Chapter

Hemocytes of the Ribbed Mussel *Aulacomya atra atra* from Nuevo Gulf (Chubut) as Biomarkers of Oxidative Stress

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ABSTRACT

Bivalve mollusks exposed to a wide variety of natural and anthropogenic environmental changes are widely used as sentinels. These factors can cause an imbalance between the generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to an oxidative stress that is manifested by alterations of the antioxidant defense system and/or oxidative damage. The hemocytes constitute the immune system in bivalves, and cell death processes have been recently described as part of the mechanism of defense against various pathogens and contaminants. Previous studies showed a higher content of trace metals Fe and Cd in the gills and digestive gland of the *Aulacomya atra atra* from Folías Wreck (impacted area) than from Punta Cuevas (control area). In order to compare oxidative stress conditions in bivalves obtained from both sites, we evaluated the production of ROS and oxidative stress biomarkers in hemocytes from the *A. atra atra* during the month of September of 2015. The results obtained by flow cytometry, using MitoSox as probe, showed that superoxide anion was 58% higher in bivalve's hemocytes from Folías Wreck than in those from the reference place Punta Cuevas. The oxidation of the dye 2´7´dichlorofluorescein diacetate (DCFH-DA), as a general indicator of oxidative stress, showed a 14% increase in bivalve's hemocytes from Folías Wreck, as compared to hemocytes from Punta Cuevas. Thiobarbituric acid reactive substances (TBARS) content showed no differences between hemocytes from animals isolated from both locations. In addition, the content of lipid radical measured by Electron Spin Resonance increased 2.1 fold in the hemocytes from Folías Wreck samples as compared to the level obtained in hemocytes from bivalves collected from Punta Cuevas. The oxidized/depleted cardiolipin was 16% higher in samples obtained from Folías Wreck than in Punta Cuevas. Based on these results, it can be concluded that hemocytes from the ribbed mussel *A. atra atra* could be used as a model to evaluate oxidative stress induced by pollutants or other environmental stressors.

Keywords: bivalves, reactive oxygen species, hemocytes, superoxide anion, cardiolipin

INTRODUCTION

Marine invertebrates represent 95% of animal species, which ecological role is determinant in the evaluation of potential routes of exposure to the toxicity of metals. Bivalves have been employed as sentinel organisms in worldwide routine biomonitoring programs based on their wide geographic distribution, their straightforward availability in the field and through aquaculture, and their suitability for caging experiments along coast lines (Viarengo et al., 2007). The ribbed mussel *Aulacomya atra atra* (Molina 1782) is a filter feeder organism with the ability to accumulate a wide range of contaminants and a typical species of the rocky shores along the Patagonian coast of Argentina forming extensive beds (Zaixso, 1999). It is an important habitat-forming species for numerous species of algae and animals and it is also a food source for gastropods, asteroids and sea birds as well as for humans (Zaixso, 2003). It has been used as biomonitor to evaluate the recovery of normal cytogenetic records through micronucleus test after cessation of urban discharges in Puerto Madryn (Nuevo Gulf) (Machado-Schiaffino et al., 2009). In ribbed mussels from the same area, several biomarkers of oxidative stress have been measured for environment quality assessment (Giarratano et al., 2013; 2014).

The physico-chemical characteristics of seawater were within the range of the previously informed data (Gil, 2001; Solís, 1998; Giarratano et al., 2013; 2014). Previous studies have shown that the presence of a high concentration of metals such as Fe Al, Cu and Cd, from Folías Wreck seemed to be caused by the ship's metal corrosion (Giarratano et al., 2013; 2014).

Metabolic homeostasis in bivalves is performed at least partially upon cells circulating in hemolymph described as hemocytes, which are routinely used to understand the impact of pollutants with accurate precision (Ladhar-Chaabouni and Hamza-Chaffai, 2016). These cells are involved in different and numerous physiological functions as well as shell and tissue repair (Sparks and Morado, 1988; Mount et al., 2004). Hemocytes also mediate cellular internal defense mechanism through

accumulation and detoxification of toxicants, phagocytosis and encapsulation of different biological toxins and inorganic material (Matozzo et al., 2001).

