosate-based herbicide tested was Glifosato II Atanor®. The PMG doses used were 0 (control tank), 1 or 10 mg PMG L^{-1} expressed as glyphosate acid equivalent. During the assays, fish were fed with commercial fish food pellets (Shulet, Buenos Aires, Argentina). The trials were carried out during 15 days. Temperature and dissolved oxygen (DO) of the assay media were determined every 2 days at 2 pm; the mean values (± SEM) were 19 ± 1 °C and 6.2 ± 0.3 mg L^{-1}, respectively. The analytical concentration of the herbicide in the media was quantified at the initial and final time.

At the end of the exposure period, fish were anesthetized with benzocaine (100 mg L^{-1}), weighted (W), the total length (L) measured and dissection was performed. Samples from the liver, muscle and brain were taken and stored at −70 °C in a freezer until nucleotides and phosphagens were extracted and determined.

2.2. Adenylate and phosphagen extraction

Samples of muscle and liver were processed according to Werner et al. (2006). These samples were pulverized in a mortar under liquid nitrogen, and extracted with HClO$_4$ 0.4 M (in a ratio of 10 mL per gram of tissue). The system was kept cold in an ice bath and after 20 min it was centrifuged at 9000g and 4 °C for 10 min in ultracentrifuge Sigma 3–18 K model with 12158 rotor. The supernatant was immediately neutralized with K$_2$CO$_3$ 4 M (67 μl mL^{-1} of supernatant) and incubated on ice for 30 min. It was centrifuged again under the same conditions, the supernatant was kept in ice to be injected into the HPLC equipment within the subsequent 3 h. The same protocol was performed for liver, but due to the low density of this tissue, the first centrifugation was performed at 15,000 g and 4 °C, for 15 min.

The extraction of brain samples was performed according to De Boeck et al. (1995), by homogenization in HClO$_4$ 0.4 M (1.2 mL per brain) with Decalab equipment, glass–Teflon rotor, at 3500–4000 rpm, 10 strokes per homogenate. Subsequently, a protocol similar to that for the other tissues was followed. In this case, centrifugation was performed at 14,000 g and 4 °C, in a Hermle Z 216MK refrigerated microcentrifuge for Eppendorf tubes during 10 min.

2.3. Adenylate and phosphagen determinations

The adenylates (AMP, ADP and ATP), creatine (Cr) and phosphocreatine (PCr) were determined by HPLC with a reverse phase C18 column (Zorbax Eclipse XDB-C18 column, 4.6 × 250 mm, 5 μm particle size, Agilent). Jasco quaternary equipment consisting of three coupled modules was used: Intelligent HPLC Pump Model PU-2089, Intelligent UV detector Model UV-2070/75, and Interface box LC-NetII/ADC, connected to a PC with ChromPass software. The quantifications were performed using gradient elution with a mobile phase containing 35 mM NaH$_2$PO$_4$, 6 mM tetra-butylammonium; mobile phase/acetonitrile gradient 95:5 to 75:25. Detection: 210 nm for 0–10 min and 254 nm after 10 min (Ally and Park, 1992; Werner et al., 2006).

The adenylates, Cr and PCr standards were prepared with milliQ water at concentrations ranging from 10 to 50 mM. The analytical concentration of adenylates and phosphagens was estimated by comparison of the area of the sample with that of standards, and adjustments were made for sample dilution.

2.4. Analytical concentration of PMG

PMG concentrations in the exposure aqueous media were analyzed by ion chromatography (Zhu et al., 1999), using a Dionex DX-100 chromatograph with a conductivity detector and a 25 μl sample loop. Dionex AG-4 and AS-4 were used as analytical columns. A mixture of NaOH/CO$_3^{2-}$ (4 mM/9 mM) was used as eluent with a flow rate of 1 ml min^{-1}. Data acquisition was performed using the Clarity Lite software. Samples from three tanks (nominal concentrations: 0, 1 and 10 mg PMG L^{-1}) and six standards (0, 0.5, 1, 5, 10, 25 mg PMG L^{-1}) freshly prepared were injected.

2.5. Results and statistical analysis

Adenylate, Cr and PCr concentrations are expressed as μmoles/gr.

