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RESEARCH ARTICLE

Multibiomarker responses in aquatic insect *Belostoma elegans* (Hemiptera) to organic pollution in freshwater system

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Abstract The present study analyzes a battery of biomarkers in the water bug *Belostoma elegans* from a stream polluted with organic matter (OMS), and another one considered as reference site (RS) during spring-summer season (December to March). Biochemical parameters of glucidic, lipidic and oxidative metabolic pathways were analyzed in males and females of this insect. In general, no significant differences were observed in all biomarkers assayed between both sexes, except lactate concentration which was higher in males than in females ($p < 0.0006$) in the first three months. About carbohydrate metabolism parameters, only pyruvate-kinase showed significant differences between insects collected in both streams ($p < 0.05$) during December. However, the total lipid content, saturated fatty acid, and mainly triacylglycerol were higher in insects from RS compared to those from OMS ($p < 0.002$) in all sampled months. Levels of lipoperoxidation, protein oxidation, reduced glutathione and glutathione-S-transferase activity showed no differences between insects collected from both streams. Nevertheless, the significant increase observed in superoxide dismutase and catalase activities ($p < 0.004$) could be due to the elevated oxidative metabolism in insects from RS compared to those from OMS with lower dissolved oxygen. Regarding those responding parameters, males accounted for the differences between the two sites during the study period. In conclusion, our results support that lipidic energetic reserves and antioxidant enzyme activities in *B. elegans* could be used as biomarkers of environmental pollution by organic matter.

Keywords Aquatic insects · Biochemical composition · Biomarkers · Ecotoxicology · Organic matter · Wastewater biological effects

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Introduction

For an effective management of water quality and aquatic ecosystems it is essential to monitor water resources (Bae et al. 2012). Since streams and rivers are among the most endangered ecosystems worldwide, there are urgent demands for comprehensive methodological approaches to evaluate the present state of these ecosystems and to monitor their rate of change (Li et al. 2010). At first, for the sustainable management of ecosystems, it is necessary to identify the cause of disturbance such as the presence of toxicants in the target ecosystem.

At the early stages of ecosystem monitoring, it is usual to assess the environmental conditions by measuring a series of physical and chemical factors such as pH, dissolved oxygen and biochemical oxygen demand (Bae and Park, 2014). However, in running waters, changes of hydrology are rapid and difficult to estimate because they cannot reflect the integration of numerous environmental factors. Thus

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biomonitoring has been proven to supplement those traditional monitoring techniques (Li et al. 2010).

Urbanization is the main factor that degrades freshwater quality by sewer effluents and industrial wastes as well as diffused pollution associated with agricultural activities (Gómez and Rodrigues Capítulo 2001). These incomes contribute with organic matter and nutrients in water bodies either in particulate and dissolved forms accelerating the process of eutrophication. The direct consequences are the increases in consumption of dissolved oxygen and primary productivity (Mariñelarena and Gómez 2008). Also, nutrient increase can lead to hypoxia, decreased species richness and increased abundance of opportunistic species that cause impact on the quality of ecosystems (Fitch and Crowe, 2011). Effluent discharges entering in freshwater systems represent a complex mixture of anthropogenic compounds. Thus it is quite difficult to relate the effects of specific pollutant on an organism due to the fact that the composition of discharges is, in general, unknown (Diodato et al. 2012). Moreover, these complex mixtures contain substances which chemical analysis is not available or extremely expensive. Thus, it is necessary to develop strategies to assess whether an environment is stressed or not (Rivadeneira et al. 2013).

Benthic macroinvertebrates are commonly used as indicators of ecological disturbance because of their sensitivity to environmental changes and easy sampling (Barbour et al. 1999; Aura et al. 2011). Exposure of organisms to contaminants may cause multiple toxic effects. The study of different effects on an exposed organism is essential for the understanding of the different biological responses and the mechanisms of toxicity of pollutants (Rivadeneira et al. 2013). Those responses to sub-individual level are known as biomarkers and their validation requires a comparison between the appropriate reference sites with polluted water bodies in the area of study. Additionally, the potential relationship of these biochemical responses with abiotic factors of the environment (the principal physicochemical variables of the water and sediment) and biotic factors (individual weight) of the ecosystem should be determined (Díaz-Jaramillo et al. 2010). The study of biomarkers related to different organism functions like antioxidant system, energy metabolism, etc., and at different levels of the biological scale like molecular, cellular, individual, etc., allows a better understanding of the mechanisms underlying the effects of stressors (Moore et al. 2004). The study of complementary biomarkers at cellular level shows the early response to environmental contamination and precedes the effects at individual level. Therefore, the analysis of dataset obtained from that study could help to synthesize and highlight stressor effects and estimate the toxicity of complex mixtures (Garaud et al. 2015).

Several pollutants inducing an increase in reactive oxygen species (ROS), which might produce oxidative stress due to the imbalance between the concentration of pro-oxidants and antioxidants (Monserrat et al. 2007). The antioxidant defense system is activated to avoid ROS harmful effects on biomolecules. These systems include several enzymes such as superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) as well as non-enzymatic compounds as reduced glutathion (GSH), ascorbic acid, uric acid, vitamin E and β-carotene (Correia et al. 2003). SOD converts O_2^- to H_2O_2, while CAT is an essential enzyme to promote the degradation of H_2O_2, a precursor of the hydroxyl radical that induces DNA damage, protein oxidation (PO) and lipid peroxidation (LPO) (Hermes-Lima and Zenteno-Savín, 2002). On the other hand, glutathione S-transferase (GST, EC 2.5.1.18) is a major family of detoxification enzymes that catalyze the conjugation of reduced glutathione (GSH) to diverse electrophilic substrates (Rauch and Nauen 2004). Therefore, all these responses are commonly employed as non-specific biomarkers in aquatic species because they are useful to characterize contaminated areas where complex mixtures of pollutants are usually present (Geracitano et al. 2004a, b; Amado et al. 2006a, b).

Due to the fact that exposed organisms may need additional energy to maintain physiological/biochemical functions at a normal level, metabolic determinations of energy-yielding substrates such as glycogen and lipids are also used as biomarkers to assess the effects of pollutants (Oliveira et al. 2013). For example, lactate dehydrogenase (LDH, EC 1.1.1.27), a key enzyme in the anaerobic pathway of energy production, is particularly important when high levels of energy may be required for a short period of time (Morris et al. 2005). Another key enzyme of the glycolytic pathway is Pyruvate-kinase (PYK, EC 2.7.1.40) which catalyzes the conversion of phosphoenolpyruvate to pyruvate and one of the two ATP producing steps in glycolysis, could be altered under stressing conditions (Kaloyianni et al. 2005). Moreover, a decrease in glycogen and lipid content in exposed organisms may be the consequence of the use of energetic reserves to meet the supplementary requirements induced by detoxification of toxic compounds, so they could be useful as biomarkers, too (Choi et al. 2001; Sánchez Paz et al., 2006; Zhou et al. 2011; Avigliano et al. 2014).

To evaluate the ecotoxicological action of organic pollution in freshwater systems, we selected the water bug *Belostoma elegans* Mayr, 1871. This aquatic insect belongs to Belostomatidae family which includes nearly 150 species distributed almost all over the world (Iglesias et al. 2008). Belostoma genus is distributed throughout the American continent and it comprises about 60 species. *Belostoma elegans* is found in Perú, Argentina and Chile (Morrone et al. 2004). Within the community, the functional role of this species is predatory, and it is widely distributed in the Pampas streams tributaries of Rio de la Plata river (Rodrigues Capítulo et al., 2003). The Río de la Plata basin concentrate the highest demographic density and industrial activities in the country, and this area is affected by increasing human pressure which

produces soil erosion and inputs of nutrients and untreated sewages to aquatic systems (Rodrigues Capítulo et al., 2010). Therefore, the aim of the present work was to evaluate antioxidant status, oxidative damage and energetic metabolism of *B. elegans* from sites under different environmental conditions. Differences between genders were also analyzed. Due to the fact that reports about the use of aquatic insects as a tool for environmental risk assessment are scarce, the present study enlarges the knowledge on the biomarker responses in these organisms from sites polluted with organic matter.

Materials and methods

Sampling sites

Two streams, both located in Buenos Aires province (Argentina) and tributaries of Río de la Plata river were selected for this study (Fig. 1). According with Labay et al. (2015), bioassessment generally relies on benchmarks ideally designed to represent pristine conditions which are difficult to be found today. In the Pampean area there are no lotic systems that could be considered pristine because land in our country has always been used in agriculture. Nevertheless, Carnaval stream has better ecological conditions than others in the region according to previous studies (López van Oosterom et al., 2015), so it could be considered as a reference site (RS). The selected site in this stream is situated in the middle section of the basin surrounded by wastelands. Agriculture (flower and fruit) is the main activity in the upper and middle section of the basin; however this stream is in good ecological conditions, so it was considered as reference site (RS). Rodriguez stream is strongly polluted with organic matter due to antropic input. The site selected in this stream (OMS) receives some clandestine discharges of organic matter. One of them is an effluent derived from the meat packing company. Another one is an artificial pond in a country club and the others represent activities of horticulture and extensive cattle rising (Bauer et al. 2002; Remes Lenicov et al. 2005; López Van Oosterom et al., 2015).