Reactive oxygen species (ROS) are molecules produced by hemocytes of different bivalve's species including oysters, mussels, scallops and clams (Donaghy et al., 2012). In order to protect themselves against the effect of toxic substances, marine organisms developed a variety of detoxification pathways including an important battery of antioxidant mechanisms against ROS, as observed in mammals. The presence of oxidative stress is quite common in marine organisms due to a wide variety of natural and anthropogenic environmental changes. Induction of the antioxidant system can be considered as an adaptation of the organisms to overcome an unsafe environment and to prevent toxicity. The use of biomarkers has been proposed as a sensitive tool for biological effects monitoring, playing an important role in the risk assessment of complex ecosystems (Monserrat et al., 2007). Since several pollutants can directly or indirectly modify the balance between the concentration of pro-oxidants and antioxidants, the determination of oxidative stress and/or antioxidant responses in aquatic species is commonly and successfully employed as a non-specific biomarker (Bainy et al., 1996).

OBJECTIVE

The production of ROS and oxidative stress biomarkers were evaluated in hemocytes of ribbed mussel *A. atra atra* obtained from two sites within Nuevo Gulf.

Materials and Methods

Zones Selected

A. atra atra is present along the Pacific Ocean from El Callao, Peru, to the Strait of Magellan in Chile, extending along the Atlantic Ocean from the south of Argentina to the south of Brazil (Caza et al., 2016). Two sites within Nuevo Gulf were selected in order to obtain the ribbed mussel samples (Figure 1). Folías Wreck is a ship of 60 m in length located at 300 m of the shore of Paraná Beach, used for scuba diving activities. Punta Cuevas is a dive site that hosts fishes, crabs, sea stars, among other, and is located at the south end of the city. The last one was considered the reference place.

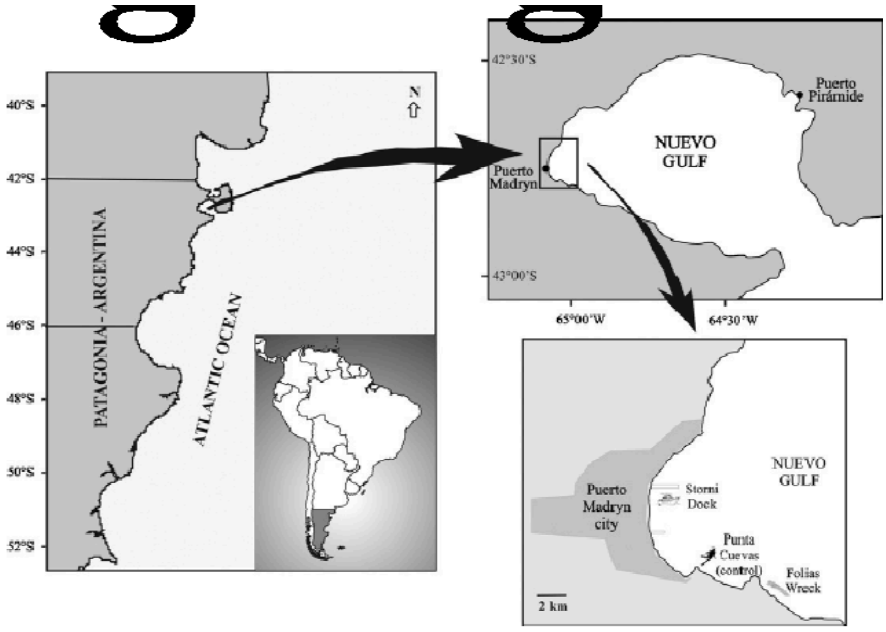


Figure 1. Map of the studied area.

Animal Collection

In September 2015, 30 individuals of *A. atra atra* were collected by scuba diving at each sampling site. Following collection, mussels were placed in isolated plastic containers previously filled with water from the sampling site and transported to the laboratory within 2 hours of collection. After hemolymph collection from the posterior adductor muscle sinus with sterile hypodermic needles, the hemocytes were filtered and maintained on ice. Each experiment was performed after pooling hemolymph from 3-5 ribbed mussels to obtain enough volume and to minimize the individual variability.

