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MODULATION OF IMMUNE AND ANTIOXIDANT RESPONSES BY AZINPHOS-METHYL IN THE FRESHWATER MUSSEL *DIPLODON CHILENSIS* CHALLENGED WITH *ESCHERICHIA COLI*

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Abstract: The aim of the present study was to characterize the immune response—total hemocyte number, cell type proportion, hemocyte viability, lysosomal membrane stability, phagocytic activity, cellular acid and alkaline phosphatase activity, and humoral bacteriolytic and phenoloxidase activity—in *Diplodon chilensis* exposed to 0.2 mg/L of azinphos-methyl (AZM), using *Escherichia coli* as immunological and pro-oxidant challenges. In addition, glutathione-S-transferase and lipid peroxidation thiobarbituric acid reactive substances were analyzed in gill tissue. Mussels from an unpolluted site were treated for 3 d as follows: 1) experimental control; 2) solvent effects control (acetone 0.01%); 3) bacterial challenge effects control (*E. coli*, 5 cells/mL $\times$ 10^4 cells/mL); 4) pesticide effects control (AZM in acetone); 5) control for combined effects of solvent and bacterial challenge; and 6) exposed to AZM, then challenged with *E. coli*. The results showed increased granulocyte proportion and phagocytic activity. Partial reversion of deleterious effects of *E. coli* on lysosomal membranes was observed in mussels exposed to AZM and then challenged with *E. coli*. Total hemocyte number and humoral bacteriolytic activity were increased only by *E. coli* challenge. Acid phosphatase activity was increased by both *E. coli* and AZM, whereas the stimulating effect of *E. coli* on alkaline phosphatase activity was negatively modulated by AZM. Azinphos-methyl inhibited phenoloxidase activity regardless of the *E. coli* challenge. Gill glutathione-S-transferase activity was increased by *E. coli* treatment either alone or pretreated with acetone or AZM and by AZM alone. Thiobarbituric acid reactive substance levels were reduced by AZM alone or combined with the *E. coli* challenge and by acetone followed by the *E. coli* challenge. Both acetone and AZM seem to be important modulators of immune and antioxidant responses in *D. chilensis*. *Environ Toxicol Chem* 2016;9999:1–10. © 2016 SETAC

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INTRODUCTION

Pesticides used in agricultural practices are often applied by ground-based spraying equipment. This methodology favors these toxicants being carried by the rain and drainage water to nearby water bodies [1]. Azinphos-methyl (AZM; phosphorodithioic acid, O,O-dimethyl S-[(4-oxo-1,2,3-benzotriazin-3 [4H]-yl) methyl] ester) is among the most persistent of the organophosphorus insecticides in water, with a half-life of almost 1 mo in aquatic environments [2]. This insecticide has been used for pest control in food crops in several parts of the world, and concentrations ranging from 4×10^{-5} mg/L to 14.9 mg/L have been reported in runoff and river surface water adjacent to agricultural fields (Table 1). In the North Patagonian region of Argentina (Valley of Río Negro and Neuquén), AZM is one of the main insecticides intensively applied in fruit production. In the Río Negro, surface water concentration varies seasonally from 4.6×10^{-5} mg/L to 0.022 mg/L [1,3]. However, Rosenbaum et al. [4] highlighted that in this region AZM had been sprayed at a recommended concentration of 400 mg/L, which means that animals living on river banks near the discharge of irrigation channels could be exposed to higher pesticide concentrations than those measured in the river. Increasing concentrations of pesticides reaching aquatic environments represent a risk for human and environmental health [5,6] and deserve further attention.

The main toxicity mechanism of organophosphorus pesticides consists in the overstimulation of neural transmission through the accumulation of acetylcholine in synapses, which is caused by the inhibition of specific serine esterases (acetylcholinesterase, carboxylesterases). Consequently, not only pest insects but nontarget species such as freshwater gastropods [7] and amphipods [8] may suffer loss of movement or paralysis and eventual death. However, before neurotoxic or lethal consequences are evident, sublethal effects are frequently monitored as early warning signals after pesticide exposure. Because other enzymes with serine residues (hydrolases) are linked to several immune functions [9], altered immune responses are expected to occur after pesticide exposure. Therefore, concern has grown to elucidate how pesticides may threaten immune competency and may also cause oxidative stress both in vertebrate and invertebrate organisms exposed to nonlethal concentrations [6,10]. In particular, bivalve immune response is a broad spectrum defense system that involves cellular and humoral components that recognize structural motifs on pathogen surfaces (pathogen-associated molecular patterns) [11]. Thus pathogen-associated molecular pattern recognition is exerted by humoral and hemocyte membrane receptors, triggering mechanisms of foreign particles elimination, such as phagocytosis, cellular aggregation and encapsulation, and reactive oxygen species production (ROS) [11,12]. In addition, enzymatic components such as acid phosphatase, alkaline phosphatase, and lysozymes participate in intracellular and/or extracellular degradation processes [13], whereas phenoloxidase plays a central role in encapsulation/melanization [14]. Both stimulation and suppression of immunological

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Table 1. Azinphos-methyl (AZM) concentrations measured in freshwater environments[a]

Sample	AZM (mg/L)	References
Lourens River	$4.0 \times 10^{-5} \pm 1.0 \times 10^{-5}$	Schulz [56], Thiere and Schulz [57]
Haraz River	$1.5 \times 10^{-3} \pm 7.6 \times 10^{-4}$	Nasrabadi et al. [58]
Unnamed tidal tributary of the North Edisto River	$7.0 \times 10^{-3} \pm$ ND	Scott et al. [59], Fulton et al. [60]
Runoff water	$0.022 \pm$ ND	Granovsky et al. [61]
Runoff water	0.261 ± 0.155	Southwick et al. [62]
Runoff water	0.417 ± 0.136	Smith et al. [63]
Qarasu River	14.60 ± 10.60	Shayeghi et al. [64]
Gorgan River	14.90 ± 11.70	Shayeghi et al. [64]

[a] Data published as mean ± standard error [56,57,61,62] or mean ± standard deviation [58–60,63,64].
ND = no data.

response variables by pesticides have been reported for the freshwater prawn *Macrobrachium rosenbergii* exposed in vivo to the organophosphorus pesticide trichlorfon (0 mg/L, 0.2 mg/L, and 0.4 mg/L during 3 h, 6 h, 12 h, and 24 h) [15] and for the blue mussel *Mytilus edulis* exposed to the organophosphorus pesticide azamethiphos (0.1 mg/L for 24 h) [16]. These effects have been associated with increased susceptibility toward pathogen infection. Similar effects have been described for pesticides with different chemical nature, such as atrazine, hexachlorobenzene, glyphosate in snails [17,18], and lindane in sea urchins [19]. However, experiments combining exposure to pesticides and subsequent bacteriological challenge have only been performed in the oyster *Crassostrea gigas* [20]. In the latter work, mortality was increased and the expression of 10 out of 19 genes involved in hemocyte functions was upregulated in oysters exposed to the pesticide and then challenged with bacteria.