Physicochemical parameters

In spring-summer (December-2012 to March-2013) water samples from each site were monthly tested to determine dissolved oxygen (DO mg l^{-1}), temperature (°C), pH, conductivity (μS cm^{-1}), and turbidity (NTU) with a portable water quality checker (HORIBA U-10). Also, other surface water

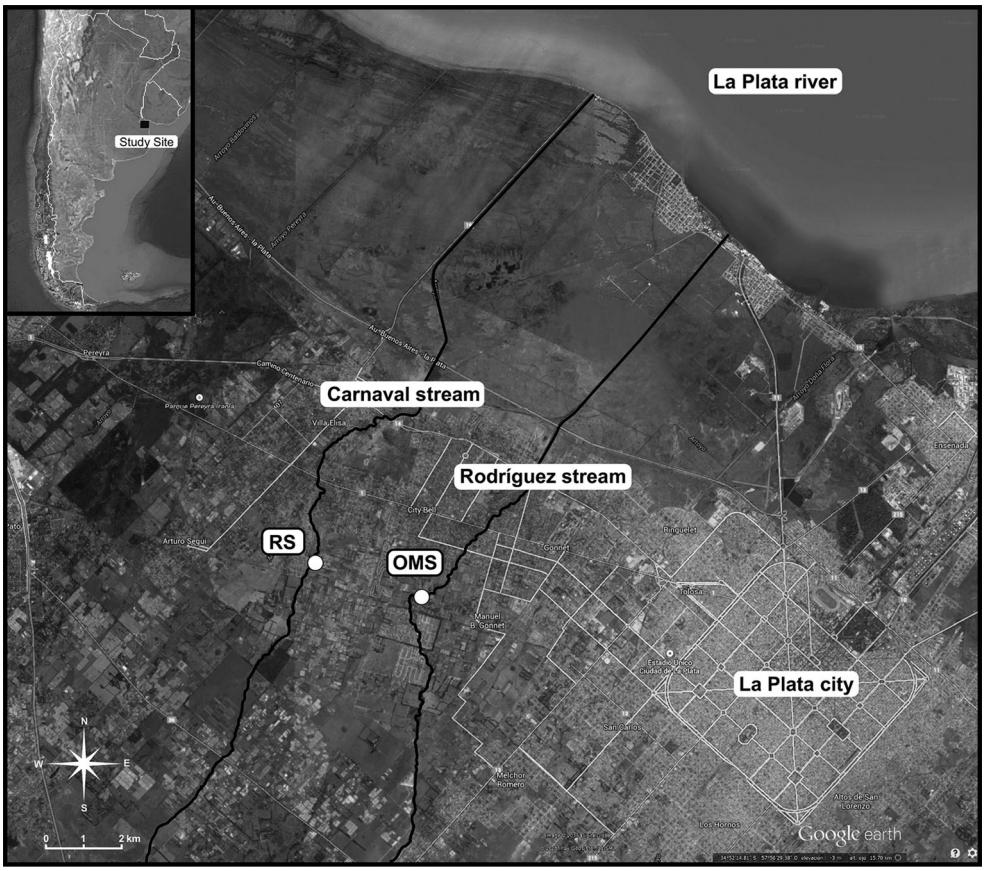

Fig. 1 Map of the study area showing the two sampling sites in Carnaval (RS) (34°53′30.73″S; 58° 5′30.44″W) and Rodríguez (OMS) (34°53′57.39″S; 58° 3′ 39.58″W) streams. Source: Image from Google Earth

samples were collected and taken to the laboratory for analysis of chemical oxygen demand (COD, mg l^{-1}), N-NH_4^+ (mg l^{-1}), N-NO_2 (mg l^{-1}), N-NO_3 1(mg l^{-1}), and P-PO_4^- (mg l^{-1}) over a period not exceeding 4 h. Furthermore, sediment samples were taken from the sites to determine the percentage of organic matter by weight loss after ignition at 500 °C for 4 h. All the analyses were performed following APHA procedures (1998).

During the last sampling period, sediment samples from RS and OMS were collected for chemical analysis. Sediment samples were kept in a cooler at 4 °C until they were taken to the laboratory. Analysis of metal content (Cd, Cu, Cr, Ni, Pb and Zn) was done by atomic absorption spectrophotometry (direct flame) following acid digestion of samples according to method 3050 (USEPA, 1996). Chemicals for sample treatments or analysis of major matrix components were analytical grade. Metal standards were purchased from AccuStandard, Inc. (1000 mg l^{-1} standard stock solutions).

Pesticides in sediments were extracted from wet samples by a solid: liquid sonication system according to method 3540 (USEPA, 1996) and analyzed by GC-ECD (Carlo Erba, 6000) by method 6630C (APHA, 1998). The pesticides analyzed were: Aldrin, p,p'-DDD, o,p'-DDE, p,p'-DDE, o,p'-DDT, p,p'-DDT, dieldrin, ß-endosulfan, endrin, heptachlor, heptachlor epoxide, a-BHC, b-BHC, g-BHC, methoxychlor, deltametrhrin, cyhalothrin-lambda, cypermethrin. Registered values were compared with the Canadian Environmental-Quality Guidelines (CCME, 2003) since no guideline levels exist for these pesticides in the benthic sediments of the area studied (Di Marzio et al. 2010).

Sample collection

Adults of *B. elegans* from RS and OMS were collected using sieves (500 μm) in selected sampling sites at the same time that water and sediment samples were taken. The sampling period (December to March) was chosen due to the increase in population and metabolic rate of insects during the summer season. About 120 water bugs from each site were taken to the laboratory and identified at species level using a stereomicroscope (Schnack, 1973). The specimens were then selected based on their weight and length (body weight: 0.22 ± 0.04 g RS, 0.22 ± 0.04 g OMS; total length: 20.86 ± 1.14 mm RS; 20.84 ± 1.19 mm OMS; media ± SD $N = 60$). The observation of the genital capsule using stereomicroscope allowed the differentiation between males and females (Schnack et al. 1989). Ovigerous females were not processed. Then, insects were anesthetized on ice for 10 min and stored at −20 °C until used for biochemical determinations.

Analyses of biomarkers

Measurements of glucose metabolism

Sample preparation Soft tissue of the whole organism was weighed, homogenized in glass potter with teflon plunger, (1:5 *w/v*) in 0.154 M KCl with 0.5 mM phenylmethylsulfonyl fluoride -PMSF- and 0.2 mM benzamidine as protease inhibitors, and centrifuged at 20,000 x*g* at 4 °C for 15 min. Supernatants were immediately used to determine the levels of glycogen and lactate, LDH and PYK activities.

Glycogen Glycogen was extracted by the method of Van Handel (1965) using 30 % KOH (1:10 w/v). Samples were placed in water bath at 100 °C for 2 h, and then glycogen was precipitated with saturated Na_2SO_4. Samples were centrifuged at 10,000 x*g* at 4 °C for 10 min, the supernatant was discarded and the pellet was resuspended in distilled water for subsequent treatment. The first treatment consisted in acid hydrolysis by 4 N HCl and the other one consisted in neutralization with Na_2CO_3. Finally, glucose was quantified by means of the glucose oxidase method using commercial kits (Wiener Laboratories, Rosario, Argentina).

Lactate concentration was measured in homogenates using commercial kits (Wiener Laboratories, Rosario, Argentina). Lactate of the sample was oxidized by lactate oxidase, and then hydrogen peroxide formed in this reaction was used as substrate by peroxidase enzyme to oxide 4-aminoantipyrine (Martí et al. 1997). Lactate content was calculated on the peak absorbance (540–550 nm) using an absorption coefficient of 35,330 M^{-1} cm^{-1}. Results were expressed as mmol lactate per mg of wet weight.

Enzimatic activity LDH activity was measured by the method of Schiedek (1997), monitoring NADH decay at 340 nm. Results were expressed as units of LDH per mg of proteins. One LDH enzymatic unit was defined as the amount of enzyme needed to catalyze the reduction of 1 μmol of piruvate per minute. PYK was assessed following the method of Bücher and Pfleiderer (1955) combined with the method of Reitman and Frankel (1957). The assay mixture contained 50 mM buffer Tris-HCl pH 7.5, 0.23 mM ADP, 8 mM MgSO4, 75 mM KCl and 0.2 mM phosphoenolpyruvate. After 20 min of incubation at room temperature, the reaction was stopped by addition of 0.78 mM DNPH. After 20 min-incubation at room temperature, 2.7 M NaOH was added. Absorbance of the pyruvate-DNPH complex was measured at 490 nm. Acid pyruvate was used as standard in a calibration curve. Results were expressed as units of PyK per mg of proteins. One PyK enzymatic unit was defined as the amount of enzyme that catalyzes the formation of 1 μmol of pyruvate per hour.

