Comparative Biochemistry and Physiology, Part A 165 (2013) 439–447

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Review

Electronic paramagnetic resonance (EPR) for the study of ascorbyl radical and lipid radicals in marine organisms ☆

Paula Mariela González, María Belén Aguiar, Gabriela Malanga, Susana Puntarulo *

Physical Chemistry-IBIMOL, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 1 December 2012
Received in revised form 18 February 2013
Accepted 19 February 2013
Available online 26 February 2013

Keywords:
Aquatic organisms
Ascorbyl radical
Electronic paramagnetic resonance (EPR)
Lipid radicals
Oxidative stress

ABSTRACT

Electron paramagnetic resonance (EPR) spectroscopy detects the presence of radicals of biological interest, such as ascorbyl radical ($A^{\bullet}$) and lipid radicals. $A^{\bullet}$ is easily detectable by EPR even in aqueous solution at room-temperature. Under oxidative conditions leading to changes in total ascorbate (AH^{-}) content, the $A^{\bullet}/AH^{-}$ ratio could be used to estimate early oxidative stress in the hydrophilic milieu. This methodology was applied to a wide range of aquatic systems including algae, sea urchin, limpets, bivalves and fish, under physiological and oxidative stress conditions as well. The $A^{\bullet}/AH^{-}$ ratio reflected the state of one part of the oxidative defense system and provided an early and simple diagnosis of environmental stressing conditions. Oxidative damage to lipids was assessed by the EPR-sensitive adduct formation that correlates well with cell membrane damage with no interference from other biological compounds. Probe instability, tissue metabolism, and lack of spin specificity are drawback factors for employing EPR for *in vivo* determination of free radicals. However, the dependability of this technique, mostly by combining it with other biochemical strategies, enhances the value of these procedures as contributors to the knowledge of oxidative condition in aquatic organisms.

Contents

1. Introduction

Soon after the electronic paramagnetic resonance (EPR) phenomenon was discovered by Zavoisky in 1945 (Borg, 1976), using a wartime radar klystron tube as a microwave source, an applied spectroscopy was rapidly developed, and its experimental utilization was quickly exploited. It was clear early on that a new sensitivity for detecting free radicals and other paramagnetic centers was available, and the further prospect of elucidating descriptive and analytical spectroscopic detail was planned (Malanga and Puntarulo, 2012). Indeed in less than 10 years the first biological samples analyzed by EPR were reported, which is a brief interval as far as applications of complex spectroscopies and physical techniques to the life sciences is concerned. EPR spectroscopy allows both characterization and quantification of free radicals in biological systems.

☆ This paper was presented at the "First International Conference on Oxidative Stress in Aquatic Ecosystems" held in San Jose del Cabo, Baja California Sur, Mexico on November 20–24, 2012.

* Corresponding author at: Fisicoquímica-IBIMOL, Facultad de Farmacia y Bioquímica, Junín 956 (C1113AAD) Buenos Aires, Argentina. Tel.: +54 11 4964 8244x101; fax: +54 11 4508 3646x102.
E-mail address: susanap@ffyb.uba.ar (S. Puntarulo).

1095-6433/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cbpa.2013.02.021

EPR techniques that report on the magnetic properties of unpaired electrons and their molecular environment (Tarpey et al., 2004), combined with spin trapping, have shown to be an appropriate tool to identify free radicals formed in tissues under normal as well as stressful conditions (Sun et al., 2008). Biologically important paramagnetic species including free radicals and many transition metals can be unequivocally identified by EPR spectroscopy as compared to routinely used standard techniques.

Redox status has historically been used to describe the ratio of interconvertible reduced/oxidized forms of a molecule. To define a cellular redox state, the ratios of important and abundant redox molecules such as NAD^+/NADH, $NADP^+$/ NADPH, and reduced glutathione (GSH)/oxidized glutathione (GSSG) can be measured. GSH/GSSG ratio is the most important and commonly measured redox couple used to obtain an estimate of cellular redox state because 1) it is found at high levels in cells, and 2) is important in determining the redox status of proteins, thus influencing protein function and activity. An assessment of the GSH/GSSG ratio therefore provides a reliable estimation of cellular redox status in cells and is thus frequently measured as an indicator of oxidative stress (Han et al., 2006). Sureda et al. (2011) showed a significant decrease in the GSH/GSSG ratio in the bivalve *Mytilus galloprovincialis* from an oil contaminated area compared to a control zone, indicating a more oxidized redox status in mussels directly affected by the spill. Also, the index of lipid radical ($LR^•$) content/α-tocopherol (α-T) content, that can be understood as indicator of the damage/protection ratio (Galleano et al., 2002), was characterized under oxidative conditions for the sea urchin *Loxechinus albus* (Malanga et al., 2009).