The gills are in direct contact with foreign particles, microorganisms, and toxicants through their filter-feeding activity [21]. Consequently, the gill epithelium and the hemolymph beneath may be continuously challenged by microbial and/or agrochemical toxic compounds, which could lead to increased production of ROS. This increase may be associated with enhanced cellular immune response and/or to an oxidative stress condition [22,23].

Organophosphorus pesticides and their metabolites may be detoxified by several enzymes including phase II biotransformation enzymes such as glutathione-S-transferase (GST), which conjugates reduced glutathione with toxic compounds, facilitating their excretion (reviewed by Sanchez-Hernandez et al. [24]). However, several studies describing biochemical effects of organophosphorus pesticides and other kinds of pesticides on aquatic organisms have reported decreased [25], increased [26], and unchanged GST activity [27,28]. In bivalves, organophosphorus pesticides can cause cell death and damage to subcellular structures of hemocytes, such as lysosomal membranes [16,29,30]; tissue damage and lipid peroxidation in gills have also been reported in freshwater mussels [31]. These authors suggest that deleterious consequences on hemocytes and tissues are related to oxidative stress and/or cellular metabolism alterations [16,29–31].

To evaluate immunosuppressive effects of diverse chemical compounds, resistance to infections is evaluated using microbiological model agents, such as parasites and bacteria (reviewed by Fournier et al. [10]). *Diplodon chilensis* is a freshwater mussel, a native of Argentina and Chile, whose ecological importance involves community structure and nutrients' recirculation in aquatic environments [32]. Immunological and antioxidant responses of *D. chilensis* have been previously evaluated after short- and long-term challenge with *Escherichia coli* [33,34]. Although these responses have been shown to be modulated by a β-glucan-rich diet [34], immune and antioxidant modulations exerted by a toxic compound have not been evaluated before. Based on these antecedents, the aim of the present study was to assess whether 72-h pre-exposure to the pesticide AZM could affect the physiological status of the freshwater bivalve *D. chilensis*, with particular emphasis on immune and antioxidant responses after challenge with *E. coli*.

MATERIALS AND METHODS

Microorganisms

Escherichia coli JM109 strain was provided by the Department of Biological Chemistry, School of the Exact and Natural Sciences, University of Buenos Aires. Working under sterile conditions, fresh *E. coli* were grown in a nutritive agar medium (CM0003; OXOID) for 18 h to 24 h (at 37 °C) before the experiments. Cultured bacteria were inoculated in sterile saline solution (NaCl 0.9%; Merck) to obtain a suspension of 1.5×10^8 cells/mL, estimated as 0.5 on the McFarland scale (0.080–0.100 absorbance at 625 nm), and then diluted to obtain an appropriate working cell suspension.

Scenedesmus vacuolatus (Chlorophyceae, Chlorophyta) BAFC CA4 strain was obtained from the Culture Collection of the Laboratory of Phycology, Department of Biodiversity and Experimental Biology, School of the Exact and Natural Sciences, University of Buenos Aires. Algae cultures were grown in Bold's basal medium based on Sabatini et al. [35]. An initial cell density of 3×10^4 cells/mL was used as inoculum. Suspension cultures were maintained in 250-mL flasks containing 110 mL of the medium at 22 ± 1 °C with continuous cool-white fluorescent light illumination (80 mmol L^{-1} photons $m^{-2} s^{-1}$) and agitation in an orbital shaker. After 25 d, cells were lyophilized and stored at –20 °C for further use in bivalves' feeding.

Diplodon chilensis

Adult individuals ($n = 108$, 69.04 ± 0.65 mm shell length) were collected in December 2014 by scuba diving (1–2 m depth) from an unpolluted area in Paimún Lake (Patagonia, Argentina; 39°44.78′ S, 71°37.48′ W), a deep, oligotrophic glacial lake in Lanín National Park, where agricultural exploitation is prohibited by Argentine federal law. The estimated age of the collected mussels was 36 yr, according to von Bertalanffy growth curves and corresponding size-at-age data obtained by counting translucent growth bands in cross-sectioned shells of *D. chilensis* from the same population [36].

To minimize transportation stress, mussels were transported in plastic aquaria that contained coolers with water from the sampling site. Then bivalves were acclimated for 20 d in aquaria containing 10 L of aerated Chimehuin River water (Patagonia, Argentina; pH, 7.6; dissolved oxygen, 8.37 mg/L; alkalinity, 34 mg/L; conductivity, 36 μS/cm), 15 mussels per aquarium. During this period, mussels were fed with 0.133 mg of lyophilized *S. vacuolatus* (3×10^6 cells) every 2 d. Water was changed before feeding.