Lipid extraction and analysis

For lipid analysis, the whole organisms (pooled two individuals) were weighed and then homogenenized using an Ultraturrax tissue disrupter (Janke and Kunkel, Ika Werk, Germany). Lipids were extracted from chloroform/methanol mixture following the method of Folch et al. (1957). All chemicals used were of analytical grade.

Total lipid concentration was determined gravimetrically in an aliquot and the rest was kept in chloroform/methanol 1:2 under N_2 atmosphere at −20 °C until further analysis for lipid classes and fatty acid composition.

Lipid class analysis was performed by thin layer chromatography (TLC) on silica gel Chromarods (type S-III) with quantization by flame ionization detection (FID) using an Iatroscan TH-10, Mark III, (Iatron Laboratories Inc., Tokyo, Japan) as described by Parrish and Ackman (1985). The separation was conducted by a sequence of two different solvent systems according to Lavarías et al. (2005). The first development was carried out in benzene/chloroform/formic acid (70:25:1) for 45 min. Chromarods were dried, partially scanned to analyze neutral lipids (NL). Then, Chromarods were developed in chloroform/methanol/water (70:25:3) for 60 min and completely scanned to reveal the different polar lipids (PL). Monoacylglycerol was used as an internal standard and quantitative analysis was performed by calibration curves of authentic standards (Sigma-Aldrich S.A.) run under the same conditions.

Fatty acid analysis fatty acids were derivatized using BF_3/methanol mixture following the procedure described by Morrison and Smith (1964). Fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) on an Omegawax 250 (30 m × 0.25 mm, 0.25 μm film) (Supelco, Bellefonte, PA) capillary column in a Hewlett Packard HP-6890, equipped with FID. The column temperature was programmed for a linear increase of 3 °C/min from 175 °C to 230 °C. Fatty acids were identified by comparison of their retention times with a FAME mixture previously identified under the same running conditions.

Oxidative stress parameter measurements

Preparation of total homogenate Each frozen insect was dissected and soft tissues were weighed and homogenized (1:9 *w*/*v*) in 125 mM Tris-base cold buffer solution, pH 6.8 containing 1 mM 2-mercaptoethanol and 0.1 mM PMSF (Vijayavel et al. 2004). Homogenates were centrifuged at 10,000 x*g* at 4 °C for 10 min (Eppendorf 5430R) and the supernatant was used for determining GST and antioxidant enzyme activities as well as LPO, OP and GSH levels. Total protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Antioxidant enzyme analyses SOD activity was determined according to Marklund and Marklund (1974). This method is based on 50 % inhibition of pirogallol auto-oxidation (26 mM, pH 2). The reaction was carried out in 50 mM Tris-cacodilate buffer (pH 8.8), and the absorbance was measured at 420 nm. Results were expressed as units of SOD per mg of proteins. One SOD unit was defined as the amount of enzyme necessary to inhibit 50 % of autocatalytic pyrogallol oxidation min^{-1}, measured at 420 nm. CAT activity was determined by following the decomposition of H_2O_2 espectrophotometrically at 240 nm, in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7) and 10 mM H_2O_2 (Aebi, 1984). Results were expressed as pmol CAT per mg of proteins. One CAT unit was the amount of enzyme required to catalyze 1 pmol of H_2O_2 min^{-1}.

Antitoxic defense GST activity was assayed as described by Habig et al. (1974) using 1-chloro-2,4- dinitrobenzene (CDNB) as substrate; the final reaction mixture contained 1 mM CDNB and 1 mM GSH. Results were expressed as units of GST per mg of proteins. One GST unit represented the amount of enzyme required to conjugate GSH with 1 μmol of 1-chloro-2,4-dinitro-benzene min^{-1} determined at 340 nm.

Non-enzymatic defense GSH levels were measured following Anderson procedure (1985) with some modifications. Briefly, the sample was acidified with 10 % sulfosalicylic acid. After centrifugation at 8000 x*g* for 10 min, supernatant (acid-soluble GSH) aliquots were mixed with 6 mM 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) in 0.143 M buffer sodium sulfate (pH 7.5), which contained 6.3 mM EDTA. Absorbance at 412 nm was measured after 30 min incubation at room temperature. GSH was used as standard to calculate the concentration expressed as nmol thiols (GSH equivalents) per g of wet weight.

Oxidative damage LPO level was measured according to Buege and Aust (1978) using the formation of thiobarbituric acid-reactive substances (TBARs). Homogenates were added to the reaction mixture (trichloroacetic acid 15 % (*w*/*v*), 2-thiobarbituric acid 0.375 % (w/v), and butylhydroxytoluene 0.147 mM) at a ratio of 1:20 (*v*/v). The mixture was vigorously shaken, maintained in boiling water for 60 min, and immediately cooled at 4 °C for 5 min (Ohkawa et al. 1979). Then, it was centrifuged at 5000 x*g* for 10 min, and the supernatant measured spectrophotometrically at 535 nm. LPO was expressed as nmol TBARs complexes per mg of wet weight. Protein oxidation measured as carbonyl content was quantified as described by Reznick and Packer (1994). Homogenates were incubated with 10 % streptomycin sulfate

at room temperature for 15 min to eliminate DNA debris. Each sample of extracted proteins was separated in two parts. One of them was treated with 10 mM DNPH in 2.5 M HCl, and the other with 2.5 M HCl. The tubes were left for 1 h of incubation at room temperature in the dark. Then, 20 % TCA (w/v) was added in both tubes, left in ice for 15 min and centrifuged at 6000 x*g* to collect the protein precipitates. Precipitates were dissolved in 6 M guanidine hydrochloride and left at 37 °C for 10 min. Carbonyl concentration was calculated from the peak absorbance (355–390 nm) using pyruvate as standard. Results were expressed as μg carbonyl per mg proteins.

Statistical analysis

Statistical comparisons of physicochemical parameters and biomarkers in each month were carried out using one-way ANOVA after checking for normality and homogeneity of variance. A post hoc Tukey test was applied. Analyses were performed using Instat v. 3.01.

A multivariate analysis was performed to examine the arrangement of the sampling sites based on the previously standardized physicochemical parameters (ln n + 1). An indirect method, the Principal Component Analysis (PCA) was selected due to the fact that the gradient length was lower than 2 units of the standard deviation. Environmental variables were automatically excluded from the analysis if the variance inflation factor was greater than 10. Significant relationships were tested by Monte Carlo test using 199 permutations (Ter Braak and Smilauer 2002).

Results

Site characterization

Physical and chemical variables of the sampling sites are shown in Table 1. From all parameters analyzed, only phosphate, nitrite, COD, conductivity and OM levels in OMS were higher than those in RS, showing significant differences between them, while DO showed the highest values in RS compared to OMS.

PCA showed the characterization of sampling sites based on registered physicochemical parameters (Fig. 2). The first axis established an organic pollution gradient, being the principal variables COD, conductivity, P-PO4 and OM toward the positive side of the axis and DO, pH and ORP toward the negative extreme. The first two axes account for 89 % of the total cumulative variance. RS was generally associated with higher values of DO and pH, while OMS corresponded to the highest concentrations of nutrients, conductivity and COD for all sampling dates. In the case of sampling corresponding to February 2013 in RS, the displacement toward axis 2 was associated with high turbidity (while the values of the other parameters were similar to the rest of samplings).

The concentrations (mg kg^{-1}) of Zn, Ni, Cr, Cd, Cu, and Pb in the sediment of the RS and OMS sites were lower than the maxima indicated in the Canadian guidelines. Those values were for RS Zn: 62.0; Ni: 16.5; Cr: 26.1; Cd: 0.26; Cu: 19.767; Pb: 25.3 being for OMS: Zn: 69.7; Ni: 13.4; Cr: 20.0; Cd: 0.22; Cu: 18.1; Pb: 23.7. Respect to pesticide values, only ß-endosulfan was detected in the sediment samples, being lower in RS (0.015 mg kg^{-1}) than in OMS (0.100 mg kg^{-1}). There are no reference values for this compound in the Canadian guidelines.

Biomarker responses

The relationship between wet weight and total length of insects *B. elegans* collected at both sampling sites was examined. Although there was a positive correlation between whole body weight and size of insects ($y = 1.9676\times - 7.4861$ r^2: 0.4341 for RS; $y = 2.5798\times - 9.3589$ r^2: 0.4991 for OMS), no significant differences between organisms collected at both sites were observed.