The adenylate energy charge (AEC) was calculated using the following formula (Atkinson, 1968):

$$AEC = \frac{[ATP] + 0.5 \times [ADP]}{[ATP] + [ADP] + [AMP]} \tag{1}$$

Total adenylate nucleotide pool (TANP), the sum of adenylate concentrations, was estimated as TANP=[ATP]+[ADP]+[AMP].

The Fulton's Condition Factor (K), a morphometric parameter, was calculated for each fish as $K=[(W \times 100)/L^3]$.

The statistical comparisons for each parameter between exposed groups and the control were performed using the Kruskal–Wallis non-parametric ANOVA, followed by Dunn's Multiple Comparison Test (GraphPad Prism Software). The minimum of statistical significance was established at $p < 0.05$.

2.6. Chemicals

All the reagents used were of analytical grade and solutions were prepared using milli-Q water. ATP, ADP, AMP, Cr and PCr standards were purchased from Sigma (St. Louis, MO, USA).

The glyphosate-based herbicide tested was Glifosato II Atanor®, which was bought from a commercial Argentinean retailer. The formulation used contained the glyphosate monopotassium salt 43.8 g /100 mL (equivalent to 35.6 g PMG/100 mL). Inerts and co-adjuvants were not specified in the label.

3. Results

The energy metabolism and the effect of sublethal exposure to glyphosate-based herbicide in different tissues of *Odontesthes bonariensis* were analyzed.

The analytical concentrations of PMG (ND, 0.9 ± 0.1 and 9.2 ± 0.4 mg PMG L^{-1}) at the initial time were similar to the nominal concentrations (0, 1 and 10 mg PMG L^{-1}), where ND means not detectable. Nevertheless, the PMG concentration strongly decrease at the end of the assay period, being no detectable in the control and the lower concentration tanks and reaching about 10% of the initial concentration in the higher concentration tank (ND, ND and 1.1 ± 0.2 mg PMG L^{-1}). This implies that there were changes in herbicide concentrations due to abiotic degradation, adsorption to dissolved organic matter or bioaccumulation processes, during the time of the trial.

The concentrations of adenylates, PCr and Cr in the brain are shown in Table 1. In this tissue ATP levels were not detectable (ND) in all cases, which may explain the low average value of AEC obtained (0.37 for controls; Fig. 1A). No significant changes regarding the control group were observed in any of these parameters in the two groups exposed to the formulated glyphosate.

In the liver of the specimens exposed to 10 mg PMG L^{-1}, a significant increase of 99% in AMP concentrations relative to the control was observed. Consistently, while the average of the AEC value for the control animals was 0.49, the mean value for the animals exposed to 10 mg PMG L^{-1} decreased by 26% (see Table 2 and Fig. 1B) and was statistically different ($p < 0.05$).

In the muscle, higher concentrations of the more energetic molecules (ATP and PCr) were found. The concentration of ATP and PCr in this tissuewere 0.18 μmol g^{-1} and 8 μmol g^{-1} respectively. The average AEC value for the control animals was 0.56 and decreased significantly by 15% for the animals exposed to the highest concentration of formulated glyphosate (see Table 3 and Fig. 1C).

TANP levels in the control fish were also high for muscle. In the control fish, the TANP relation between tissues was 1:3:15 for brain, liver and muscle respectively (Table 4), showing a higher adenylate concentration for muscle than for the other tissues. An increasing trend in TANP levels in fish exposed to the herbicide was observed in brain and liver. However, the differences in this parameter were not statistically significant.

Table 1
Effects of sublethal exposure to a formulated glyphosate on the concentration of ATP, ADP, AMP, PCr and Cr in *O. bonariensis* brain.

Herbicide concentration (mg PMG L^{-1})	ATP	ADP	AMP	PCr	Cr
0 (control)	ND	0.029 ± 0.001	0.011 ± 0.003	0.21 ± 0.04	6 ± 2
1	ND	0.036 ± 0.006	0.018 ± 0.003	0.47 ± 0.13	10 ± 2
10	ND	0.039 ± 0.010	0.014 ± 0.005	0.43 ± 0.16	8 ± 2

Data in μmol g^{-1} ($n=6$); ND=not detectable.