However, over time there was an increasing interest in the use of ascorbyl radical ($A^•$) content, as an informative, non-invasive and natural indicator of oxidative stress in the hydrophilic medium in biological tissues (Roginsky and Stegmann, 1994; Galleano et al., 2002; Malanga et al., 2012). On the other hand, in the cellular lipophilic medium, lipid peroxidation, defined as the oxidative deterioration of lipids containing two or more carbon-carbon double bonds (Hummel et al., 2006), seems as a common feature of oxidative deterioration effects. Polyunsaturated fatty acids (PUFA) are critical targets for potent oxidizing species such as reactive oxygen species (ROS). Peculiarities of membrane lipids in many marine organisms, particularly high content of PUFA (Joseph, 1982), suggest a special pattern for lipid peroxidation in these tissues as compared to mammals (Halliwell and Gutteridge, 1984; Malanga et al., 2004). Peroxidation of lipids has important consequences, e.g. amplification of ROS reactions, alteration of integrity, fluidity, permeability, and functional loss of biomembranes; disturbance of ion-gradients, and impairment of lipid–protein interactions (Halliwell and Gutteridge, 1989). Among the wide range of products generated by lipid peroxidation (Halliwell and Gutteridge, 1989), lipid alkyl radical ($R^•$), peroxyl radicals ($ROO^•$) and alcohoxyl radical ($RO^•$) could be assessed by EPR.

The principal aims of this review are the following: i) to provide a basic understanding of the capabilities and limitations of the EPR methods for assessing $A^•$ and $LR^•$ content in marine systems, ii) to show examples of EPR measurements used to assess oxidative stress and damage in cells and tissues from photosynthetic and non-photosynthetic marine organisms, and iii) to provide a starting point for those scientists in the field who may want to use EPR in their own experiments.

2. General features of the EPR technique

Just as the energy of spinning nucleus depends on its orientation in a magnetic field, so does the energy of spinning electron (e^-). The e^-, like protons, has only two spin quantum states, and thus two orientations in a magnetic field (Eisenberg and Crothers, 1979). Resonance between the two states can be induced by applying an alternating electromagnetic field of the correct frequency. EPR is not observed with most substances, because the spins of e^- usually are paired, so that an atom has no net magnetic moment. But for paramagnetic substances, including free radicals, triplet ground states such as the O_2 molecule, paramagnetic ions of transition metals and photochemical intermediates in the triplet state, e^- spin resonance is observed. The energy differences between the parallel or anti-parallel orientations with respect to an applied magnetic field (β) is shown by Eq. (1).

$$\Delta E = g\beta H \tag{1}$$

For the free e^- the value of g resulting from the e^-'s spin is very close to 2, but because the e^-'s unit negative charge spins at relativistic velocities there is a small correction so that the actual g value resulting from e^- spin is 2.002319278 (Borg, 1976). The quantity H is called the Bohr magneton and has a value of $9.2732\ 10^{-24}$ J/T. The H is a physical constant and the natural unit for expressing an e^-'s magnetic dipole moment. The Bohr magneton is defined in SI units and in Gaussian units (G) and depends on the elementary charge, the reduced Planck's constant, the e^- rest mass and the speed of light. The magnitude of an e^-'s spin magnetic moment is approximately one Bohr magneton (Eisenberg and Crothers, 1979). In a magnetic field of 20 kG, the energy ΔE corresponds to the microwave region of the electromagnetic spectrum calculated applying Eqs. (2) and (3), with h being Planck's constant and ν the frequency of the radiation field oscillation ($\nu = 5.6\ 10^{10}$ Hz).

$$\Delta E = h\nu = g\beta H \tag{2}$$

$$\nu = (g\beta H)/h \tag{3}$$

Magnetically aligned e^- are in resonance with the radiation field, so they can take energy from it or give energy to it. Electronic transitions will occur if the spin ensemble is irradiated simultaneously with oscillating electromagnetic radiation whose photon energy, E, equals $h\nu$, such that $h\nu$ exactly corresponds to the energy difference, ΔE, between the antiparallel and parallel electronic magnetic moments. Thus, the net absorption of energy from the applied electromagnetic field provides the sample signal in EPR spectroscopy. Since most radicals contain one or more atoms with magnetic nuclei, an unpaired e^- in a radical fragment is apt to interact with internal fields due to nuclear magnetic dipolar moments as well as with the applied field. This is termed "the nuclear hyperfine interaction". This interaction results in the splitting of resonance lines into two or more components, so-called hyperfine splitting (hfs). Nuclear hfs can enhance the informational content of an EPR spectrum, because many characteristic patterns of lines may result, and these may aid in the identification of free radicals. In addition, their full resolution and analysis may permit accurate determination of the orbital distributions of unpaired e^-, and hence of reactivities, of spatial orientations, and of structural information (Borg, 1976). The e^- spin resonance spectral lines have shape, width, intensity and position (g value), and hyperfine spectral line splitting from the interaction of unpaired e^- with magnetic nuclei that can determine the structure or positions of free radical components, and is a powerful aid in free radical identification.