Flow Cytometry Studies

Flow cytometry assays were performed in a FACS calibur (Becton-Dickinson, US) equipped with a 488 nm argon laser and a 615 nm red diode laser. The studied hemocytes population with high FSC (Forward Scatter) and high SSC (SideScatter) characteristics was chosen as the gate (R1) according to the typical cellular hemocyte size, calibrated for all the cytometric studies.

Superoxide Anion Production

Superoxide anion (O_2^-) production was determined in unstimulated isolated hemocytes. Cells suspended in 1ml PBS and incubated at 25°C for 20 min in the presence of 12.5µM MitoSox, a potentiometric probe that is used for direct measurement of mitochondrial superoxide by flow cytometry. Samples were protected from light until acquired by the cytometer FACS calibur (Becton-Dickinson, US (Bustamante et al., 2004)). After auto-fluorescence evaluation, MitoSox fluorescence was analyzed using the median value of the fluorescence events distribution from each treatment. Differences in MitoSox fluorescence histograms were evaluated

in the gated R1 population from three independent experiments as the number of events that drop under a common marker. The results were expressed as the percentage of control MitoSox fluorescence from hemocytes obtained from animals located in Punta Cuevas (reference place), which was considerd as 100%.

DCFH-DA Oxidation Rate

The membrane-permeable non-fluorescent DCFH-DA oxidation has been used for detecting several ROS in biological media (McDowell et al., 2013). DCFH-DA was initially thought to be useful as a specific indicator for hydroperoxides. It was already demonstrated that DCFH is oxidized by different reactive molecules, including superoxide anion (O_2^-), hydroxyl radical, peroxyl, alkoxyl, hydroperoxyl and peroxynitrite which are products of normal metabolism (Halliwell and Gutteridge, 2007). DCFH-DA is a fluorogenic probe that passes through cell membranes and is cleaved by cellular esterases. During incubation, DCFH-DA is hydrolyzed by intracellular hydrolytic deacetylation to DCFH, which is trapped inside the cell due to its polarity. This substance is then rapidly oxidized to the highly fluorescent compound, DCF that allows the evaluation of oxidant cellular levels. Hemocytes were incubated *in vivo* in the dark for 30 min in 2 ml of 40 mM Tris-HCl buffer (pH 7.0) in the presence of 5 µM DCFH-DA at 27°C (Bass et al., 1983; Malanga et al., 2001). Fluorescence was measured with λ_{ex} = 498 nm and λ_{em} = 525 nm.

Content of Thiobarbituric Acid Reactive Substances (TBARS)

The hemocytes were homogenized in relation 1:3 of 0.1 M Tris buffer pH 7.8. The homogenates were treated with 30% (w/v) trichloroacetic acid and 50 mM potassium phosphate buffer (pH 7.0). After centrifugation, the content of TBARS was determined in the supernatant

spectrophotometrically at λ = 535 nm, using the acid hydrolysis of 1, 1, 3, 3 tetraethoxy propane as standard, according to Malanga et al., (2004).

Quantification of Lipid Radicals by Electron Paramagnetic Resonance (EPR)-Spin Trapping

Lipid radical ($LR^{\bullet}$) content in hemocytes was detected by a spin trapping technique using N-t-butil-α-phenyl nitrone (PBN). A 40 mM PBN stock solution was prepared in DMSO immediately prior to use. The homogenates were prepared in DMSO-PBN (stock solution). EPR spectra were obtained at room temperature using a Bruker spectrometer ECS 106, operating at 9.81 GHz with 50 kHz modulation frequency. EPR instrument settings for the spin trapping experiments were: microwave power, 20 mW; modulation amplitude, 1.194 G; time constant, 81.92 ms; scans number, 5; center fields, 3480 G; modulation frecuency, 50 kHz; and receiver gain, 2 104 (Lai et al., 1986). Quantification was performed according to Kotake et al., (1996).