A

B

C
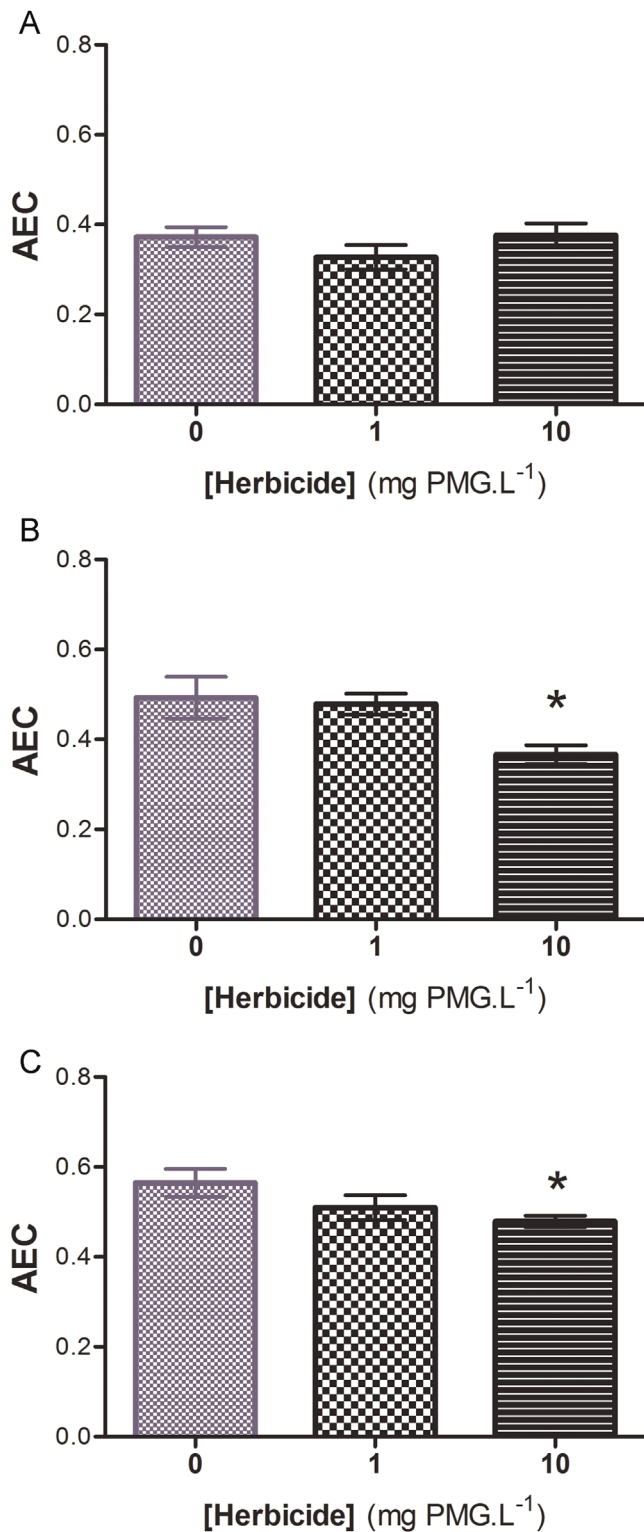
Fig. 1. Effect of sublethal exposure to a glyphosate-based herbicide on adenylate energy charge (AEC) in brain (A), liver (B) and muscle (C) of *O. bonariensis*. *$p < 0.05$ compared to the control group.

At the end of the experiment, no significant changes in fish morphometric parameters were found. Condition Factor (K) values (mean ± SEM) were 0.78 ± 0.05; 0.83 ± 0.05 and 0.83 ± 0.04 for the control group and for the groups exposed to 1 and 10 mg PMG L^{-1}, respectively.

4. Discussion

The levels of adenylates (ATP, ADP and AMP) that allowed the calculation of the AEC and complementary Cr (creatine) and PCr (phosphocreatine) concentrations were evaluated in brain, liver and muscle of *Odontesthes bonariensis,* a native freshwater teleost. Regarding adenylate and phosphagen basal levels determined in control fish, it should be noted that the muscle showed higher concentrations of the more energetic molecules (ATP and PCr). The concentration of ATP in this tissue ($0.18 \, \mu mol \, g^{-1}$) was four times higher than that determined in liver, whereas in brain it was ND. At the same time PCr concentration was 29 times higher in muscle than in the brain, whereas in the liver it was ND. Similar results were reported by van den Thillart and van Raaij (1995). AEC value was also higher than that the other tissues analyzed. These results are consistent with the high and fluctuating energy requirements of the muscle tissue.