Usual EPR spectra are the first derivatives of microwave power absorbed plotted vs. the applied magnetic field strength. It is conventional in EPR spectroscopy to present first derivative spectra rather than the integrated absorption or emission spectra characteristic of most other applied spectroscopies used by chemists and life scientists (Simontacchi et al., 2011).

The limiting factor for direct observation of EPR is the concentration of free radicals. About 10^{-13} mole of a paramagnetic substance gives an observable signal, so this technique is one of the most sensitive of all spectroscopic tools (Eisenberg and Crothers, 1979). With the usual experimental apparatus and optimal conditions for X-band (9 Ghz) spectroscopy, the limit is approximately $2\ 10^{-9}$ M, whereas for Q-band (35 Ghz) spectroscopy, it is approximately $6\ 10^{-9}$ M. Although these numbers at first glance may seem to indicate extraordinary

sensitivity, in practice it may be difficult to detect free radicals in many biochemical systems because of their reactivity. Because free radicals are highly reactive entities whose lifetimes in chemical or biochemical reactions tend to be very short, a common problem in making EPR measurements is to find experimental techniques that will allow time consuming EPR spectra to be recorded from detectable levels of short-lived paramagnetic intermediates. Either one of the following approaches serve to retain intrinsically short-lived intermediates at constant concentrations during the relatively long periods for EPR measurements: i) trapping, in which normally reactive species are sequestered in an unreactive matrix, ii) regenerative procedures, which produce dynamic steady states whose stationary concentrations can be maintained over suitable prolonged intervals, and iii) fast perturbation methods that can be cycled repeatedly when only kinetic EPR data are sought (Malanga and Puntarulo, 2012). Chemically reactive species may be trapped if they are formed by physical means in a dehydrated matrix, in solid samples, in aprotic or apolar solvents, at low temperatures, or under some other conditions that suppress their characteristic reactivity. Stable radical species are also formed by adding exogenous "spin traps"-molecules that react with primary radical species to give more enduring radicals adducts, with characteristic EPR "signatures". These spin traps, frequently nitroxide and nitrone derivatives, can also be used to label biomolecules and probe basal and oxidative induced molecular events in protein and lipid environments (Borbat et al., 2001). At room temperature, spin traps had been successfully used to detect hydroxyl radical ($^{\bullet}$OH) and LR$^{\bullet}$. In some other cases, such as nitric oxide detection, spin trapping and low temperature are routinely employed. However, quantitation in EPR experiments requires considerable care and it is very often that the devised experiments require relative more than absolute quantitation.

On the other hand, under favorable circumstances EPR spectroscopy can provide information for unambiguous identification of a free radical. In the most straightforward situation, this is done on the basis of the hyperfine structure of the spectra. In such circumstances the number and relative intensity of the hyperfine lines provide detailed information on the various atoms with which the unpaired e^- is associated. In less favorable conditions a complete description of the molecule still can be obtained by the use of several different techniques such as a double resonance and isotopic substitution in model compounds, plus correlation with results of classical biochemical experiments. Even if complete biochemical identification cannot be achieved, EPR spectroscopy may provide considerable knowledge about the nature of the free radicals. For example, the nature of the power-saturation curve may be sufficient to indicate whether or not the free radical is in a close association with a paramagnetic metal. Identification of free radicals in complex biological systems is usually difficult, because many of the free radicals of interest are located on macromolecules. Techniques to aid in the interpretation of such spectra have been developed within the last few years, and it is likely that such development will continue at an accelerated pace in the near future, enabling to achieve more precise identification of free radicals in such systems (Swartz and Swartz, 2006). Even more, although probe instability, tissue metabolism, lack of spin specificity are drawback factors for employing EPR for the *in vivo* determination of free radicals, the dependability of this technique, mostly by combining it with other biochemical strategies, drastically enhance the value of these procedures.