Concerning biochemical parameters related to carbohydrate metabolism, PyK activity was significantly lower in insects collected in OMS in December compared to those of RS ($p < 0.05$), but in subsequent months significant differences were not observed between insects from both sites (Fig. 3A). Either LDH activities (range of values 0.54–3.55 U. mg^{-1} protein) or glycogen content (range of values 1.36–4.33 mg. g^{-1} wet wt) were not affected in organisms from OMS compared to those from RS (Supplementary material Figs. 1A and B, respectively). In all these parameters (PyK, LDH and Glycogen levels) no differences between sexes in each site were observed. However, as shown in Fig. 3B, lactate content was greater in males than in females ($p < 0.0006$) during the first three months of study in both sites when reproductive activity was higher. The differences were up to 56 and 61.7 % for males from RS and OMS with respect to females, respectively. It was observed that there were many males carrying eggs in the first three months compared to March (personal observation). Although no significant differences in lactate levels were observed between males or females between both sites at any period of sampling except in March when females from RS showed significant increase in lactate concentration compared to the rest of the insects collected in the same month ($p < 0.003$) (Fig. 3B).

In each sampling month it was observed that total lipid content in male insects from RS was significantly higher than in insects from OMS ($p < 0.002$) (Fig. 4). In general, lipid content of female insects was lower than that of males ($p < 0.002$), but differences among females from both sites were observed only in December and March (Fig. 4).

Table 1 Physicochemical characteristics of the reference and polluted streams

Physicochemical parameters	December/ 2012		January/ 2013		February/ 2013		March/ 2013	
	RS	OMS	RS	OMS	RS	OMS	RS	OMS
Temperature (°C)	25.62 ± 0.53*	24.37 ± 0.06*	28.13 ± 1.26	26.16 ± 0.18	31.69 ± 0.81	28.84 ± 0.12	17.39 ± 0.5**	19.59 ± 0.03**
pH	9.66 ± 0.16**	8.09 ± 0.05**	8.86 ± 0.18**	8.22 ± 0.04**	8.95 ± 0.2**	8.19 ± 0.11**	8.96 ± 0.26	8.03 ± 1.32
Conductivity (μS)	522 ± 9.85**	1579 ± 1.92**	301 ± 2.77**	1090 ± 0**	282 ± 60.39**	773 ± 1.84**	317 ± 9.45**	1055 ± 5.09**
DO (mgL^{-1})	10 ± 0.46**	1 ± 0.06**	8.6 ± 0.39**	3.3 ± 0.28**	8 ± 0.23**	2.8 ± 0.53**	8.4 ± 0.92**	3.1 ± 0.23**
$P\text{-}PO_4^{-}$ (mgL^{-1})	0.55 ± 0.3	2.92 ± 0.12	1.28 ± 0.04**	2.65 ± 0.04**	1.37 ± 0.007**	2.06 ± 0.03**	1.39 ± 0.004**	2.62 ± 0.03**
$N\text{-}NO_3^{-}$ (mgL^{-1})	0.12 ± 0.15	0.53 ± 0.03	0.007 ± 0.01	0.053 ± 0.04	0.07 ± 0.03	0.08 ± 0.01	0.003 ± 0.003	0.018 ± 0.01
$N\text{-}NO_2^{-}$ (mgL^{-1})	0.002 ± 0	0.128 ± 0.05	0.006 ± 0.003**	0.207 ± 0.008**	0.052 ± 0.001	0.042 ± 0.001	0.015 ± 0.003**	0.034 ± 0.004**
$N\text{-}NH_4^{+}$ (mgL^{-1})	0.074 ± 0.03**	0.761 ± 0.10**	0.01 ± 0.005**	1.45 ± 0.13**	0.04 ± 0.014**	2.52 ± 0.12**	0.01 ± 0.004**	0.84 ± 0.03**
NH_3 (mgL^{-1})	0.1 ± 0.019	0.05 ± 0.006	0.003 ± 0.002**	0.1 ± 0.012**	0.02 ± 0.0065**	0.3 ± 0.013**	0.003 ± 0.001**	0.03 ± 0.001**
COD (mgL^{-1})	16 ± 1**	124 ± 6**	26 ± 7**	59 ± 1**	54 ± 5**	71 ± 2**	48 ± 2*	202 ± 104*
Organic Matter (%)	4.5 ± 1.1*	28.5 ± 15.99*	3.5 ± 0.2**	44.9 ± 1.6**	4.9 ± 0.9**	10.8 ± 3.4**	3.3 ± 0.1**	13.8 ± 1.6**
ORP	132 ± 9.9**	59 ± 10.5**	149 ± 3.3*	129 ± 8.4*	140 ± 1.9	123 ± 27.0	178 ± 14.8**	111 ± 17.9**

Data are shown as mean + SD ($n = 3$). RS: reference site; OMS: site polluted with organic matter. Significant differences between sites of each month of sampling are indicated by * ($p < 0.02$) and ** ($p < 0.005$)

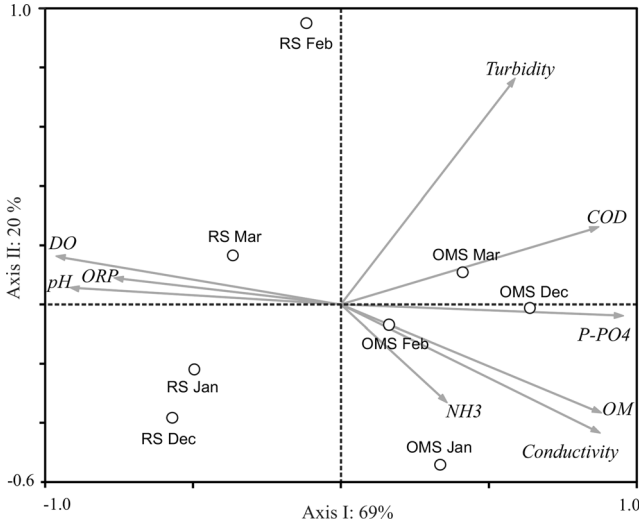

Fig. 2 Biplot of the sampling sites and environmental parameters in relation to the first ordination axes of PCA based on water quality parameters. RS: reference site; OMS: organic matter polluted site; dec: December; jan: January; feb: February; mar: March

The analysis of total lipid content of insects from different sampling months showed a significant decrease ($p < 0.0001$) at the same time as breeding season was finishing; this effect

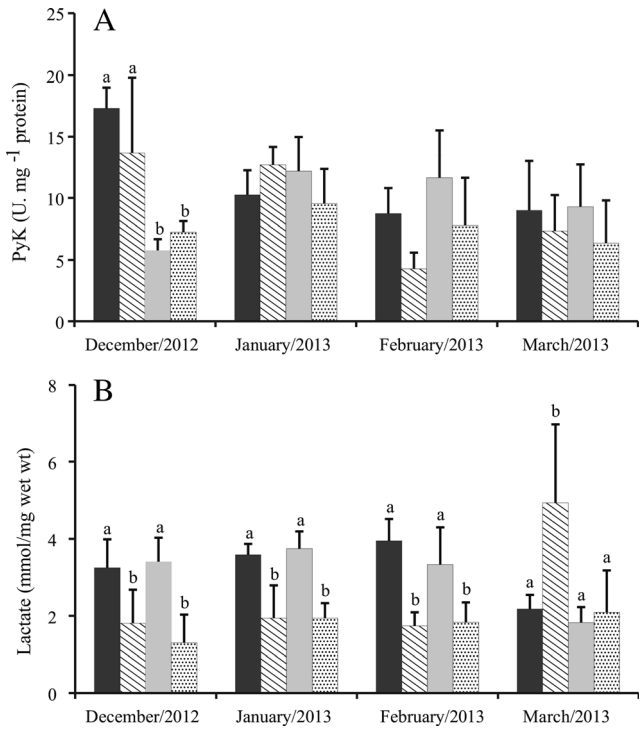

Fig. 3 Parameters of carbohydrate metabolism: PyK activity (**A**) and lactate levels (**B**) in females and males of *B. elegans* from reference site and site polluted with organic matter. Data are shown as mean + SD ($n = 5$ per sex and per site). Each sample corresponds to an individual organism. Significant differences among insects of each month of sampling ($p < 0.05$ (**A**); $p < 0.0006$ (**B**)) are indicated by different letters. RS: reference site; OMS: site polluted with organic matter; F: females; M: males. RS M RS F OMS M OMS F

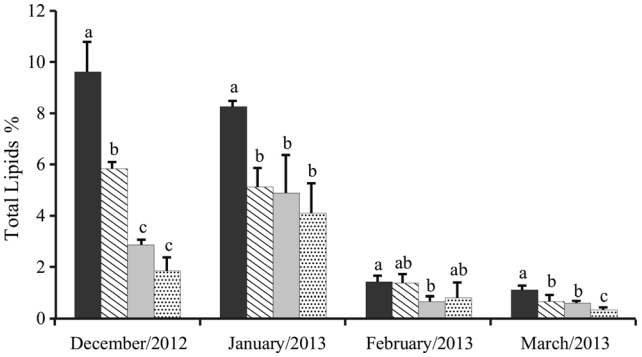

Fig. 4 Total lipid content in females and males of *B. elegans* from reference site and site polluted with organic matter. Data are shown as mean + SD (n = 5 per sex and per site). Each sample corresponds to two individuals pooled. Significant differences among insects of each month of sampling ($p < 0.002$) are indicated by different letters. RS: reference site; OMS: site polluted with organic matter; F: females; M: males. RS M RS F OMS M OMS F

was observed in both males and females from the two sites (Fig. 4). Lipid content of male insects from RS showed a decrease to 88.5 % from December to March while females showed a similar behavior evidencing a decrease to 88.6 % in the period studied. Although insects from OMS showed a significant increase in the lipid content in January (70.6 % for males and 122.7 % for females), the tendency to decrease the lipid content at the end of the breeding season showed a 79.3 and 81.9 % diminution in males and females, respectively.