Cardiolipin Content

Acridine orange 10-nonyl bromide (nonyl acridine orange, NAO, λ_{em} = 525 nm) binds specifically to cardiolipin providing a direct and reliable method for evaluating the cardiolipin content in mitochondria (McMillin and Dowhan, 2002; Petit et al., 1992; Wright et al., 2004). Nomura et al., (2000) reported that NAO is associated selectively with monolayer of cardiolipin. Also, they described that the binding of NAO to cardiolipin decreased by oxidation/depletion of cardiolipin; NAO is notable to bind to peroxidated cardiolipin (CL-OOH) producing a decreased NAO fluorescence. Previous observations indicate that low NAO fluorescence would be due to oxidation/depletion of cardiolipin (Nomura et al., 2000; Paradies et al., 2000; 2001). This can explained by the fact that free radicals-induced cardiolipin damage leads to decreased cardiolipin accessibility to NAO. After suspension of hemocytes in PBS, samples were

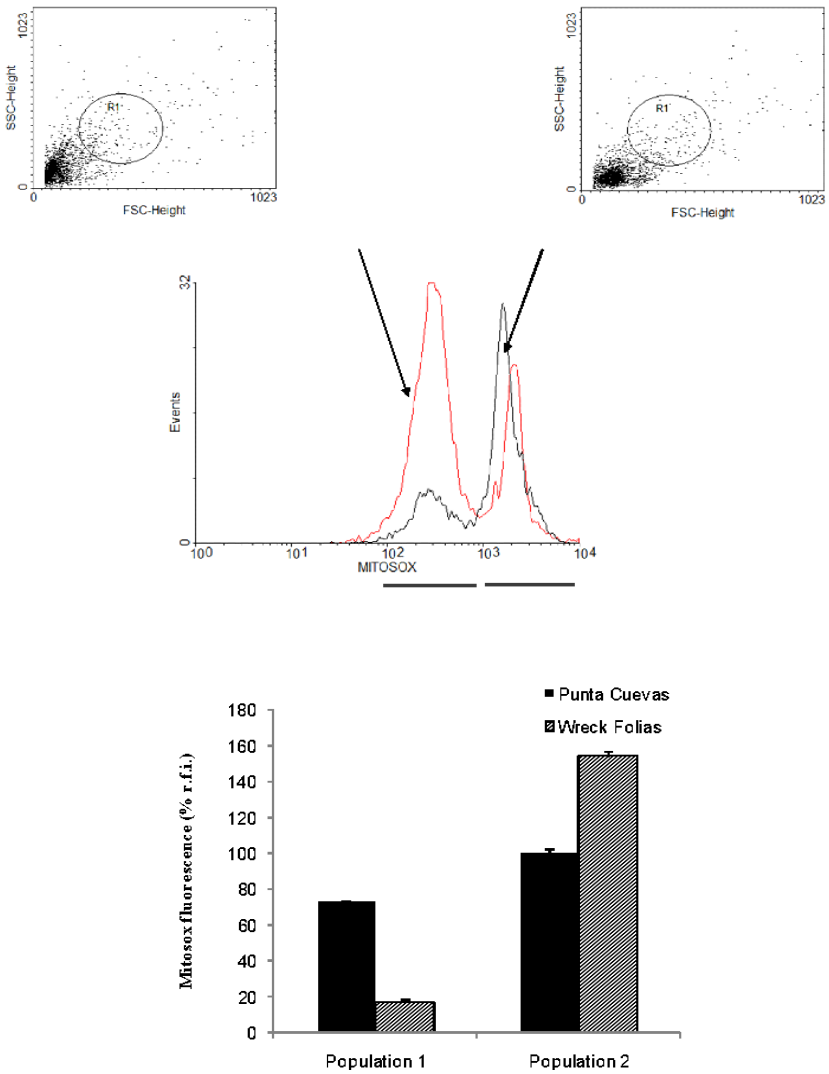
loaded with 100 nM NAO during 20 min at 25°C in a dark room and data acquired by the cytometer as previously described. NAO relative fluorescence intensity (r.f.i.) of hemocytes evaluated in three independent experiments as the number of events, which drop under a common and specific marker were evaluated in the presence or absence of antimycin (0.5 μM). Quantification is shown by cardiolipin oxidation/depletion for each hemocyte sample. Hemocytes autofluorescence without probe was also evaluated.