Experimental design

Mussels were placed in 1.5-L glass receptacles (2 individuals each), containing 1 L of aerated river water at 17 ± 1 °C and were fasted for 48 h to favor filtration rate. Then mussels were acclimated to these receptacles for 3 d. During the acclimation and experimental periods, each receptacle received 2.66×10^{-4} g of lyophilized *S. vacuolatus* (6×10^6 cells), every 2 d. Six groups were set, 5 of which were controls: 1) experimental control, aerated river water for 3 d (hereafter called "control"); 2) control for solvent effects (hereafter called "acetone"; 0.01% in water) for 3 d; 3) control for bacterial challenge effects (*E. coli*; 5×10^4 cells/mL) for 3 d; 4) control for pesticide effects (AZM), 0.2 mg/L of AZM (Pestanal 97.2% pure; Sigma–Aldrich), dissolved in acetone 0.01%, for 3 d; 5) control for combined effects of solvent and bacterial challenge, exposed to acetone for 3 d and then challenged with *E. coli* for 3 d (hereafter called "acetone + *E. coli*"); and 6) exposed to AZM for 3 d and then challenged with *E. coli* for 3 d (hereafter called "AZM + *E. coli*"). In all cases, water containing freshly added *E. coli*, acetone, or AZM, according to the experimental group, was changed every 24 h. The AZM and acetone concentrations used in the experiment were selected on the basis of in vivo laboratory [4] and in situ [37] studies. A second exposure was carried out using 0.1 mg/L of AZM to verify that the initial concentration of AZM in the experimental receptacles was maintained in between water changes. Samples ($n = 2$) were analyzed by gas chromatography at $t = 0$ h and $t = 24$ h. After liquid-liquid extraction with methylene chloride [38], the extract was injected into an Agilent 6890 GC with a nitrogen-phosphorus detector (HP-5 column) and was quantified by external standard with a detection limit of 0.02 μg/L. Mean AZM concentrations in the experimental receptacles were 0.0935 mg/L and 0.085 mg/L at 0 h and 24 h, respectively (Student's t test, $p > 0.05$). Thus, the remaining pesticide concentration after 24 h was 91.44%.

Sample processing

Because only 2 hematological variables in each group could be measured in a working day, 6 mussels (2 per flask, $n = 3$ for each treatment/variable, each dataset was the mean of 2 individual measurements) from control and treatment groups were exposed and then processed in the same order during 3 consecutive days. In the same way, 1 gill pair per flask was randomly selected at each processing day for gill oxidative balance analysis ($n = 9$ for each treatment).

Working on ice, shell length (mm) was measured and 2 mL of hemolymph were withdrawn from the anterior adductor muscle, using a sterile syringe. Hemolymph was aliquoted and placed in sterile microcentrifuge tubes for immediate analysis.

Mussels were then opened by adductor muscle incision. Gills were separated, weighed, and homogenized (Omni 1000 motorized homogenizer at 20 000 rpm) in a cold 100-mM sodium phosphate buffer, pH 7.0, 1:5 w/v, with a protease inhibitor (0.2-mM phenylmethylsulfonyl fluoride; Sigma). Gill homogenates were centrifuged for 15 min at 11 000 *g* at 4 °C, and supernatants were used for biochemical analysis.

Immune response

Total number of hemocytes. Cells from fresh hemolymph were counted on a Neubauer chamber in 4 replicates. The total number of hemocytes was expressed as cells/mL of hemolymph [39].

Hemocyte population. Hemolymph (50 μL) was smeared onto clean microscope slides, and cells were fixed by heating for 10 min at 26 ± 1 °C. Smears were covered with May–Grünwald stain (Biopack) for 6 min and then counterstained with Giemsa stain (Biopack) for 30 min. Slides then were carefully washed with distilled water and mounted. Hemocytes were observed under light microscopy (100–400×), and a minimum of 300 cells were counted in duplicate and identified according to Kuchel et al. [40] classification. Hyalinocytes were differentiated as cells without granules or containing few granules and with a round or irregular nucleus, whereas granulocytes were identified as cells with abundant granules in their cytoplasm and a round nucleus. Results were expressed as percentage of cells of each type with respect to the total number of hemocytes observed.

Phagocytosis. Hemolymph (100 μL) was mixed and incubated at room temperature for 30 min, with a volume of Congo-red (Fluka) stained yeast suspension, which contained twice the number of cells with respect to the number of viable hemocytes in the sample. After incubation, 50 μL of the sample were loaded onto clean microscope slides, and cells were fixed by heating for 10 min at 26 ± 1 °C. Cells were stained for 10 min with 10% Giemsa solution. Slides were carefully washed with distilled water and mounted, and a minimum of 300 cells were counted in duplicate under light microscopy (100–400×). Nuclei appeared blue, cytoplasm appeared light blue or violet (due to metachromasia), and yeast cells appeared black or red. Phagocytic activity was calculated as phagocytized yeast cells/viable hemocytes (modified from Kuchel et al. [40]). Hemocytes' viability was measured as explained in the section *Cytotoxicity*.

Cellular enzyme activity. Hemolymph (50 μL) was smeared onto clean microscope slides, and cells were fixed by heating for 10 min at 26 ± 1 °C. After fixation, the smears were stained, as described below, according to the cytochemical methods of Cima et al. [41]. Then slides were carefully washed with distilled water and mounted. Hemocytes were observed under light microscopy (100–400×) and at least 300 cells were counted in duplicate. Results were expressed as percentage of positive cells (with red reaction) with respect to total hemocyte number.

For acid phosphatase staining, hemolymph smears were incubated in a wet chamber (2 h at 37 °C) adding 400 μL naphthol AS-BI phosphate (2.5% in dimethylformamide; Sigma) as substrate, plus 400 μL of 0.4 g fuchsin (Fluka) in 2 mL of HCl 36% and 8 mL of distilled water, 400 μL of 4% $NaNO_2$ (Anedra) in distilled water, and 20 mL of sodium acetate buffer 0.1 M, pH 5.2. Results were expressed as percentage of positive cells (with red reaction) with respect to total hemocyte number. For alkaline phosphatase staining, the reaction mixture and the incubation time were similar to those used in the acid phosphatase method, except that sodium acetate buffer was replaced by Tris-HCl (Serva) buffer 0.1 M, pH 9.

Humoral response. Humoral enzymatic activities were measured according to Bianchi et al. [42]. Whole hemolymph

was centrifuged at 500 *g* for 20 min at room temperature. The cell-free supernatant was mixed with *E. coli* JM109 strain (0.5 on the McFarland scale), and bacteriolytic activity was measured by recording changes in absorbance at 625 nm for 5 min. Phenoloxidase activity was measured by recording changes in absorbance at 490 nm for 20 min after 4 h of incubation of cell-free supernatant with L-DOPA, Sigma (3 mg/mL in anticoagulant solution; 3 g/L glucose and 0.36 g/L trisodium citrate, 60 mOsm/L, pH 7). Negative and spontaneous substrate oxidation controls were performed. In both cases, 1 unit of activity was defined as a 0.001 change in absorbance. Results were expressed as milliliter of sample instead of protein concentration, because protein content in free-cell supernatant samples varied significantly among treatments, without a clear pattern.