Differences in the NL/PL ratio were observed ($p < 0.0001$). This ratio was found to be higher in RS than in OMS, due to a higher proportion of triacylglycerol (TAG), and a lower proportion of the PL phosphatidylethanolamine and phosphatidylcholine (PE, PC) (Table 2). A significant increase in TAG values was clearly observed in insects from RS compared to OMS ($p < 0.002$). Then, males and females also showed differences in the content of TAG in coincidence with the energy demand during the reproductive period (Table 2). The content of free fatty acids (FFA) and cholesterol (CHO) resulted significantly higher ($p < 0.002$) in males from OMS compared to RS, while females showed greater variation in the content of both lipid classes (Table 2). The proportion of diacylglycerol (DAG) presented significant differences between sites only in the first two months of sampling as shown in Table 2.

Concerning PLs, PC was the majority in all animals. PC and PE were significantly higher in insects from OMS compared to RS as mentioned above. In some months there were differences of lipid classes between males and females at each site ($p < 0.002$); however the values found in males accounted better for the differences between the two sites (Table 2).

Total FA composition from the water bugs showed significant differences between sites ($p < 0.05$) (Table 3). Proportions of saturated fatty acids (SAFA), particularly 16:0 and mono-unsaturated fatty acids (MUFA) 18:1 *n*-9 were

Table 2 Lipid composition in females and males of *B. elegans* from reference site and site polluted with organic matter

	December/2012				January/2013			
	RS		OMS		RS		OMS	
	M	F	M	F	M	F	M	F
TAG	58.01 ± 1.00^{a}	37.99 ± 1.14^{b}	24.86 ± 4.03^{c}	39.11 ± 0.07^{b}	51.92 ± 3.34^{a}	58.44 ± 2.00^{b}	39.85 ± 5.19^{c}	38.07 ± 1.76^{c}
FFA	1.31 ± 0.06^{a}	2.23 ± 1.27^{ab}	3.03 ± 0.16^{b}	2.34 ± 1.30^{ab}	1.41 ± 0.12^{a}	1.19 ± 0.11^{a}	2.18 ± 0.19^{b}	2.96 ± 0.32^{b}
CHO	1.39 ± 0.02^{a}	2.29 ± 0.06^{b}	2.12 ± 0.01^{c}	2.90 ± 0.06^{c}	1.48 ± 0.12^{a}	1.21 ± 0.13^{a}	2.28 ± 0.13^{b}	2.33 ± 0.13^{b}
DAG	3.41 ± 0.17^{a}	2.70 ± 0.06^{a}	6.46 ± 2.69^{b}	2.16 ± 0.10^{a}	1.62 ± 0.09^{a}	1.36 ± 0.15^{a}	2.46 ± 0.29^{b}	2.57 ± 0.13^{b}
PE	4.90 ± 0.13^{a}	5.65 ± 0.34^{a}	11.36 ± 2.68^{b}	8.55 ± 1.51^{b}	9.25 ± 2.05^{ab}	8.66 ± 0.45^{a}	12.02 ± 4.10^{b}	15.94 ± 3.29^{b}
PC	30.81 ± 0.01^{a}	45.89 ± 0.87^{b}	44.50 ± 11.33^{b}	44.74 ± 0.93^{b}	34.86 ± 1.12^{a}	28.81 ± 1.62^{b}	42.11 ± 7.76^{c}	35.72 ± 0.86^{a}
NL/PL	1.80 ± 0.03^{a}	0.88 ± 0.12^{b}	0.66 ± 0.08^{b}	0.87 ± 0.27^{b}	1.29 ± 0.16^{a}	1.66 ± 0.13^{a}	0.88 ± 0.21^{b}	0.89 ± 0.10^{b}

	February/2013				March/2013			
	RS		OMS		RS		OMS	
	M	F	M	F	M	F	M	F
TAG	54.72 ± 0.98^{a}	52.05 ± 1.09^{a}	39.01 ± 2.72^{b}	38.90 ± 1.85^{b}	48.64 ± 0.92^{a}	47.94 ± 3.35^{a}	35.82 ± 1.53^{b}	40.02 ± 1.71^{c}
FFA	2.96 ± 0.56^{a}	4.25 ± 0.80^{b}	7.62 ± 2.22^{c}	8.94 ± 6.28^{c}	11.65 ± 0.11^{a}	11.52 ± 2.58^{a}	15.41 ± 3.82^{b}	13.75 ± 3.89^{ba}
CHO	1.54 ± 0.05^{a}	1.57 ± 0.11^{a}	1.84 ± 0.15^{b}	1.87 ± 0.03^{b}	1.35 ± 0.11^{a}	1.34 ± 0.02^{a}	1.50 ± 0.05^{b}	1.41 ± 0.01^{c}
DAG	1.84 ± 0.03	1.67 ± 0.04	1.83 ± 0.16	1.86 ± 0.23	1.40 ± 0.14	1.39 ± 0.05	1.66 ± 0.10	1.55 ± 0.03
PE	13.29 ± 1.69^{a}	14.64 ± 1.02^{a}	19.18 ± 1.52^{b}	19.07 ± 3.03^{b}	15.15 ± 0.61^{a}	15.16 ± 0.43^{a}	18.30 ± 0.52^{b}	17.15 ± 0.56^{b}
PC	26.34 ± 0.21^{a}	25.23 ± 0.20^{a}	30.36 ± 2.94^{b}	28.80 ± 2.08^{b}	22.56 ± 1.50^{a}	22.82 ± 1.78^{a}	26.23 ± 1.03^{b}	24.94 ± 0.26^{b}
NL/PL	1.55 ± 0.07^{a}	1.49 ± 0.10^{a}	1.03 ± 0.19^{b}	1.09 ± 0.20^{b}	1.67 ± 0.06^{a}	1.64 ± 0.04^{a}	1.22 ± 0.10^{b}	1.35 ± 0.08^{b}

Data are shown as mean + SD (n = 5 per sex and per site). Each sample corresponds to two individuals pooled. CHO: cholesterol; DAG: diacylglycerol; FFA: free fatty acids; NL: neutral lipids; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PL: polar lipids; TAG: triacylglycerol. Significant differences among insects of each month of sampling ($p < 0.002$) are indicated by different letters. RS: reference site; OMS: site polluted with organic matter; F: females; M: males

Table 3 Major fatty acids of total lipids in females and males of *B. elegans* from reference site and site polluted with organic matter