Statistical Analyses

Data in the text and tables were expressed as mean ± S.E.M. of three independent experiments, with two replicates in each experiment. Significant differences were tested by factorial analysis of variance ANOVA ($P < 0.05$).

RESULTS

Ribbed mussels *A. atra atra* from Punta Cuevas and Folías Wreck (Nuevo Gulf) were analyzed by flow cytometry using the probe MitoSox to measure their ability to produce O_2^-. Unstimulated hemocytes loaded with MitoSox were used for the flow cytometry assays. Typical histograms of FL-1MitoSox fluorescence for both samples are presented in Figure 2 a. Histograms under the respective markers showed two different O_2^- producing hemocytes populations with: low r.f.i (population 1) and high r.f.i. (population 2). Quantification of three different experiments was shown in the bar graph of Figure 2 b indicating a significant 58% increase ($P < 0.05$) in O_2^- level in Folías Wreck hemocytes as compared to Punta Cuevas hemocytes from population (2).



* Significant different as compared to values obtained in hemocytes of ribbed mussel collected at Punta Cuevas ($P < 0.05$).

Figure 2. Determination of superoxide anion levels in hemocytes of ribbed mussel *A. atra atra* from Punta Cuevas and Folías Wreck. a. Overlay of MitoSox fluorescence histograms from both hemocytes populations. b. Quantification of MitoSox fluorescence as a bars graph representing the mean ± SEM of 3 different experiments.

Table 1. DCF-DA oxidation rate, TBARS content and $LR^{\bullet}$ content in hemocytes isolated from ribbed mussels from Punta Cuevas and Folías Wreck

Zone	Punta Cuevas	Folías Wreck
DCF-DA oxidation rate (u.a./ min 10^6 cells)	148 ± 23	169 ± 15
TBARS content (nmol/ 10^6 cells)	29 ± 2	31.3 ± 0.9
$LR^{\bullet}$ content (nmol/ 10^6 cells)	0.27 ± 0.04	0.92 ± 0.01*

Values represent the mean ± S.E.M. of 3 different experiments.
*Significant different as compared to values obtained in hemocytes of ribbed mussel collected at Punta Cuevas values ($P < 0.05$).

In addition, ROS were also determined using DCFH-DA. The results obtained by this procedure showed a high rate of DFC oxidation by hemocyte loading. As shown in Table 1, hemocytes from Folías Wreck samples showed a 14% increase as compared to hemocytes from Punta Cuevas in agreement with the results obtained by flow cytometry using MitoSox as a probe.

The TBARS content showed in Table 1 was similar in hemocytes from Folías Wreck as compared to the values obtained in samples collected from Punta Cuevas.

Oxidative damage to lipid in the hemocytes of *A. atra atra* was also estimated in the present study, as the content of $LR^{\bullet}$ assessed by EPR. $LR^{\bullet}$ content in hemocytes combined with the spin trap PBN resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of a_N = 15.8 G and a_H = 2.6 G, in concordance with computer spectral simulated signals obtained using the overall mentioned parameters (Figure 3). A solution of PBN and DMSO itself was examined and no PBN spin adduct was observed (Figure 3d). $LR^{\bullet}$ content showed significant differences between the zones (Table 1), being 70.6% higher in Folías

Wreck samples than in hemocytes isolated from ribbed mussel collected in Punta Cuevas ($P < 0.05$).