Cytotoxicity

Hemocyte viability was measured by mixing 100 μL of hemolymph with 50 μL of Trypan blue (0.2%) dissolved in sterile anticoagulant solution (see the *Humoral response* section in *Materials and Methods*) and incubated for 5 min at 4 °C. After incubation, live and dead cells (undyed and dyed, respectively) were counted 4 times within 15 min, on a Neubauer chamber. Hemocyte viability was presented as the percentage of total hemocytes [42].

Lysosomal membrane stability of hemocytes was measured according to Bianchi et al. [42]. First, 50 μL of hemolymph were pipetted onto glass slides and allowed to adhere for 5 min in a wet chamber, at room temperature. Adhered cells then were stained with neutral-red (Fluka) solution (50 mL, 0.002% in dimethyl sulfoxide), and the number of red cells was counted under light microscopy every 10 min (in duplicate), until stained cells reached 50% of total cells. Results were expressed as neutral red retention time 50% (min).

Gill oxidative balance

Gill GST activity was measured using 10 μL of supernatant, 5 μL of 100-mM reduced glutathione (glutathione in phosphate-buffered saline; Sigma) and 5 μL of 1-chloro-2,4-dinitrobenzene (100 mM, in ethanol; Sigma) in a final volume of 1 mL phosphate-buffered saline, 100 mM, pH 6.5. The change in absorbance at 340 nm was followed for 5 min [43]. One unit of activity was defined as the amount of enzyme needed to catalyze the formation of 1 μmol of GS-DNB per min, at 25 °C. Lipid peroxidation was estimated by the thiobarbituric acid reactive substances (TBARS) method. Gill supernatant was mixed with thiobarbituric acid 0.37% (Sigma) solution in 50% trichloroacetic acid and incubated at 95 °C to 100 °C for 15 min. Then the mixture was cooled and centrifuged for 15 min at 11 000 g, at room temperature. Absorbance was read at 535 nm, and TBARS concentration was estimated using an extinction coefficient of 156 $mM^{-1}cm^{-1}$ [44]. Results were referred to milligrams of protein, which, according to our preliminary data, is the reference variable with lowest variability.

Statistical analysis

Data are presented as mean ± standard error. Normal distribution and homogeneity of variance were checked by Kolmogorov's and Bartlett's tests, respectively. Data were transformed by square root arcsine, when appropriate. Experimental results were tested by one-way ANOVA and Newman–Keuls post hoc comparisons. Significant differences were assumed when $p < 0.05$.

RESULTS

Immune response

Total and viable hemocyte counts. Total hemocyte number was significantly increased by *E. coli* challenge with respect to control mussels (Newman–Keuls, $p < 0.01$). Applied alone, AZM did not significantly affect the hemocytes' number and did not modulate the response against *E. coli* (no significant differences between AZM + *E. coli* and acetone + *E. coli*; Figure 1). Hemocyte viability ranged from 96.72 ± 0.47% to 98.37 ± 0.39%, with no significant variation among treatments (data not shown).

Hemocyte population. Hemocyte proportion significantly changed after experimental treatment (one-way ANOVA, $p < 0.001$ for both hyalinocytes and granulocytes). Hyalinocytes accounted for 88.9% to 95.5% of the identified hemocytes. The *E. coli* challenge had a slight negative effect on the proportion of these cells, which was reinforced by AZM pre-exposure (AZM + *E. coli*) causing a further decrease compared with all the control groups (Newman–Keuls, $p < 0.001$ for all comparisons; Figure 2A). Granulocytes' population ranged between 2.2% and 9.3%, 2-fold higher in AZM + *E. coli* (Newman–Keuls, $p < 0.001$) with respect to all control groups (Figure 2B).

We computed hyalinocytes' and granulocytes' numbers as the product of each cell type proportion by the total hemocytes' count of the same treatment. The estimated hyalinocyte number was 2-fold and 1.5-fold increased by both *E. coli* and AZM, respectively, compared with their corresponding controls (control and acetone; Newman–Keuls, $p < 0.0001$) but the response against *E. coli* was only slightly modulated by AZM (10% increase; Newman–Keuls, $p < 0.0001$). Granulocytes' number was also increased by *E. coli* and AZM, and the effect of *E. coli* was positively modulated by AZM; the granulocytes' number was more than 2-fold higher in AZM + *E. coli* than in any of the other groups (Newman–Keuls, $p < 0.0001$; Figure 3).

Phagocytic activity. Both hyalinocytes and granulocytes of *D. chilensis* were able to phagocytose Congo-red stained yeast, and this cellular function was affected by the applied treatments (one-way ANOVA, $p < 0.001$). Phagocytic activity of hyalinocytes increased in both the *E. coli* and AZM groups compared with the respective controls (Newman–Keuls, $p < 0.001$) but the effect of *E. coli* was not modified by pre-exposure to AZM (AZM + *E. coli* vs acetone + *E. coli*, $p > 0.05$; Figure 4A). In turn, granulocytes' phagocytic activity decreased in *E. coli*-challenged mussels with respect to control (Newman–Keuls, $p < 0.01$) but this effect was reversed in the AZM + *E. coli*

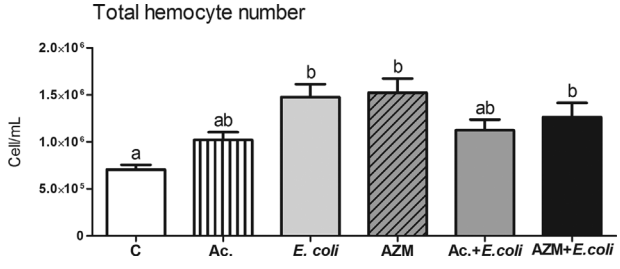

Figure 1. Total hemocyte number in hemolymph of *Diplodon chilensis* exposed to azinphos-methyl (AZM) and/or challenged with *Escherichia coli* (mean ± standard error; $n = 3$). Different letters denote significant differences among groups (Newman–Keuls, $p < 0.05$). C = control; Ac. = acetone (solvent control).