Moreover the basal adenylates values were relatively low (mainly, as expected, in the brain) (see Tables 1–3). The literature shows great heterogeneity in AEC values, suggesting that the direct correspondence between AEC values and the environmental (season, temperature, chemical profile) and physiological state of the organisms (stage, reproductive state) require further experimental analysis.

Several reports have described seasonal variations in the levels of AEC in bivalves (Giesy and Dickson, 1981; Picado et al., 1988; Giesy and Graney, 1989; Moal et al., 1991); crustaceans (Dickson and Giesy, 1982) and other invertebrates that have been associated with the reproductive cycle of molluscs and crustaceans. Subsequently Dehn (1992) found a seasonal pattern in the levels of adenylates and phosphocreatine in the muscle and liver of the fish *Lepomis microlophus*. Particularly, this author observed minimal levels of ATP, PCr and AEC (0.49) in muscle during the winter months and established a positive correlation between these parameters and temperature. Also, a minimum level of ATP, PCr and AEC (0.17) in liver was established in the spring (when the environmental temperature was in the range of 23–28 °C), although no correlations with the temperature were determined. Considering that the assay in the present work was conducted in the spring (late November in Southern Hemisphere), the seasonal factor may explain the lower basal levels of AEC determined. However, other factors cannot be discarded. AEC values in control animals lower than optimal could be due to the particular assay conditions that do not represent optimal conditions for the organisms (static test, tank volume). Manipulation of both animals and tissues should also be considered as a stressor that may affect the stability of these molecules (Vogel, 1993; Pottinger and Calder, 1995).

Phosphocreatine levels are provided for the phosphate groups of ATP synthesis in the reaction catalysed by CK. PCr in skeletal muscle concentration is about 10 times higher than that of ATP; while in other tissues (smooth muscle, brain, kidney) it is 3–6 times less (Nelson and Cox, 2000). PCr values indicate that the concentration determined in silverside muscle (Table 3) was 46 times greater than that of ATP, suggesting a critical role of this phosphagen in energy regulation of *O. bonariensis*.

The evaluation of the health status of fish exposed to pollutants that induce sublethal stress requires appropriate biomarkers. Chronic exposure to environmental stressors, as may occur in the case of water bodies near to areas of intensive crops, may provoke subcellular changes in the energy metabolism of aquatic animals. These subcellular changes expressed as changes in the energy balance may occur by a toxic mechanism (i.e. inhibition of oxidative phosphorylation) or as part of a stress response to toxic exposure. The integrated study of metabolism energy changes is a tool that can help to understand the physiological consequences of

Table 2
Effects of sublethal exposure to a formulated glyphosate on the concentration of ATP, ADP, AMP, PCr and Cr in *O. bonariensis* liver.

Herbicide concentration (mg PMG L^{-1})	ATP	ADP	AMP	PCr	Cr
0 (control)	0.043 ± 0.009	0.035 ± 0.009	0.043 ± 0.006	ND	0.92 ± 0.03
1	0.043 ± 0.004	0.049 ± 0.014	0.046 ± 0.004	ND	1.25 ± 0.26
10	0.040 ± 0.006	0.037 ± 0.004	0.085 ± 0.013*	ND	1.01 ± 0.08

Data in μmol g^{-1} ($n=6$). * $p < 0.05$ compared to the control group; ND=not detectable.

Table 3
Effects of sublethal exposure to a formulated glyphosate on the concentration of ATP, ADP, AMP, PCr and Cr in *O. bonariensis* muscle.

Herbicide concentration (mg PMG L^{-1})	ATP	ADP	AMP	PCr	Cr
0 (control)	0.18 ± 0.05	0.33 ± 0.06	0.09 ± 0.01	8.27 ± 1.9	26 ± 2
1	0.16 ± 0.04	0.36 ± 0.05	0.13 ± 0.02	9.95 ± 0.6	29 ± 1
10	0.12 ± 0.02	0.22 ± 0.06	0.14 ± 0.02	6.88 ± 1.4	21 ± 4

Data in μmol g^{-1} ($n=6$).