% FA	December/2012				January/2013				February/2013				March/2013			
	RS		OMS		RS		OMS		RS		OMS		RS		OMS	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
14:00	2.04 ± 0.05	1.59 ± 0.18	2.39 ± 0.11	3.23 ± 0.28	1.96 ± 0.33	1.35 ± 0.28	1.32 ± 0.05	2.32 ± 0.21	1.99 ± 0.09	1.86 ± 0.56	1.86 ± 0.38	2.16 ± 0.73	1.11 ± 0.43	1.67 ± 0.62	2.13 ± 0.46	1.66 ± 0.27
16:00	**33.80 ± 0.37^{a}**	**31.04 ± 0.54^{ba}**	**21.22 ± 2.39^{c}**	**27.28 ± 4.65^{bc}**	**31.88 ± 0.15^{a}**	**31.15 ± 0.78^{a}**	**24.46 ± 3.31^{b}**	**27.59 ± 2.95^{ab}**	**32.16 ± 1.66^{a}**	**31.20 ± 0.62^{a}**	**24.33 ± 3.76^{b}**	**25.79 ± 9.88^{ba}**	**27.79 ± 0.90^{a}**	**23.13 ± 1.63^{b}**	**22.44 ± 3.07^{b}**	**17.09 ± 1.86^{b}**
16:1*n*- 7	7.13 ± 0.21^{a}	5.71 ± 1.24^{a}	11.14 ± 1.31^{b}	13.73 ± 1.09^{b}	7.02 ± 1.71^{a}	5.86 ± 0.23^{a}	10.31 ± 2.00^{b}	9.48 ± 1.63^{b}	6.35 ± 1.73	5.59 ± 0.83	7.33 ± 1.00	9.37 ± 2.42	5.12 ± 0.34^{a}	7.68 ± 2.61^{ba}	10.56 ± 1.72^{b}	10.09 ± 0.27^{b}
16:2*n*-4	0.50 ± 0.40	0.36 ± 0.10	0.63 ± 0.03	0.64 ± 0.07	0.80 ± 0.58	1.26 ± 0.27	0.44 ± 0.11	0.58 ± 0.17	0.53 ± 0.13	0.51 ± 0.12	0.42 ± 0.04	0.40 ± 0.08	0.54 ± 0.07	0.70 ± 0.08	0.53 ± 0.21	0.61 ± 0.02
16:3*n*-4	0.70 ± 0.10	0.80 ± 0.16	1.11 ± 0.07	1.00 ± 0.08	0.77 ± 0.15	0.85 ± 0.13	0.89 ± 0.22	0.88 ± 0.03	0.84 ± 0.17	0.87 ± 0.05	0.97 ± 0.14	0.89 ± 0.16	0.86 ± 0.23	1.24 ± 0.35	0.96 ± 0.26	1.19 ± 0.02
16:3*n*-3	0.58 ± 0.08	0.58 ± 0.06	0.76 ± 0.08	0.74 ± 0.03	0.64 ± 0.05	0.56 ± 0.12	0.82 ± 0.11	0.79 ± 0.12	0.62 ± 0.13	0.49 ± 0.01	0.63 ± 0.09	0.59 ± 0.07	0.70 ± 0.02	0.81 ± 0.02	0.68 ± 0.18	0.71 ± 0.07
18:00	7.10 ± 0.01^{a}	7.16 ± 0.07^{a}	10.03 ± 0.73^{b}	8.18 ± 0.95^{b}	7.11 ± 0.46	6.98 ± 0.15	8.56 ± 1.41	7.81 ± 0.80	8.99 ± 1.12	9.13 ± 0.14	10.07 ± 1.48	9.23 ± 2.27	9.35 ± 0.82^{a}	10.09 ± 0.26^{a}	9.08 ± 1.79^{a}	12.56 ± 0.51^{b}
18:1*n*-9	**30.70 ± 3.13^{a}**	**31.75 ± 1.01^{a}**	**20.71 ± 2.10^{b}**	**21.47 ± 4.61^{b}**	**29.40 ± 0.44^{a}**	**31.21 ± 1.44^{a}**	**24.84 ± 2.66^{b}**	**25.67 ± 0.22^{b}**	**29.49 ± 0.80^{a}**	**28.77 ± 3.2^{ab}**	**23.94 ± 3.14^{b}**	**23.86 ± 8.51^{ba}**	**31.44 ± 1.08^{a}**	**23.51 ± 3.17^{b}**	**22.19 ± 4.43^{b}**	**18.80 ± 0.86^{b}**
18:1*n*-7	2.36 ± 0.73	2.21 ± 0.61	3.21 ± 0.12	2.41 ± 0.09	3.42 ± 0.68	3.48 ± 0.39	2.82 ± 1.02	2.76 ± 0.18	2.51 ± 0.41	2.60 ± 0.12	2.73 ± 0.58	2.42 ± 0.35	2.54 ± 0.38	3.96 ± 1.29	2.76 ± 0.86	3.31 ± 0.28
18:2*n*-6	**6.31 ± 1.17^{a}**	**7.85 ± 0.65^{a}**	**17.81 ± 4.63^{b}**	**14.73 ± 5.23^{b}**	**6.01 ± 0.05^{a}**	**6.59 ± 0.82^{a}**	**16.67 ± 0.84^{b}**	**11.07 ± 0.61^{c}**	**6.62 ± 1.46^{a}**	**6.96 ± 0.30^{a}**	**15.15 ± 4.25^{b}**	**13.97 ± 9.68^{ba}**	**8.75 ± 0.11^{a}**	**10.92 ± 1.60^{a}**	**19.08 ± 4.12^{b}**	**24.92 ± 0.64^{b}**
18:3*n*-6	0.54 ± 0.01^{a}	0.57 ± 0.02^{a}	1.10 ± 0.14^{b}	0.74 ± 0.09^{b}	0.48 ± 0.02	0.48 ± 0.08	0.79 ± 0.26	0.79 ± 0.22	0.46 ± 0.01	0.53 ± 0.03	0.87 ± 0.24	0.79 ± 0.38	0.57 ± 0.01	0.68 ± 0.09	1.16 ± 0.65	1.50 ± 0.05
18:3*n*-3	3.48 ± 1.20	4.50 ± 0.81	3.56 ± 0.60	2.90 ± 0.47	4.05 ± 0.39	3.88 ± 0.19	3.27 ± 1.39	3.87 ± 0.68	3.74 ± 0.56	4.91 ± 0.46	5.90 ± 0.55	4.80 ± 1.22	4.37 ± 0.10	5.67 ± 1.71	3.32 ± 0.99	2.49 ± 0.28
18:4*n*-3	**0.33 ± 0.18^{a}**	**0.32 ± 0.03^{a}**	**0.74 ± 0.10^{b}**	**0.63 ± 0.44^{ba}**	**0.26 ± 0.04^{a}**	**0.31 ± 0.03^{b}**	**0.47 ± 0.17^{bc}**	**0.43 ± 0.03^{c}**	**0.24 ± 0.03^{a}**	**0.34 ± 0.10^{a}**	**0.84 ± 0.12^{b}**	**0.98 ± 0.84^{ba}**	**0.30 ± 0.05^{a}**	**0.36 ± 0.07^{ba}**	**0.59 ± 0.20^{b}**	**0.62 ± 0.14^{cb}**
20:0	0.26 ± 0.01^{a}	0.31 ± 0.01^{b}	0.46 ± 0.04^{c}	0.33 ± 0.03^{b}	0.22 ± 0.01	0.22 ± 0.03	0.33 ± 0.09	0.32 ± 0.06	0.30 ± 0.04	0.37 ± 0.02	0.43 ± 0.11	0.42 ± 0.15	0.37 ± 0.05	0.43 ± 0.11	0.36 ± 0.12	0.68 ± 0.01
20:3*n*-6	0.31 ± 0.25	0.33 ± 0.06	0.22 ± 0.03	0.13 ± 0.01	0.49 ± 0.47	0.85 ± 0.12	0.17 ± 0.06	0.37 ± 0.30	0.35 ± 0.05	0.39 ± 0.15	0.31 ± 0.21	0.26 ± 0.13	0.46 ± 0.07	0.47 ± 0.44	0.24 ± 0.11	0.38 ± 0.01
20:4*n*-6	0.37 ± 0.18	0.38 ± 0.09	0.33 ± 0.05	0.23 ± 0.04	0.43 ± 0.08	0.40 ± 0.22	0.47 ± 0.14	0.39 ± 0.10	0.35 ± 0.04	0.38 ± 0.11	0.33 ± 0.09	0.38 ± 0.04	0.24 ± 0.13	0.46 ± 0.23	0.29 ± 0.05	0.42 ± 0.11
20:5*n*-3	2.64 ± 1.02	3.14 ± 1.34	3.55 ± 1.14	1.34 ± 1.03	4.05 ± 1.23	4.20 ± 1.32	2.26 ± 0.96	3.82 ± 2.05	3.41 ± 0.71	3.46 ± 0.71	3.43 ± 0.42	3.10 ± 0.57	4.35 ± 0.12	6.45 ± 3.28	3.61 ± 0.69	3.75 ± 0.07
22:2*n*-6	2.32 ± 0.72	2.98 ± 0.11	3.12 ± 0.80	1.72 ± 0.12	2.64 ± 0.11	2.31 ± 0.63	2.87 ± 1.69	2.93 ± 0.21	2.51 ± 0.23	3.31 ± 0.63	2.40 ± 0.22	2.45 ± 0.14	2.76 ± 0.17	3.81 ± 0.44	2.07 ± 0.09	2.19 ± 0.37
SAFA	**42.94 ± 0.43^{a}**	**39.80 ± 0.98^{a}**	**33.65 ± 3.24^{b}**	**38.69 ± 2.96^{a}**	**40.94 ± 0.94^{a}**	**39.49 ± 0.65^{a}**	**34.33 ± 1.85^{b}**	**37.72 ± 2.37^{ba}**	**43.14 ± 2.07^{a}**	**42.19 ± 1.03^{a}**	**36.26 ± 2.58^{b}**	**37.18 ± 6.89^{b}**	**38.26 ± 0.35^{a}**	**34.90 ± 2.51^{b}**	**33.66 ± 1.52^{b}**	**31.31 ± 1.30^{b}**
MUFA	40.19 ± 4.07	39.67 ± 0.88	35.06 ± 3.53	37.61 ± 2.90	39.84 ± 1.48	40.54 ± 1.59	37.98 ± 3.64	37.91 ± 1.23	38.35 ± 1.81^{a}	36.95 ± 2.49^{ab}	34.00 ± 1.85^{b}	35.65 ± 5.74^{b}	39.10 ± 0.36^{a}	35.15 ± 0.73^{b}	35.51 ± 1.85^{b}	32.19 ± 0.31^{c}
PUFA	**16.86 ± 4.87^{a}**	**20.53 ± 1.20^{a}**	**31.29 ± 7.45^{b}**	**23.70 ± 5.87^{ba}**	**19.21a ± 2.42^{a}**	**19.97 ± 0.95^{a}**	**27.69 ± 5.49^{b}**	**24.37 ± 3.60^{ab}**	**18.51 ± 2.28^{a}**	**20.85 ± 1.45^{a}**	**29.74 ± 4.40^{b}**	**27.18 ± 12.62^{ba}**	**22.64 ± 0.01^{a}**	**29.95 ± 3.24^{ba}**	**30.83 ± 3.37^{b}**	**36.49 ± 1.61^{b}**
n6/n3	1.50 ± 0.53	1.42 ± 0.34	2.71 ± 0.95	3.13 ± 1.2	1.13 ± 0.12^{a}	1.21 ± 0.22^{a}	3.23 ± 0.80^{b}	1.80 ± 0.37^{a}	1.29 ± 0.06	1.26 ± 0.09	1.77 ± 0.39	1.81 ± 0.58	1.32 ± 0.03^{a}	1.23 ± 0.03^{a}	2.40 ± 1.14^{ba}	3.90 ± 0.13^{b}