3. A$^{\bullet}$ content and A$^{\bullet}$/AH$^-$ ratio in aquatic systems

3.1. A$^{\bullet}$ detection in photosynthetic and non-photosynthetic aquatic organism

Ascorbic acid is a water-soluble antioxidant present in aquatic organisms (Malanga et al., 2012). Ascorbic acid has low redox potential, which allows it to donate one single e^- to almost any free radical occurring in a biological system or to reduce oxidized biological radical scavengers, such as α-T (Vergely et al., 2003). The A$^{\bullet}$ is the intermediate in the oxidation of ascorbate (AH$^-$) to dehydroascorbate (DHA) (Hubel et al., 1997). It has an unpaired e^- in a highly delocalized π-system, giving it stability as the "terminal small-molecule antioxidant" (Buettner, 1993). The concentration of A$^{\bullet}$ is a steady state value, determined by the rates of generation and decay of this species. A$^{\bullet}$ is formed by AH$^-$ oxidation processes mediated or not by metal catalysts such as Fe and Cu (Martell, 1982). The reaction that leads to A$^{\bullet}$ generation is shown by Reaction 1.

$$AH^- + Fe^{3+} \rightarrow A^{\bullet} + Fe^{2+} \quad (1)$$

The production of A$^{\bullet}$, a resonance stabilized tricarbonyl species, is easily observable by EPR. It is a relatively stable radical with a biologic half-life of 30–60 min at room temperature but highly stable at freezing temperature (Ahola et al., 2004). This half-life is longer compared to other free radicals generated in biological systems (Buettner and Kiminyo, 1992). Therefore, A$^{\bullet}$ is directly detectable by EPR in aqueous solutions at room temperature (Buettner and Jurkiewicz, 1993). To determine the optimal experimental conditions for the detection of A$^{\bullet}$, an ascorbic acid solution containing ferrous sulfate in an alkaline pH is prepared. Pietri et al. (1990) optimized the EPR method for the detection of the A$^{\bullet}$ by the addition of dimethylsulfoxide (DMSO). The basic properties of DMSO lead to the kinetic stabilization of A$^{\bullet}$, and DMSO has been used now for several years for the EPR analysis of A$^{\bullet}$ in many biological systems (Pietri et al., 1994; Barbehenn et al., 2003). EPR spectra obtained present a duplet with the following spectral parameters: $g = 2.005$ and $a_H = 1.8$ G, consistent with the EPR spectrum of A$^{\bullet}$ (Fig. 1b).

Mytilus edulis platensis, a filter feeder from the South America East Atlantic coast, is one of the most important aquaculture sources of the area with major economic relevance. The mussel populations at San Matías Gulf are distributed along the coast from 15 to 50 m depth, forming population units called banks, occupying rocky surfaces. Digestive gland (DG) was selected as the tissue under study since in molluscs, it is the major site of uptake of natural (dietary) and organic xenobiotic pro-oxidant chemicals and, as a consequence, the main target for oxidative disruption. Adult specimens of *M. edulis platensis* (7.82 ± 0.11 cm shell length and 3.34 ± 0.08 cm shell width) were brought to the laboratory from the Argentine Sea (San Antonio Oeste, San Matías Gulf, Río Negro) during the 2012 summer, and were kept in aquaria at 10 °C, at least one week prior to dissection. To assess A$^{\bullet}$ in DG from *M. edulis platensis*, homogenates were prepared in pure DMSO (1:30, p/v) and immediately transferred to a Pasteur pipette. Experimental measurements were performed at room temperature (18 °C) by EPR using a Bruker (Karlsruhe, Germany) spectrometer EMX plus 080, employing the following instrument settings: 9.86 GHz microwave frequency, 20 mW microwave power, 50 kHz modulation frequency, 1 G modulation amplitude, 3487 G center field, 327.68 ms time constant, 81.92 ms conversion time, 1 10^5 receiver gain and 15 G sweep width. The trace d in Fig. 1 shows the recorded signal, coincident with both the computer simulated (Fig. 1a) and the spectrum recorded in the chemical system (Fig. 1b). The diatom *Navicula* sp. from Antarctica (Potter Cove, King George Island /Isla 25 de Mayo, 62° 14′ S, 58° 38′ W) was selected as a model of photosynthetic organism. Stock cultures of Antarctic *Navicula* sp. were collected during the summer (2010). Cultures were grown for 15 days in filtered seawater plus sterile F/2 culture medium (10 ml/l seawater) (Guillard, 1975) at 4 °C under light/dark cycles of 12:12 h. The irradiance at the surface of the culture was approximately 38 W/m^2 of photosynthetically active radiation. Cellular algae suspensions (6 ml) were prepared in 200 μl DMSO and the spectra were scanned according to Estévez et al. (2001a). The trace e in Fig. 1 shows the recorded signal, in fully agreement with previously described spectra. Quantification of the spin adduct was performed using an aqueous solution of 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL) introduced into the same sample cell used for the samples. EPR spectra for both, sample