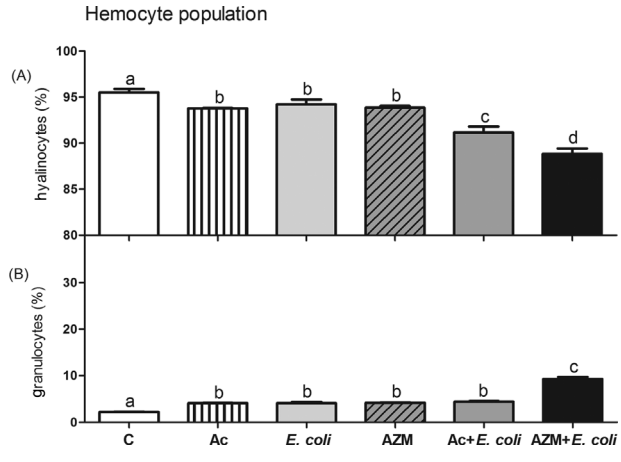

Figure 2. Percentage of (**A**) hyalinocytes, and (**B**) granulocytes, with respect to total hemocytes in hemolymph of *Diplodon chilensis* exposed to azinphos-methyl (AZM) and/or challenged with *Escherichia coli* (mean ± standard error; $n = 3$). Different letters denote significant differences among groups (Newman–Keuls, $p < 0.05$). C = control; Ac. = acetone (solvent control).

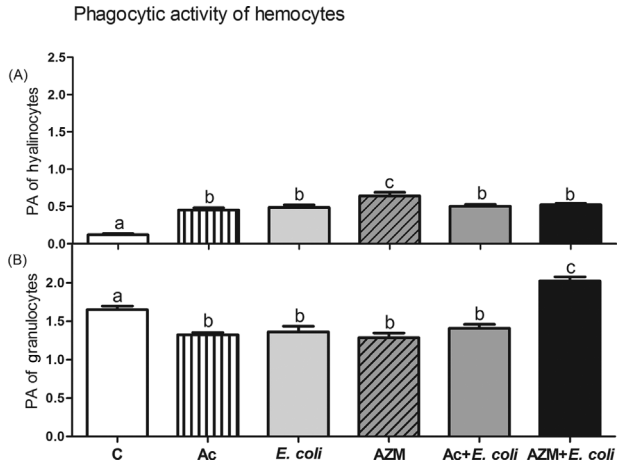

Figure 4. Phagocytic activity (PA) of (**A**) hyalinocytes, and (**B**) granulocytes in hemolymph of *Diplodon chilensis* exposed to azinphos-methyl (AZM) and/or challenged with *Escherichia coli* (mean ± standard error; $n = 3$). Different characters denote significant differences among groups (Newman–Keuls, $p < 0.05$). C = control; Ac. = acetone (solvent control).

group, where phagocytic activity was higher than in all the other groups (Newman–Keuls, $p < 0.001$; Figure 4B).

Cellular enzyme activity. Both acid phosphatase and alkaline phosphatase activities responded to the applied treatments (one-way ANOVA, $p < 0.001$, for both enzymes). Acid phosphatase activity was increased more than 2-fold by *E. coli* and AZM compared with the respective controls (Newman–Keuls, $p < 0.05$). However, there was no significant effect of AZM + *E. coli* compared with its solvent control (acetone + *E. coli*; Figure 5A). Alkaline phosphatase activity was also increased by *E. coli* and AZM compared with the respective controls (Newman–Keuls, $p < 0.01$) but was strongly reduced (Newman–Keuls, $p < 0.001$) by AZM + *E. coli* with respect to the acetone + *E. coli* control (Figure 5B).

Humoral response. Humoral enzyme activities were significantly affected by the applied treatments (one-way ANOVA, $p < 0.001$ for both bacteriolytic and phenoloxidase activities). Bacteriolytic activity was significantly increased by the *E. coli* challenge (Newman–Keuls, $p < 0.001$ compared with control, acetone, and AZM for all comparisons). This effect was not modulated by AZM because there were no significant differences between AZM + *E. coli* and acetone + *E. coli* (Figure 6A). Phenoloxidase activity was inhibited by AZM and was 50% lower in AZM + *E. coli* than in the acetone + *E. coli* group (Newman–Keuls, $p < 0.01$; Figure 6B).

Lysosomal membrane stability

Lysosomal membrane stability, as neutral red retention time, was significantly reduced by both, *E. coli* and AZM (Newman-–Keuls, $p < 0.001$). However, the effect of *E. coli* was partially reversed by pre--exposure to AZM (AZM + *E. coli* vs acetone + *E. coli,* Newman–Keuls, $p < 0.001$; Figure 7).

Gill oxidative balance

Enzyme activity and lipid peroxidation significantly changed in the gills of treated mussels (one-way ANOVA, $p < 0.0001$ for both GST activity and TBARS content). Gill GST activity was significantly increased by about 2-fold by both *E. coli* and AZM

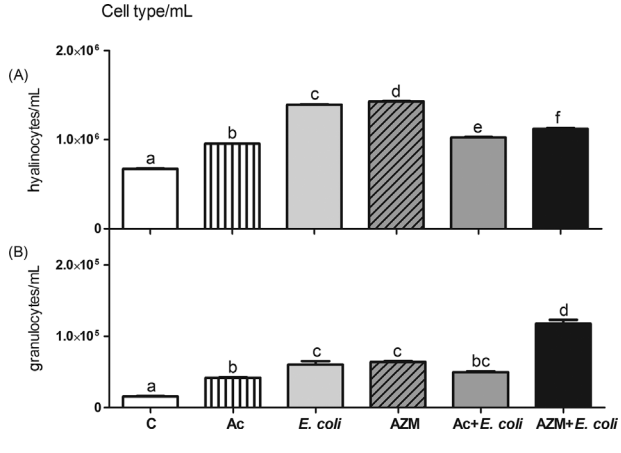

Figure 3. Cell type proportion related to the total number of hemocytes: (**A**) hyalinocytes, and (**B**) granulocytes of *Diplodon chilensis* exposed to azinphos-methyl (AZM) and/or challenged with *Escherichia coli* (mean ± standard error; $n = 3$). Different letters denote significant differences among groups (Newman–Keuls, $p < 0.05$). C = control; Ac. = acetone (solvent control).