Data are shown as mean + SD (n = 5 per sex and per site). Each sample corresponds to two individuals pooled. SAFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: polyunsaturated fatty acids. Significant differences among insects of each month of sampling ($p < 0.05$) are indicated by different letters. Those FA that showed significant differences in all sampled months were highlighted in bold. RS: reference site; OMS: site polluted with organic matter; F: females; M: males

higher in RS than in OMS insects ($p < 0.05$), so the proportion of polyunsaturated fatty acids (PUFA) was lower in insects from RS than that from OMS. The main PUFAs that significantly increased ($p < 0.05$) in insects from OMS were 18:2*n*-6 and 18:4*n*-3 (Table 3). The *n-6/n-3* ratio was always >1, it showed little variation between sites (Table 3). In general, there was no significant difference in FA composition between males and females.

Figure 5 shows the activities of antioxidant defense enzymes in water bugs from both sites. Males collected from RS showed a significant increase in SOD and CAT activities ($p < 0.004$) compared to the OMS ones (Figs. 5A and B, respectively). In some cases, females from both sites showed significant differences in SOD and CAT activities ($p < 0.05$) as well as when compared with males (Fig. 5).

Water bugs did not show significant differences in GSH levels (range of values 1.53–3.83 nmol. g^{-1} wet wt) and GST activity (range of values 65.79–183.06 mU. mg^{-1} protein) between sites and among males and females in the studied period (Supplementary material Figs. 2A and B, respectively). However, insects from OMS showed a slight trend to increase the GST activity from December to March (Supplementary material Fig. 2B).

Regarding to oxidative damage in insects from both sites, levels of lipid peroxidation measured as TBARs (range of values 21.5–172.7 nmol. g^{-1} wet wt) and damage to proteins measured as oxidative carbonyl groups (range of values 0.81–2.37 μg. mg^{-1} protein) showed no significant variation as well as no difference between males and females (Supplementary material Figs. 3A and B, respectively).

Discussion

Rodriguez stream presents low water quality compared to that of Carnaval stream. This situation was evidenced by low values of DO and high values of organic matter, nutrients, conductivity and COD, as well as the presence of β- endosulfan in OMS. Most physicochemical parameters analyzed helped to differentiate one site from another in relation to organic matter contamination. These results are supported by previous studies (López Van Oosterom et al., 2015). It is evident that water quality has decreased the enlargement of agriculture activity (Bauer et al. 2002; Rodrigues Capítulo et al. 2010). It means that agrochemicals are strongly used such as urea, ammonium sulfate, dibasic ammonium phosphate, ammonium nitrate, and potassium nitrate leading to eutrophication freshwater bodies (Hernandez and Gonzalez, 1993). For this reason, high nutrient concentrations could damage aquatic invertebrates as observed in *B. elegans*.

Regarding the potential use of macroinvertebrates as bioindicators, aquatic insects have been used to biomonitore stream and river ecosystems by several environmental stress types (Aura et al. 2011; Friberg et al. 2011; Li et al. 2010). When organisms are under environmental stress conditions, the first effects are at the subcellular level causing disruption of normal metabolic pathways (Ansaldo et al. 2006). In this context, energy balance of organisms can be affected by the additional energy required to restore and maintain homeostasis. These conditions lead to the disruption of the systems involved in the acquisition, conversion and conservation of energy (Sokolova et al. 2012). Glycogen level is one of the parameters that evidences the energetic status of organisms when they are under stress (Vasseur and Cossu-Leguille, 2003). It has been reported that insects *Chironomus anthracinus* (Hamburger et al. 1995) and *Chironomus riparius* (Choi et al. 2001) exposed to hypoxic conditions for short periods of time showed a decrease of glycogen content due to the fact that this is the only source of energy in anaerobic metabolism. In that metabolic state, lactate is accumulated as final product of glycolysis and glycogenolysis pathways. However, studies on progressive hypoxia revealed that respiration was otherwise unperturbed and lactate concentrations were unchanged in freshwater crayfish *Cherax destructor* (Morris et al. 2005). These insects showed no difference in glycogen and lactate content compared to the reference ones, demonstrating that this type of energy reserves is not affected by organic pollution. These results are coincident with those observed in *B. elegans* collected from chronically eutrophic site. Regarding biochemical glycolytic pathways, there are few studies on enzymatic response to hypoxia in invertebrates. In crustaceans *Lithodes santolla* (Paschke et al. 2010) and *Litopenaeus vannamei* (Soñanez-Organis et al. 2012) acute hypoxia induced an increased LDH activity indicating a raise of anaerobic metabolism. However, Dupont-Prinet et al. (2013) reported no increased activities of LDH and PyK in shrimp *Pandalus borealis* under hypoxia conditions as observed in *B. elegans.*

In insects, lipids are stored as TAG which serves as energetic reserve for reproduction, diapause, starvation, and long periods of flight (Arrese and Soulages 2010). Metabolic lipid activity could be higher in females than in males due to high energy demand for reproduction. Thus lipids are the primary source of energy for the developing embryo (Toprak et al. 2014). In crustacean *Gammarus roeseli,* energy reserves are influenced by season and gender (Gismondi et al. 2012). These authors observed that total lipid and glycogen contents are higher in females than in males during the reproductive period and, in both genders, energy reserves were higher in autumn and winter than in spring and summer. Although *B. elegans* were not analyzed during cold seasons, the total lipid content showed a progressive decrease toward the autumn demonstrating that insects tend to accumulate lipids during the reproductive period without any difference between males and females. As regards the effect of organic matter on energetic reserves in

invertebrates, Rocchetta et al. (2014) observed in clams *Diplodon chilensis* exposed to sewage water pollution that their glycogen reserve was increased, but they did not show significant differences in total lipid content compared to those collected in the clean area. *B. elegans* presented an opposite behavior to that observed in *D. chilensis* since their glycogen content was not changed but their total lipid content was decreased in insects from OMS. Anyway it is important to note feeding differences between both organisms; bivalves have filter capacity reducing nutrient loads (Sabatini et al., 2011) while water bugs are predators (Armúa de Reyes and Estévez 2006). Therefore, the high NL/PL ratio in insects from reference site as resulting from the high proportion of TAG, probably represents a great capacity to store lipids, as proposed by Pontes et al. (2008).

Although the main functions of FA are energy storage and structural components of membranes, they have very important functions in insects as precursors of waxes, pheromones, defensive secretions, and biosynthesis of eicosanoids, which are involved in reproduction (van Dooremalen and Ellers 2010). The SAFA/PUFA ratio remained unchanged in *B. elegans*, but it was higher in insects from reference site, probably to preserve FA from oxidative damage caused by high DO. Also, SAFAs could be stored in the TAG as energy reserve evidenced by a great proportion of palmitic acid. That FA was the most abundant in other freshwater invertebrates (Sushchik et al. 2003). The increase of PUFAs 18:2*n*-6 and 18:4*n*-3 observed in water bugs from OMS could be proposed as a response or an adaptive mechanism to increase their resistance and immunity under chronic exposure to urban discharges as suggested by Rocchetta et al. (2014). Also, it has been proposed that increased proportions of these FA, as C18-*n*3, are related to eutrophication processes (Desvilettes et al. 1994). Although no significant differences were observed in Σ MUFA between insects from both sites, oleic acid was higher in insects from RS than those from OMS ones. Oleic acid is the main MUFA in insects (Raksakantong et al. 2010) and it could also be stored as reserve in TAG of water bugs from reference site. The ratio of essential FA, *n*-6/*n*-3 remained stable in insects from RS, though those from OMS showed a great variation due a tendency to increase in both genders. These variations in FA composition might be due to the fact that aquatic insects are indirectly affected by nutrient loading due to diversity/species richness that generally decreases under eutrophic conditions (Whatley et al. 2014). This could be the main cause to explain the changes in FA composition in *B. elegans* since this variation would not be associated with lipid peroxidation as observed in *D. chilensis* (Rocchetta et al. 2014).