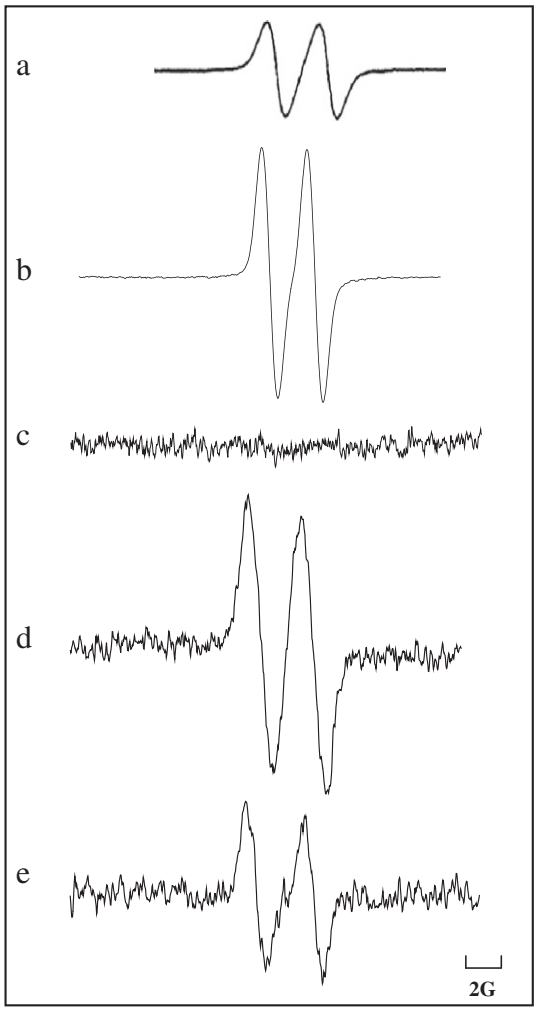

Fig. 1. A$^{\bullet}$ detection by EPR. Spectra from: a) computer-simulated employing the following spectral parameters, $g = 2.005$ and $a_H = 1.8$ G, b) AH^- 60 μM in DMSO, c) DMSO, d) DG from *M. edulis platensis* (with a 80 times higher scale to the rest of the spectra) and e) *Navicula* sp. cells.

and TEMPOL solutions, were recorded at exactly the same spectrometer settings and the first derivative EPR spectra were double integrated to obtain the area intensity, from which the concentration of the radical was calculated according to Kotake et al. (1996). A$^{\bullet}$ content resulted 14 ± 1 pmol/mg FW and 16 ± 7 nmol/10^7 cells for *M. edulis platensis* and *Navicula* sp. cells, respectively.

3.2. A$^{\bullet}$/AH$^-$ ratio under physiological conditions

A$^{\bullet}$ signal intensity is a function of pH, temperature, catalytic metal concentration, oxygen concentration and AH^- concentration. Only when these variables can be controlled, the intensity of the EPR signal of the steady-state concentration A$^{\bullet}$ serves as a marker for the oxidative stress in a system (Buettner and Jurkiewicz, 1993). However, in biological systems it is very difficult to control all these variables, especially the total AH^- content, which can be modified by the pro-oxidative condition in a studied tissue. Thus, the A$^{\bullet}$/AH^- ratio was established as an appropriate indicator of oxidative condition in the hydrophilic cellular space (Galleano et al., 2002). The content of AH^- was measured by reverse phase HPLC with electrochemical detection. *M. edulis platensis* DG and *Navicula* sp. samples were homogenized in metaphosphoric acid 10% (w/v) according to Kutnink et al. (1987). Commercially available AH^- (Sigma, St. Louis, USA) was used as standard. AH^- content resulted 293 ± 37 pmol/mg FW and 40 ± 20 μmol/10^7 cells for *M. edulis platensis* and *Navicula* sp. cells, respectively.

Data shown in Fig. 2 indicate that algae cells, from different origins, but all studied at their natural environment condition, keep the A$^{\bullet}$/AH^- ratio approximately constant since all of the values range from (0.5–40) 10^{-3} AU. This seems as a very interesting result, since it could indicate that this value for the ratio represents the best steady-state condition that an unicellular, photosynthetic organism could achieved to limit oxidative damage. Among previously reported values, A$^{\bullet}$/AH^- ratio for gonads of the sea urchin *L. albus*, DG of the limpets *Nacella magellanica* and *Nacella deurata*, and the liver of the fish *Odontesthes nigricans* are also in a common range from (3.8–6.5) 10^{-5} AU (Table 1). These data suggest that in metabolic active tissues, such as DG or gonads, from complex subantarctic animals, the oxidative status achieved under physiological conditions is carefully regulated at a lower level than in the photosynthetic algae tested. Even though they are ecologically similar molluscs, *M. edulis platensis* is exposed in the natural environment to water temperatures that could reach 20–22 °C over the summer (Esteves et al., 1996), meanwhile the bivalve *Mya arenaria* in the Wadden Sea affords a temperature range from 5 to 18 °C (Abele et al., 2002). Accordingly, the DG of *M. edulis platensis* showed a higher value for the A$^{\bullet}$/AH^- ratio as compared to DG from the bivalve *M. arenaria* (Table 1).