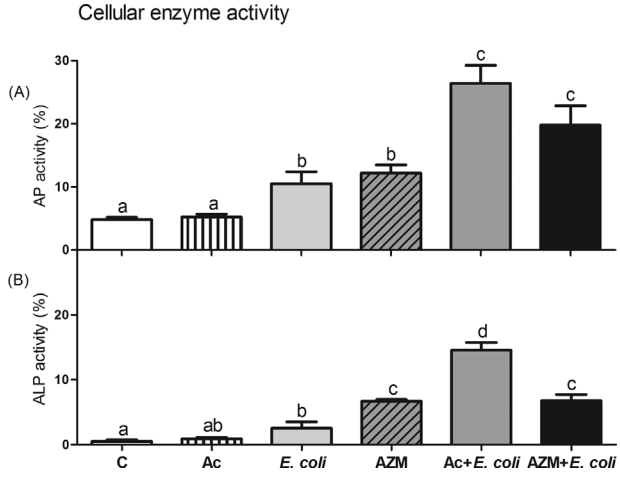

Figure 5. (**A**) Acid phosphatase (AP), and (**B**) alkaline phosphatase (ALP) activities in hemocytes of *Diplodon chilensis* exposed to azinphos-methyl (AZM) and/or challenged with *Escherichia coli* (mean ± standard error; $n = 3$). Different characters indicate significant differences among groups (Newman–Keuls, $p < 0.05$). C = control; Ac. = acetone (solvent control).

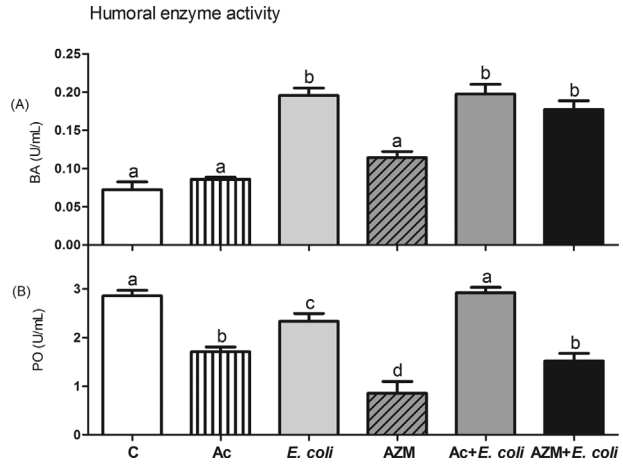

Figure 6. (**A**) Humoral bacteriolytic (BA), and (**B**) phenoloxidase (PO) activities in *Diplodon chilensis* exposed to azinphos-methyl (AZM) and/or challenged with *Escherichia coli* (mean ± standard error; $n = 3$). Different characters show significant differences among groups (Newman–Keuls, $p < 0.05$). C = control; Ac. = acetone (solvent control).

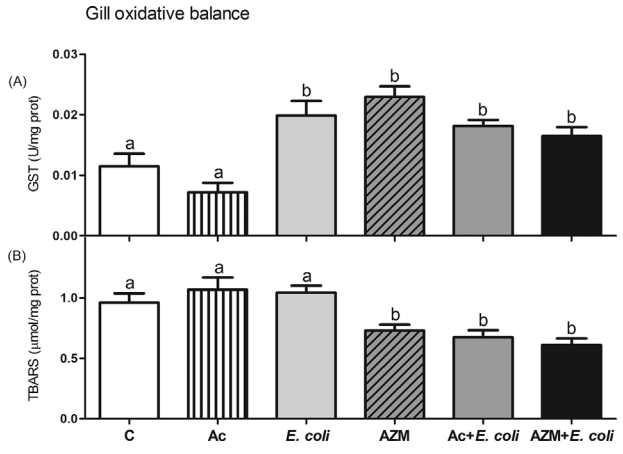

Figure 8. (**A**) Glutathione-S-transferase activity (GST), and (**B**) lipid peroxidation (thiobarbituric acid-reactive substances [TBARS]) in gills of *Diplodon chilensis* challenged with *Escherichia coli* (mean ± standard error; $n = 9$). Different characters demonstrate significant differences among groups (Newman–Keuls, $p < 0.05$). C = control; Ac. = acetone (solvent control).

compared with the respective controls (Newman–Keuls, $p < 0.05$ for both comparisons). Azinphos-methyl + *E. coli* did not differ from acetone + *E. coli* (Figure 8A). In turn, gill TBARS content was 25% lower in AZM and acetone + *E. coli* mussels than in the respective controls, acetone and *E. coli* (Newman–Keuls, $p < 0.05$ for both comparisons), whereas AZM + *E. coli* did not differ from acetone + *E. coli* (Figure 8B). The effects and modulation of the immunological and oxidative balance responses to *E. coli* by AZM are summarized in Table 2.

DISCUSSION

The present study provides data on the modulation of immune and oxidative responses of the long-lived freshwater mussel *D. chilensis* against *E. coli* by the pesticide AZM. To our knowledge, the effects of pre-exposure to agrochemicals on the immune response against sewage water bacteria have not yet been addressed in freshwater bivalves. Although a dose–response study would have been more appropriate to characterize *D. chilensis* immune variables as biomarkers, in the present work we have preferred the study of multiple variables to identify the AZM-sensitive variables for the present and further studies. As a result, we have identified 6 immune response variables whose responses against *E. coli* are modulated by AZM.

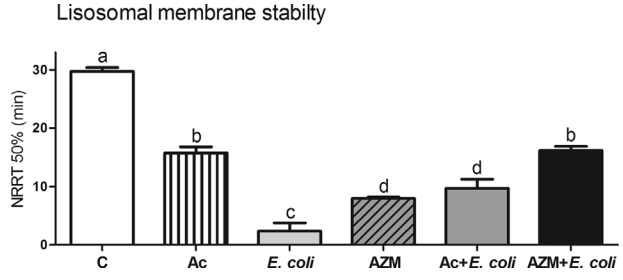

Figure 7. Lysosomal membrane stability (neutral red retention time [NRRT] 50%) in hemocytes of *Diplodon chilensis* exposed to azinphos-methyl (AZM) and/or challenged with *Escherichia coli* (mean ± standard error; $n = 3$). Different characters denote significant differences among groups (Newman–Keuls, $p < 0.05$). C = control; Ac = acetone (solvent control).