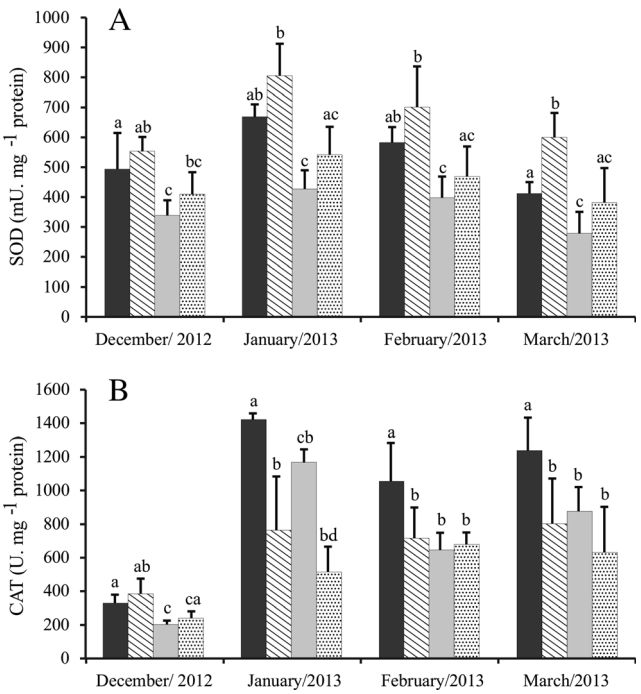

Fig. 5 Antioxidant enzymes activities Superoxide dismutase (A) and Catalase (B) in females and males of *B. elegans* from reference site and site polluted with organic matter. Data are shown as mean + SD (n = 5 per sex and per site). Each sample corresponds to an individual organism. Significant differences among insects of each month of sampling ($p < 0.004$) are indicated by different letters. RS: reference site; OMS: site polluted with organic matter; F: females; M: males.

Organisms have some sites of ROS formation such as the 'leaky' mitochondrial respiratory chain, P450 systems, soluble oxidases and autoxidation of some small molecules. The defense mechanism against ROS included key enzymatic players as SOD and CAT while GSH works as GST substrate and non-enzymatic antioxidant defense (Hermes-Lima and Zenteno-Savín 2002). Organisms are able to adapt themselves to some chronic situations of high exposure to ROS by increasing the expression of antioxidant enzymes.

Antioxidant defense has often been observed to be stimulated in hypoxia-resistant animals. In fish *Cyprinus carpio* (Lushchak et al. 2005) and *Percottus glenii* (Lushchak and Bagnyukova 2007) under hypoxic conditions, hypoxia increased the activities of SOD and CAT. Vidal et al. (2002) reported in the freshwater clam *Corbicula fluminea* that hypoxia increased the activities of CAT and glutathione peroxidase. However, in shrimps *Pandalus borealis* located in areas with low DO levels, Dupont-Prinet et al. (2013) observed a decrease of SOD activity in females and males while CAT activity remained stable, suggesting that this species could be particularly tolerant to hypoxia. Females and males of *B. elegans* showed a similar pattern as eutrophication causes a decrease in these antioxidant enzymes compared with insects from the site in normoxia conditions. On the other hand, hypoxia leads to oxidative stress in fish *Oryzias latipes* as evidenced by the increase of GST activity (Oehlers et al. 2007) while *B. elegans* did not show any changes in its activity during the period studied. It must be emphasized that GST

activity is essential not only for detoxification of xenobiotics but also for maintaining the normal physiological metabolism (Strange et al. 2000). Possibly, water bugs from OMS are adapted to low xenobiotic concentrations in this site. Therefore, these conditions did not induce GST activity, as they did not change GSH concentration.

On the other hand, GSH is commonly used as a biomarker of oxidative stress due to the fact that its decrease of concentration is usually associated to enhanced peroxidation processes in cell membrane. However, cells tend to maintain a constant level of GSH, so this biomarker could be considered not sufficiently sensitive for biomonitoring programs. (Viarengo et al. 2007).

Concerning gender differences, it has been reported that females of amphipod *Gammarus roeseli* have high GSH concentrations and lower cell damage than those of males (Gismondi et al. 2012). The authors proposed that these differences between genders could induce differential sensitivity in a contamination context, mainly during breeding period; thus GSH concentration could affect the population. However, *B. elegans* did not show any difference in GSH concentration and in the cell damage measured by TBARs and PO between genders during the reproductive period. These results could indicate that females and males of water bugs have similar sensitivity to environmental stress.

It is known that DO concentration puts a limit on aerobic metabolic rate in aquatic animals, this fact affects their survival. Some studies have shown that hypoxia modifies physiological performance by means of increased ventilation and heart rate, slow digestion, decreased food consumption, and reduced growth in invertebrates (Dupont-Prinet et al. 2013). To avoid anaerobiosis, organisms can lower the rate of respiration as a survival mechanism to endure severe degradation of water quality. This hypometabolic response could be energetically conservative, though it limits physiological processes and behavior as suggested by Morris et al. (2005). This metabolic situation could explain the low activity of antioxidant enzymes SOD and CAT observed in insects from eutrophic site. On the other hand, insects from reference site are exposed to higher DO which increases ROS generation due to an enhanced probability of electrons escaped from electron-transport chains to be combined with molecular oxygen (Lushchak 2011).

Concerning breathing, it must be noted that water bugs from Belostomatidae family are usually kept hold to aquatic vegetation by hind legs remaining plunged; emerging only to breathe atmosphere air using their caudal retractile appendices (Schnack 1976). On the other hand, thorax and abdomen show waterproof hair that allows maintaining an air film when the animal is plunged (Bachmann 1981). Such a bubble is in contact with water and could affect the physiology of the insects.

In parallel with the use of biochemical and physiological stress parameters as biomarkers of anthropogenic pollution, recently many authors suggested the detection of changes in behavioral traits as a useful diagnostic tool and a reliable early-warning biomarker (Amaral et al. 2009; Bartolini et al. 2009; Culbertson et al. 2007; Johnson and White 2009). For example, crayfish *Austropotamobius italicus* exposed to nitrate showed a mortality increase as a result of the decrease in their escape response and food consumption (Benítez-Mora et al. 2014). It is evident that food consumption is an important endpoint because a negative change in this behavioral response can decrease the survival chance of affected animals as suggested by authors. Therefore, it could be interesting to study the behavior of *B. elegans* in both characterized sites.

A particular feature of the Belostomatidae species is the reproductive behavior. The males incubate the eggs, which are attached on their dorsal surface by females during mating (Schnack et al. 1990). According to Munguía-Steyer and Macías-Ordóñez (2007), Belostomatidae males perform diverse parental care behavior including some body movement to increase oxygen diffusion into the eggs, maintaining optimal oxygen and humidity levels required for egg viability. Paternal care in these insects involves a cost in terms of feed efficiency, mobility, longevity and the brood pumping which may be an energy loss due to strong muscular activity (Crowl and Alexander 1989; Kight et al., 1995; Gilg and Kruse 2003). Although no significant difference in LDH activity was observed, its product lactate was the only parameter that showed a clear differential behavior between males and females of *B. elegans,* mainly during the reproductive peak. This period has been identified from August to February in this area (Schnack et al. 1981). Probably this fact is the result of energy loss produced by males fighting for females and parental care of the offspring as mentioned above.

Conclusions

In conclusion, the effects of organic matter in *B. elegans* were evident in several biomarkers. The most affected biological functions were the lipidic metabolism and antioxidant defenses. Therefore, these responses suggest a greater ability to store energy reserves as TAG by insects from the reference site compared to those ones from the eutrophic site due to their aerobic metabolic status which may be in better conditions. That situation was evidenced by the high activity of antioxidant enzymes due to elevated respiration rate attributable to high DO. Even though females showed significant differences in some biomarkers evaluated in comparison with males or from each other, males accounted better for the differences between the two sites in each month studied. These biological responses, mainly in males of *B. elegans*, could be useful to assess these biomarkers in organisms collected from sites with

similar characteristics to the ones described in this work. Therefore, lipidic composition and antioxidant systems could be used as a tool for monitoring organic matter contamination in freshwater environments.

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