Moreover, Lattuca et al. (2009) reported that the *in situ* A$^{\bullet}$/AH^- ratio of silverside, *O. nigricans*, from the Beagle Channel was (6 ± 2) 10^{-5} and (5 ± 2) 10^{-6} AU, for gills and liver, respectively. Thus, it is suggested that there is an organ-specific oxidative status even in the same animals since the gill that is in direct contact with the oxygenated surrounding seawater showed a significantly higher oxidative stress than the liver, even though it is a strongly vascularized organ.

3.3. A$^{\bullet}$/AH$^-$ ratio under oxidative stress conditions

Assuming the A$^{\bullet}$/AH^- ratio as an oxidative index in the cellular aqueous medium, it is reasonable to expect that when cells are exposed to oxidative stress, either related to the metabolic activity or to the environmental changes, it would reflect these changes. As a matter of fact, several reported examples indicate that this effectively occurs. Many cellular constituents like AH^-, can reduce Fe^{3+} to Fe^{2+} generating catalytically active Fe that fuels $^{\bullet}OH$ and A$^{\bullet}$ formation. Data from Estévez et al. (2001a) indicate that the pro-oxidant effect of Fe supplementation on the *Chlorella vulgaris* cultures significantly increased both A$^{\bullet}$ and the A$^{\bullet}$/AH^- ratio in cells supplemented with

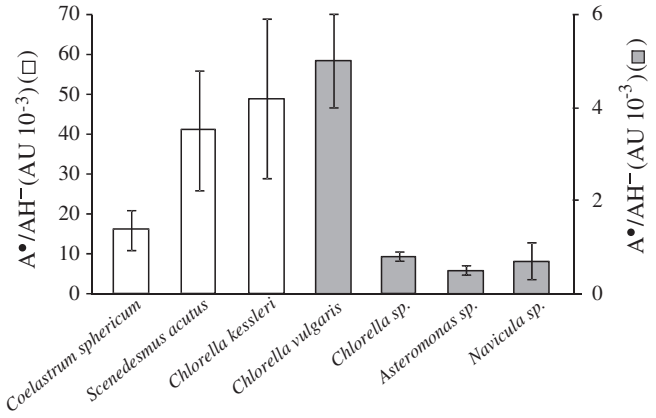

Fig. 2. A$^{\bullet}$/AH^- ratio in different algae cultures. Data are expressed as AU. Data for *C. vulgaris* (University of Buenos Aires), *C. kessleri* (Neuquén, Argentina), *C. sphericum* (CCAP217/2, SAG217/2) and *S. acutus* (CCAP276/3a, SAG276/3a) in log phase of development (day 12) were taken from Malanga and Puntarulo (2012) and Malanga et al. (2001). Data for *Chorella* sp. (Antarctic) and *Asteromonas* sp. (Subantarctic) in log phase of development (day 18) were taken from Estévez et al. (2001b) and Hernando et al. (2008), respectively.

Table 1
$A^{\bullet}/AH^{-}$ ratio and LR content in different species from different geographic locations.

Species	Geographic location	$A^{\bullet}/AH^{-}$ ratio (AU)	$LR^{\bullet}$ content (pmol /mg FW)	References
Fish (liver)				
O. nigricans[a]	Subantarctic	$(5 \pm 2)\ 10^{-6}$	0.94 ± 0.08	Lattuca et al. (2009)
Bivalves (DG)				
L. elliptica[b]	Antarctic	nd	422 ± 47	González and Puntarulo (2011)
M. arenaria[b]	Wadden Sea	$(8 \pm 1)\ 10^{-5}$	114 ± 21	González et al. (2008)
M. edulis platensis[b]	Argentinean Sea	$(5.2 \pm 0.7)\ 10^{-2}$	6.2 ± 0.7	
Limpets (DG)				
N. magellanica[a]	Subantarctic	$(4.6 \pm 0.9)\ 10^{-5}$	122 ± 29	Malanga et al. (2004)
N. deaurata[a]	Subantarctic	$(3.8 \pm 0.6)\ 10^{-5}$	235 ± 27	Malanga et al. (2004)
Sea urchin (gonads)				
L. albus[a]	Subantarctic	$(6.5 \pm 0.4)\ 10^{-5}$	0.7 ± 0.2	Malanga et al. (2009)

nd: Not determined.
Data are expressed as means ± E.S.M. of 4 to 6 independent experiments.
[a] Organisms measurements under physiological conditions were done during winter.
[b] Organisms measurements under physiological conditions were done during summer.