Immune response

Exposure to anthropogenic pollutants may alter cellular immune function and the consequent release of humoral components, threatening the individual's capacity to cope with immunological challenges [20]. In *D. chilensis*, total hemocyte number was increased in response to the *E. coli* challenge, whereas neither AZM nor *E. coli* affected the hemocytes' viability. This immune reaction against *E. coli* was not affected by previous exposure to AZM. Chang et al. [15] found that exposure to the organophosphorus insecticide trichlorfon at the same concentration used for AZM in the present study (0.2 mg/L) increased the hemocyte count in the giant freshwater prawn *M. rosenbergii* after 12 h of exposure. They suggested that this cellular response may reflect increased hematopoiesis and/or cellular mobilization to maintain homeostasis against possible tissue damage. In the *D. chilensis* hemolymph, the change in cell type proportion in response to the *E. coli* challenge (decreased proportion of hyalinocytes and increased proportion of granulocytes) is positively modulated by previous exposure to AZM. In particular, the granulocytes' proportion in *E. coli*-challenged mussels is 2-fold higher in AZM + *E. coli* than in the corresponding control (acetone + *E. coli*). Seiler and Morse [45] have reported a higher number of kidney granulocytes in individuals of *Mya arenaria* from a polluted site (New Bedford, MA, USA) than in those collected from an unpolluted site (Orleans, MA, USA). The authors have attributed this difference to a hemolymph cell-level response to pollution stress because granulocytes would be incorporating and transporting toxic compounds into their granules to later be excreted.

When the numbers of hyalinocytes and granulocytes per milliliter of hemolymph, estimated from the cell type proportion and the total hemocyte numbers, are considered, the numbers of both cell types are increased after *E. coli* challenge. This effect is notably enhanced by the AZM + *E. coli* treatment in granulocytes and only slightly enhanced in hyalinocytes. This suggests that the proportion of granulocytes is not increased at the expense of a reduction in hyalinocytes but through an augmented rate of granulocyte production.

Table 2. Effects and modulation of the immunological and oxidative balance responses by azinphos-methyl (AZM) pre-exposure (72 h) in *Diplodon chilensis* challenged with *Escherichia coli*

Variable	Effects[a]
Cell type	(+) Reduction in hyalinocyte proportion
	(+) Increase in granulocyte proportion
	(+) Increase in granulocyte number/mL
Phagocytic activity	(–) Increase of PA in granulocytes
Cellular enzyme activity	(#) Increase in acid phosphatase activity
	(–) Reduction in alkaline phosphatase activity
Lysosomal membrane stability	(–) Partial reversion of *E. coli* effect
Humoral enzyme activity	(#) Reduction in phenoloxidase activity
Oxidative balance in gills	(#) Increase in GST activity
	(#) Reduction in TBARS levels

(+) = positive modulation (augmentation of the *E. coli* effect); (–) = negative modulation(reversion of the *E. coli* effect); (#) = effect with or without *E. coli* challenge; PA = phagocytic activity; GST = glutathione-S-transferase; TBARS = thiobarbituric acid-reactive substances.

Diplodon chilensis hyalinocytes' phagocytic activity was induced by an *E. coli* challenge and, to a lesser extent, by AZM. However, no modulation of the response of PA to *E. coli* by AZM is evident in this cell type. In contrast, AZM has no effect by itself on granulocyte phagocytic activity, but applied before the *E. coli* challenge (AZM + *E. coli*) it significantly induces this function. These results demonstrate a differential modulatory effect of AZM on the phagocytic activity in *D. chilensis*, depending on the type of hemocyte examined. The hemocyte phagocytic activity of the blue mussel *M. edulis* decreases after exposure to the organophosphorus pesticide azamethiphos for periods of up to 24 h [16], whereas exposure to the fungicide hexachlorobenzene increases phagocytic activity of granulocytes over *E. coli* in the snail *Lymnaea palustris* [17]. In addition, Gagnaire et al. [20] observed that exposure of the oyster *Crassostrea gigas* to a mixture of pesticides and posterior challenge with *Vibrio splendidus* upregulates the gene expression of galectin, which is related to phagocytosis mechanisms. However, considering the increase of mortality in oysters treated with pesticide and bacteria at 48 h and 72 h (postinjection), these authors hypothesize that this upregulation of defense genes could be harmful.

Because granulocytes have approximately 4-fold higher phagocytic activity than hyalinocytes, the positive modulation by AZM of granulocytes' number and phagocytic activity enhances the phagocytic response of *D. chilensis* against bacteria. Whether this modulatory effect of AZM increases the resistance of this species to sewage pollution or it is harmful, as hypothesized by Gagnaire et al. [20], it deserves further experiments. The simultaneous modulation of granulocytes' number and phagocytic activity suggests that AZM activates a cell-signaling mechanism (e.g., calcium, cyclic adenosine monophosphate) that, combined with other signals probably initiated by lipopolysaccharide receptors, triggers a defensive cellular reaction.

Cellular enzymes from different groups of organisms may be differentially affected by pesticide exposure. For example, high levels of acid phosphatase activity have been reported in the plasma of sprayers chronically exposed to mixed pesticides [46], whereas this enzyme's activity is negatively affected in the snail *Biomphalaria alexandrina* after atrazine and glyphosate exposure for 4 wk [18] and remains unchanged in tissues of the freshwater fish *Piaractus mesopotamicus* during trichlorfon (organophosphorus) exposure. Activation of cellular acid phosphatase has been related to intensive lysosomal activity without leakage to plasma [47], which may be responding to immune stimulation and/or intracellular digestion of nutrients or foreign particles [48]. Iummato et al. [26] have found that alkaline phosphatase activity is increased in soft tissues of the golden mussel *Limnoperna fortunei* after glyphosate exposure. In *D. chilensis*, acid phosphatase and alkaline phosphatase activities show mostly coincident patterns, with activation by both *E. coli* and AZM applied individually and enhanced response to *E. coli* when the challenge is applied after exposure to acetone (acetone + *E. coli* group). However, a negative modulation by AZM is observed only for alkaline phosphatase activity (decrease of activity in AZM + *E. coli* with respect to acetone + *E. coli*). In this sense, Barky et al. [18] and Venturini et al. [47] suggested that pesticides may have a direct inhibitory effect, phosphorylating active sites (serine residues) of alkaline phosphatase, or may affect enzyme synthesis processes. According to this, it could be speculated that if AZM affected the phosphorylation of serine residues of enzymes such as phosphatases, its modulatory effect would be explained by changes in regulatory ways that involve serine/threonine protein kinases, such as protein kinase A and protein kinase C, among others.