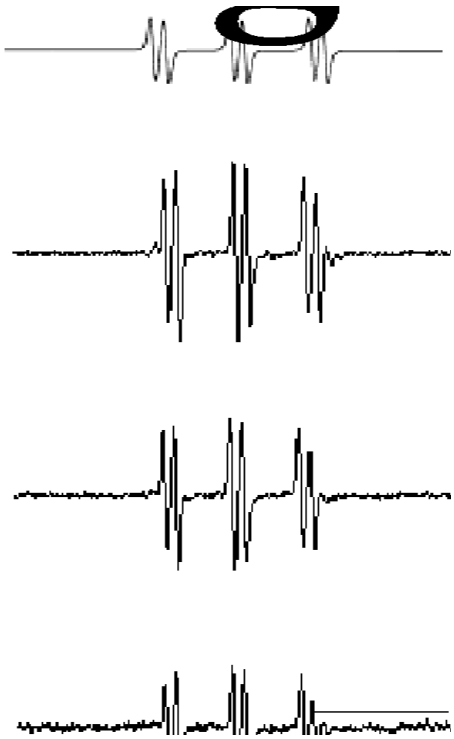
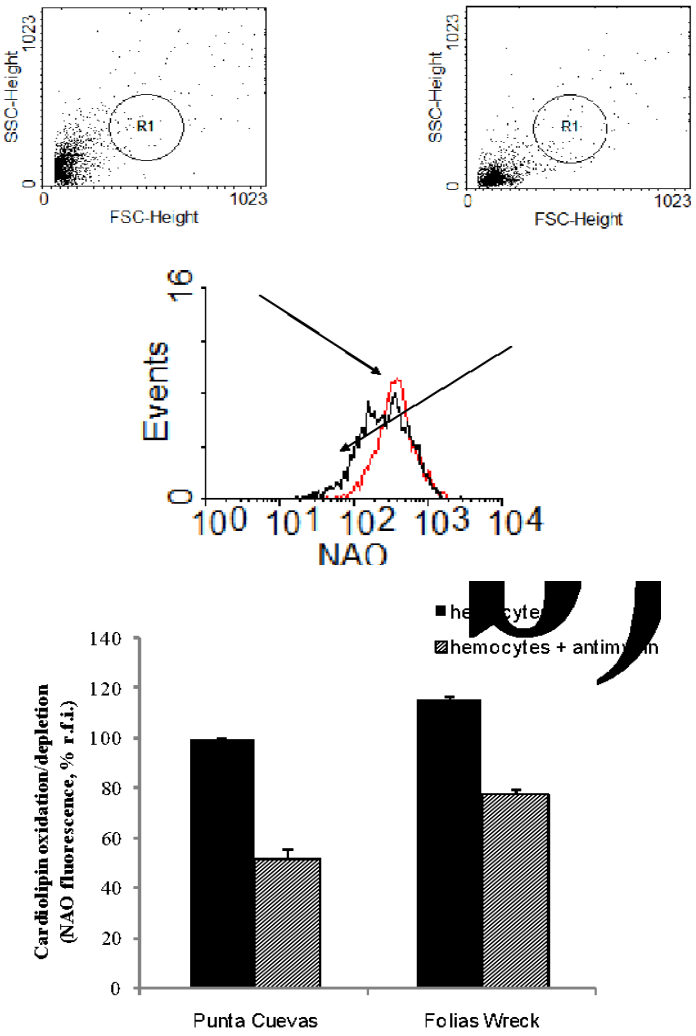


Figure 3. $LR^{\bullet}$ detection by EPR in hemocytes of the ribbed mussel *A. atra atra.* Spectra from: a. computer-simulated EPR spectra exhibiting hyperfine splittings that are characteristic of $PBN/LR^{\bullet}$, $a_N = 15.8$ G and $a_H = 2.6$ G, b. hemocytes from Folías Wreck, c. hemocytes from Punta Cuevas and d. EPR spectra of PBN itself, are shown.

Immediately after hemocytes isolation, they were stained with NAO to detect mitochondrial cardiolipin oxidation/depletion by flow cytometry as shown in Figure 4 a. We can observe in the overlay histograms that Folías Wreck samples presented higher amount of events with lower NAO-fluorescence indicating a higher rmitochondrial cardiolipin oxidation/depletion as compared with the samples collected in Punta Cuevas samples. In Figure 4 b the quantification of the cardiolipin histograms showed a significant increase ($P < 0.05$) of 16% in cardiolipin oxidation/depletion from Folías Wreck hemocytes as compared to

hemocytes of ribbed mussel from Punta Cuevas. When hemocytes from both zones were stimulated with antimycin, the levels of mitochondrial cardiolipin oxidation/depletion were decreased in both samples.