500 μM Fe as compared to cells grown at 90 μM Fe. This study, confirming Fe as a major catalyst of oxidative stress in algal cultures, may have implications in the context of Fe fertilization in oligotropic and "anaemic" open oceanic waters (Pollard et al., 2009). Further, other interesting examples of natural oxidative stress conditions in marine invertebrates were studied using the $A^{\bullet}/AH^{-}$ ratio as stress indicator. DG of intertidal limpets *N. magellanica* from the Beagle Channel were investigated for the effect of the natural oxidative stress in response to seasonal change (Malanga et al., 2007). The $A^{\bullet}/AH^{-}$ ratio indicated significantly lower oxidative stress levels (4.9 fold) in DG of intertidal limpets during winter, as compared to the rest of the year. Another seasonal survey was carried out with the gonads of the sea urchin *L. albus* (Malanga et al., 2009). The $A^{\bullet}$-EPR spectrum was lower (64%) in gonads isolated from sea urchins in spring and summer, as compared to winter; and the AH^{-} content remained unchanged in tissues from gonads collected in winter, spring and summer, but significantly decreased in gonads from sea urchins collected in fall. The low values of the $A^{\bullet}/AH^{-}$ ratio in summer as compared to winter collected sea urchin gonads can be related to the low level of maturation in summer, when the sea urchins feed to store reserve intermediates for gametogenesis in the fall. Moreover, the $A^{\bullet}/AH^{-}$ ratio was indeed significantly increased in fall, indicating that gonad maturation state may have a significant effect on the natural levels of oxidative stress in this tissue. These results highlight the need of taking into account the seasonal variations of oxidative stress conditions in invertebrates when analyzing other stress effects, such as contamination.

In this regard, reported studies from González et al. (2010) on the effect of exposure to excess Fe under laboratory conditions of *M. arenaria*, showed that the AH^{-} content in the DG from treated animals was not significantly different from the content in control bivalves. However, the $A^{\bullet}/AH^{-}$ ratio was increased by almost 15-fold over control values after 9 days of exposure of excess Fe.

On the other hand, Malanga et al. (2001) showed that the $A^{\bullet}/AH^{-}$ ratio was not significantly affected by the growth of intact cells of the green alga *C. vulgaris*, under laboratory conditions. The measurements of $A^{\bullet}$ showed no difference between log and stationary growth phase, but a significant increase in AH^{-} content was observed in stationary phase compared to the log growth phase (log phase: 1.3 ± 0.2 and stationary phase: 1.8 ± 0.2 nmol/10^7 cell). A very mild and indeed non-significant increase in the $A^{\bullet}/AH^{-}$ ratio from $(5.7 \pm 0.8)\ 10^{-3}$ in the log phase to $(6.1 \pm 0.5)\ 10^{-3}$ AU in the stationary phase indicates no oxidative stress under the respective *C. vulgaris* cultures conditions in the aqueous phase (Malanga, 2001). Thus, it could be postulated that since the growth is a physiological programmed event in the cell development, a synchronized increase in the content of oxidants and antioxidants in the hydrophilic phase avoided stress conditions.

4. Detection of $LR^{\bullet}$ in aquatic systems

As stated above, endogenous produced $LR^{\bullet}$ have extremely short half-lives and are present in low concentrations making detection difficult, however these difficulties are solved by applying spin trapping EPR analysis. Even though EPR detection of $LR^{\bullet}$ could be considered a finger-print of radical presence, spin trapping studies cannot really distinguish among $ROO^{\bullet}$, $RO^{\bullet}$ and $R^{\bullet}$ adducts owing to the similarity of the corresponding coupling constants (Buettner, 1987). The use of spin traps such as phenyl-tert-butylnitrone (PBN) and α-4-pyroyl-1-oxide-N-tert-butylnitrone (POBN) have been widely used for detecting organic radical products of lipid peroxidation (Detcho et al., 1999).