Humoral enzyme activity may suffer alterations in organisms exposed to pesticides, affecting the immune response capacity. For example, in the sea urchin *Paracentrotus lividus*, antibacterial activity decreases after 24 h to 48 h of exposure to the organochlorine insecticide lindane [19]. In mice exposed to atrazine, serum lysozyme decreases after 28 d from injection [49]. Contrarily, AZM does not affect humoral bacteriolytic activity in *D. chilensis* and does not alter the stimulation of bacteriolytic activity after an *E. coli* challenge. Thus no modulatory effect is observed. These differences could be explained in terms of the nature and dose of the pesticide used and on the exposure time. For example, lysozyme activity in plasma of *Nile tilapia* is not altered after 96 h of exposure to a low concentration (0.39 mg/L) of the organophosphorus pesticide diazinon [50]. *Diplodon chilensis* phenoloxidase activity is inhibited by AZM with or without *E. coli* challenge, which suggests that, at least for this enzyme, the dose/exposure time applied is adequate to detect effects on humoral response. Scarce antecedents exist about the effect of pesticides on invertebrates' phenoloxidase activity. In hemocytes of the giant freshwater prawn *M. rosenbergii*, phenoloxidase activity increases after 3 h of exposure to 0.4 mg/L of the organophosphorus pesticide trichlorfon, but this activity and the expression of the prophenoloxidase system significantly decrease after 12 h to 24 h of exposure [15]. Chang et al. [15] relate this downregulation to the disruption of neural and endocrine signaling, which increases the influx of Ca^{2+} into the cell, causing oxidative stress and resulting in the decrease of phenoloxidase activity and the resistance to pathogen infection.

Cytotoxicity

Exposure to pesticides may produce deleterious effects on hemocytes, diminishing immune defense in aquatic invertebrates. In adult Pacific oysters *C. gigas*, 12 d of exposure to 1.0 mg/L of lindane decreased hemocyte viability [29]. In the blue mussel *M. edulis,* hemocyte death increased when it was exposed for a few hours to 1.3 mg/L of the organophosphorus pesticide azamethiphos [16]. However, hemocyte viability in *D. chilensis* was not affected by AZM exposure, regardless of *E. coli* challenge, whereas lysosomal membrane destabilization as neutral red retention time 50% is evident in mussels exposed to Ac, *E. coli,* and AZM. These results suggest that the concentration/time of exposure combination used in the present study is enough to affect lysosomal membrane stability but not enough to produce significant cellular death. Lysosomal membrane stability reflects the health status of mussels; its reduction is associated with physiological stress caused by the exposure to mixtures of pollutants, organophosphorus pesticides among them [51]. In spite of the deleterious effect of AZM alone on neutral red retention time 50%, pre-exposure of *D. chilensis* to AZM reverses part of the reduction of neutral red retention time 50% caused by bacterial challenge (negative modulation). This protective effect could be related to activation of antioxidant and detoxification defenses by AZM, as the stimulation of GST activity recorded in the present work for gill tissue (discussed in the section *Gill oxidative balance* in the *Discussion* section). Accordingly, Russo and Lagadic [17] have reported a decrease in ROS production in hemocytes of snails exposed to atrazine for 3 h.

Gill oxidative balance

In *D. chilensis*, gill GST activity increases, whereas lipid peroxidation decreases after AZM exposure. In contrast, *E. coli* challenge also increases GST activity but does not reduce lipid peroxidation levels, unless the mussels have been previously exposed to AZM or to acetone. Antioxidant and detoxifying enzymes have been widely studied in aquatic organisms in relation to pesticide toxicity. For example, Ferrari et al. [27,52] have found that GST activity in the liver of the rainbow trout *Oncorhynchus mykiss* is not affected by AZM exposure for at least 96 h, whereas this activity is increased as a detoxifying response in developing embryos of the toad *Rhinella arenarum*. In particular, GST has been related to tolerance of amphibian larvae to AZM [53]. Increased GST activity has also been reported as leading to elevated detoxification rates, by elimination of bacterial lipopolysaccharides and lipid peroxidation products from the cell [54,55]. Finally, the results for lysosome membrane stability of hemocytes (neutral red retention time) are coincident with those obtained for gill GST and TBARS, in that pre-exposure to AZM reduces the negative effect caused by *E. coli* and, to a lesser extent, by acetone.

Solvent effect considerations

To our knowledge, there are no studies regarding the effects of Ac used as a cosolvent on freshwater mussels. Tsarpali et al. [37] investigated the toxic effects of 2 imidazolium ionic liquids on the marine mussel *Mytilus galloprovincialis*, using either water or acetone as a cosolvent. Treatment with acetone alone (0.06%) for 4 d did not produce significant effects on hemocytes' lysosomal membrane stability, ROS production, lipid peroxidation, or DNA damage. Nevertheless, Tsarpali et al. [37] also reported increased cytotoxic, oxidative, and genotoxic effects of imidazolium ionic liquids applied with acetone as a cosolvent compared with imidazolium ionic liquids applied with water, and suggested that acetone favored imidazolium ionic liquids' toxicity by altering imidazolium ionic liquids' molecular structure. Accordingly, in the present study, acetone, at a concentration 5-fold lower than that used by Tsarpali et al. [37] had slight to no significant effects on *D. chilensis* hemocytes and gills but modulated the response to *E. coli* challenge effects in several immune and oxidative variables.

CONCLUSIONS

Exposure to AZM before an *E. coli* challenge increases 2-fold the proportion of granulocytes, which are the most phagocytic hemocytes in *D. chilensis* and, at the same time, enhances their phagocytic activity. Applied alone, this insecticide increases hemocyte acid and alkaline phosphatase and inhibits humoral phenoloxidase activity. Both AZM and, to a lesser extent, acetone reduce the damage caused by *E. coli* to the lysosomal membrane of hemocytes.

In gills, AZM stimulates GST activity and reduces TBARS level when applied alone or preceding *E. coli* challenge. Acetone also has significant effects on some immune variables, which should be considered in future experiments in which it is used as a cosolvent.

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Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (castro.juanmanuel6@gmail.com).

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