*Significant different as compared to values obtained in hemocytes of ribbed mussel collected at Punta Cuevas ($P < 0.05$).

Figure 4. Determination of the cardiolipin content in hemocytes of the ribbed mussel *A. atra atra* from Punta Cuevas and Folías Wreck. a. Overlay of NAO fluorescence histograms. b. Quantification of the level of cardiolipin oxidation/depletion present in both hemocytes samples with and without antimycin. The bars represent the mean ± SEM of 3 different experiments.

DISCUSSION

Effective O_2^- detection has been performed using flow cytometry with a variety of different probes such as dihydroethidium (Ding et al., 2007) and MitoSox (Velez-Pardo et al., 2010; Bustamante et al., 2014). These fluorophores are readily oxidized by O_2^- but not by other ROS and specifically bind to the mitochondria, which is an important place of O_2^- generation. In this work, two different hemocytes populations were observed in agreement with previous studies where hemocytes after characterization by flow cytometry and morphological studies presented also two different groups of cells (Soares da Silva et al., 2002). Both hemocytes populations observed in our study presented different ability to produce O_2^-. The results obtained showed that hemocytes from Folías Wreck (population 2) showed higher levels of FL-1 fluorescence as compared to the population 2 from Punta Cuevas. Interesting to note was the fact that hemocytes from the reference place Punta Cuevas presented the highest population (1) with low O_2^- production. Different types of radicals can mediate DCFH oxidation (Curtin et al., 2002; Rao et al., 1992); by reacting with different types of hydroperoxides after intracellular de-esterification and oxidation. Hemocytes from Folías Wreck showed a higher level of DCFH fluorescence than hemocytes isolated from samples collected in Punta Cuevas. These results indicated an enhanced hydroperoxide metabolism in *A atra atra*, potentially due to a higher exposure to metals such as Fe and Cd (Giarratano et al., 2013). Toxicological studies at cellular level have shown that metals such as Fe and Cd can affect mitochondrial metabolism and induce ROS production (Wang et al., 2004; Taze et al., 2016).

Although the measurement of TBARS is rather an unspecific test, it was used with other method to unequivocally characterize the lipid

peroxidation process (Rice-Evans et al., 1991). Since endogenous lipid radicals show short half-lives and are present in low concentrations, detection is a difficult task. Spin trapping-EPR analysis overcomes the limit of sensitivity of endogenous radical content in biological systems, being the best method available to detect short-lived reactive free radicals generated in low concentrations in biological systems (Luo et al., 2006). Even though EPR detection of lipid radicals could be considered a fingerprint of radical presence, spin trapping studies cannot really distinguish among peroxyl, alcohoxyl and alkyl adducts owing to the similarity of the corresponding coupling constants (Jurkiewicz and Buettner, 1994). Coincidently with TBARS results, the EPR analysis showed higher $LR^{\cdot}$ content in hemocytes from Folías Wreck than in those from Punta Cuevas. This fact could be in agreement with observations of Giarratano et al., (2013, 2014), in which gills and digestive glands of bivalves presented higher $LR^{\cdot}$ by EPR procedure.

The high efficiency of the mitochondrial electron transport is mainly due to the presence of the phospholipid cardiolipin at the inner mitochondrial membrane, which is required for the proper structure and activity of mitochondrial respiratory chain complexes. NAO is a mitochondrial probe that binds specifically to cardiolipin. Cardiolipin content has been used as a parameter of mitochondrial function in marine bivalves (Barmo et al., 2013; Ciacci et al., 2012). This fluorescent dye binds tightly to the acidic phospholipid cardiolipin, which is found exclusively in inner mitochondrial and bacterial membranes (Petit et al., 1994). In this work the level of cardiolipin oxidation/depletion measured as low NAO fluorescence, was higher in Folías Wreck hemocytes than in Punta Cuevas organisms. Contrary to mammalian cells, incubation of ribbed mussel's hemocytes from Punta Cuevas and Folías Wreck with antimycin (complex III inhibitor) resulted in a decrease ROS production, coincident with the results reported for the Pacific oyster *Crassostrea gigas* (Donaghy et al., 2012). Antimycin effect in mitochondrial mammalians induces an increase in ROS, mediated by its ability to inhibit the respiratory chain at complex III between cytochrome b and cytochrome c1 (Alexandre and Lehninger, 1984; Maguire et al., 1992). The decrease in