Table 4
Effects of sublethal exposure to a formulated glyphosate on the total adenylate nucleotide pool (TANP) in *O. bonariensis* tissues.

Herbicide concentration (mg PMG L^{-1})	TANP		
	Brain	Liver	Muscle
0 (control)	0.039 ± 0.004	0.12 ± 0.01	0.60 ± 0.07
1	0.054 ± 0.009	0.14 ± 0.01	0.66 ± 0.09
10	0.053 ± 0.014	0.16 ± 0.02	0.48 ± 0.10

Data in μmol g^{-1} ($n=6$).

the pollutants exposure. In this sense, the analysis cannot be addressed from a reductionist approach because of the nature of the processes and the multiplicity of organs involved in the energy balance of the animals (Suarez, 2012).

There are several reports describing sublethal effects in fish exposed to glyphosate (see introduction section). Among them, some reported effects at cellular level such as antioxidant defences (changes of antioxidant enzymes) and/or oxidative stress (lipid peroxidation and DNA damage) (Menéndez–Helman et al., 2013 and the references cited therein; Uren Webster et al., 2014).

Changes in glycogen energetic metabolism, more specifically glycogen depletion in fish after exposure to formulated of PMG were found (Ortiz–Ordoñez et al., 2011; Shiogiri et al., 2012). Glycogen content is a parameter that reflects the state of energy reserves, while ATP and PCr are the molecules that represent the immediate sources of energy at the subcellular level.

Energy transduction is based on the use of adenosine triphosphate (ATP); since it cannot be stored, changes in the cellular capacity for energy production as a consequence of the impact of an environmental stressor may be reflected in the levels of ATP. It should be noted that the use of AEC was evaluated as an ecotoxicity biomarker in a small number of studies, especially in fish. The AEC of gill, muscle, liver and stomach of *Leponis macrochirus* after exposure to the insecticide carbofuran for 10 days was studied by Hohreiter et al. (1991). Other reports indicate changes in ATP levels in fish muscle and brain under hypoxic conditions (Vetter and Hodson, 1982; Renshaw et al., 2002). Also, changes

have been reported in the adenylate levels of *Onchorynchus mykiss* in response to heat stress (Werner et al., 2006) and to environmental pH in juvenile *Salmo salar* (Waiwood et al., 1992).

Since the energy metabolism is affected by many non-chemical factors, a general index of energetic processes, such as AEC, must be considered as a non-specific biomarker. Toxic effects from natural factors could be difficult to discriminate. Thus, to use AEC as a biomarker for wild fish should be necessary to know in advance how energy balance is affected between individuals at different lifestages, reproductive state, seasons, etc. However, the results presented in this work suggest that AEC could be a useful biomarker of the fish stress condition that points out the animal energetic balance. The effects of glyphosate on the subcellular energy balance of *Odontesthes bonariensis* after subchronic exposure to sublethal concentrations of a commercial formulation of the herbicide were evaluated. No significant variation was determined in the brain (Table 1, Fig. 1A). Furthermore, a tendency to decrease ATP and increased AMP levels by exposure was observed in both liver and muscle. However, this tendency was only significant in the case of increased AMP in the liver (Table 2). On the other hand, a significant decrease in AEC was determined in both tissues (Fig. 1B and C). The pertinence of the results obtained for glyphosate exposure is supported by the fact that the concentrations used have environmental relevance.

Some authors have reported high levels of glyphosate and its metabolite AMPA in sediment, soil and water, particularly in the surroundings of cultivation areas in Argentina (Peruzzo et al., 2008, Ronco, 2010). In particular, PMG concentrations between 0.1 and 0.7 mg L^{-1} have been reported by Peruzzo et al. (2008) in streams of the Rolling Pampa Region, 10.9 mg L^{-1} on a tributary of Pescado Stream, Buenos Aires Province was informed by Ronco (2010). Also, 5.2 mg L^{-1} in runoff waters in Ohio USA was found by Edwards et al. (1980); concentrations of 1.2 and 1.7 mg L^{-1} in streams and ponds of USA were reported by WHO (1994).

Finally, it should be noted that among all the energy parameters determined, AEC was the most sensitive biomarker. Therefore, adenylate energy charge could be a useful biomarker of the physiological condition of fish exposed to environmental pollutants in toxicity tests and in environmental risk assessments.

Acknowledgements

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