ROS production observed in bivalves could be due to the fact that in invertebrates the electron transport chain is branched with the presence of additional terminal oxidases (Buchner et al., 2001; Tschischka et al., 2000). The occurrence of these terminal oxidases and its genetic basis could indicate an alternative pathway for the electron transport, accepting electron directly from ubiquinol and reducing O_2 to H_2O (Hoffman and Brookes, 2009).

Monitoring of mixed beds containing *A. atra atra* has been proposed as an important opportunity to study the effect of climate change and pollution on the marine ecosystems in this area (Caza et al., 2016). In this sense, we can conclude that Folías Wreck bivalves seem to be exposed to higher stressing factors than those from Punta Cuevas, such as the high metal content that would induce an oxidative pressure. This fact could have a deleterious effect against organisms itself but could lead to important ecological alterations. Thus, we could conclude that hemocytes represent a sensitive model for the rapid screening of the cellular effects of different contaminants and pathogens. Furthermore, they could be useful to generate valuable tools for pollution monitoring and for establishing preventive measures that tend to protect the environment. Finally, we can state that this is the first work that indicates that two populations of *A. atra atra* hemocytes show different level of O_2^- content. It is interesting to note that the source of production is not already known and, requires future deeper studies.

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Professional Appointments: Doctor

Publications from the Last 3 Years:

1. Pérez AF, Boy C, Calcagno J and Malanga G. 2015. Reproduction and oxidative metabolism in the brooding sea star Anasterias antarctica (Lütken, 1957), *Journal of Experimental Marine Biology and Ecology* 463, 150-157. ISSN: 0022-0981. http://dx.doi.org/10.1016/j.jembe.2014.11.009.
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crab *Neohelice granulata* from San Antonio bay, *Ecotoxicology and Environmental Safety*, 132, 68-76.

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12. González PM, Puntarulo S and Malanga G. 2017. Natural adaptation to the environmental conditionsaffects the oxidation-dependent processes in limpets, *Int. J. Environment and Health*, 8(4), 302-315.
13. Fraysse C, Malanga G and Pérez AF. 2017. Effects of artificial diets with different carotene content on the organoleptic characteristics of the gonads and reproductive condition of *Loxechinus albu*s (Echinodermata: Echinoidea). *Rev. Biol. Trop. (Int. J. Trop. Biol.* ISSN-0034-7744) Vol. 65(Suppl. 1): S207-S220.

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Professional Appointments: Doctor

Publications from the Last 3 Years:

1. Gil M. N, I. A. Torres, M. G. Commendatore, C. H. Marinho, A. Arias, E. Giarratano, G. N. Casas, 2015. Nutritive and xenobiotic compounds in the alien algae *Undaria pinnatifida* from Argentine Patagonia. Arch. Environ. Contam. Toxicol. 68: 553-565.
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Professional Appointments: Doctor

Publications from the Last 3 Years:

1. Czerniczyniec, A., La Padula, P., Bustamante, J., Karadayian, A. G., Lores-Arnaiz, S., Costa, L. E. (2015) Mitochondrial function in rat

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Professional Appointments: Doctor

Publications from the Last 3 Years:

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19. Piloni N. E., Caro A. A. and Puntarulo S. Iron Overload Prevents Oxidative Damage to Rat Brain after Chlorpromazine Administration. Submitted.

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Professional Appointments: Doctor

Publications from the Last 3 Years:

1. Czerniczyniec, A., La Padula, P., Bustamante, J., Lores-Arnaiz, S., Costa, LE. Mitochondrial function in rat cerebral cortex and hippocampus after short and long term hypobaric hypoxia. *Brain Research*, 1598: 66–75, 2015. ISSN 0006-8993. Eds Elsevier.
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