4.1. EPR measurements of $LR^{\bullet}$ content

EPR techniques can be successfully applied to several unrelated aquatic systems. *M. edulis platensis* DG homogenates were prepared using potassium phosphate buffer pH 7.4, and 130 mM POBN. Intact *Navicula* sp. algae were suspended in 40 mM PBN stock solution (prepared in DMSO immediately prior to use) (Malanga et al., 2009). Measurements were performed at room temperature (18 °C) by EPR. Instrument settings were as follows: 9.81 GHz microwave frequency, 20 mW microwave power, 50 kHz modulation frequency, 1.232 G modulation amplitude, 3515 G centered field, 81.92 ms time constant, 81.92 ms conversion time, 1 10^4 receiver gain (Jurkiewicz and Buettner, 1994). $LR^{\bullet}$ combined with the spin trap 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 agreement with computer simulated signals obtained using those parameters (Fig. 3a). No spin adducts were observed when POBN was tested by itself, as shown in Fig. 3b, and the same lack of signal was seen when PBN was studied by itself (data not shown). The EPR signals obtained for isolated DG from *M. edulis platensis* and algae cells from Antarctica are shown in Fig. 3, traces c and d, respectively. Quantification was done using TEMPOL according to Kotake et al. (1996), and the content of $LR^{\bullet}$ resulted of 3.3 ± 0.6 pmol/10^7 cells in *Navicula* sp. cells. The value obtained for *Navicula* sp. cells was in agreement with data from *C. vulgaris* that showed a $LR^{\bullet}$ content of 6 ± 1 pmol/10^7 cells at the log phase of development (Malanga and Puntarulo, 2012).

However, as previously described for the $A^{\bullet}/AH^{-}$ ratio, other more complex organisms showed a variable $LR^{\bullet}$ content, even growing under natural environmental conditions. Data included in Table 1 show the content of $LR^{\bullet}$ in the DG of the bivalves *M. arenaria* from the Wadden Sea, and *Laternula elliptic*a from Antarctica, along with the $LR^{\bullet}$ content reported here for *M. edulis platensis* obtained from the Argentine sea. These values showed a big variation among them.

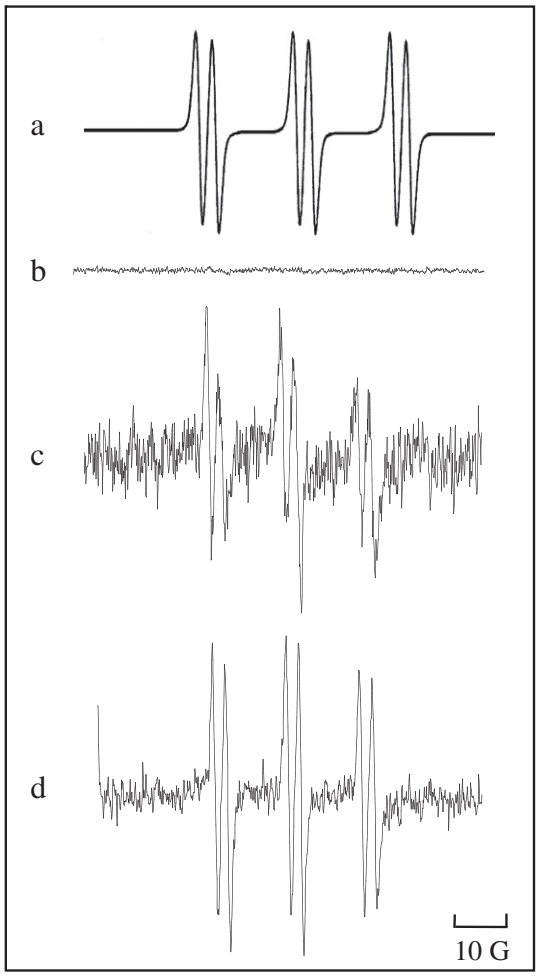

Fig. 3. LR• detection by EPR. Spectra from: a) computer-simulated exhibiting hyperfine splittings that are characteristic of POBN/LR•, $a_N = 15.8$ G and $a_H = 2.6$ G, b) POBN, c) DG from *M. edulis platensis* and d) *Navicula* sp. cells were the scale was 1×10^5 times lower than the scale in *M. edulis platensis*. The appropriate spin trap was selected for each system, according to the features of the cellular environment under study. POBN was selected for DG from *M. edulis platensis* and PBN for algae cells.

However, the higher content of LR• in *M. arenaria* is accompanied by a lower value in the $A^{\bullet}/AH^{-}$ ratio, as compared to values of *M. edulis platensis*. These observations could suggest that either antioxidant capacity in aqueous and lipid soluble media are not equivalent in these animals, and/or stressors in both phases are different for each animal due to the nature of their respective environments. Even among the subantarctic animals listed in Table 1, there is a wide variety in the content of LR• when comparing the limpets at one side and the fish and the sea urchin on the other side.

Metabolic rates, locomotory activity and growth rates in marine ectotherms are a function of temperature and of the seasonality of food availability in temperate and Antarctic waters (Kirchin et al., 1992). As food resources for most animals are more abundant during spring and summer in seasonality variable environments, many benthic grazers and detritus feeders cease feeding for weeks to months in winter (Brockington and Peck, 2001). Lipid peroxidation in the DG from *N. magellanica* was assessed as the content of LR• showing that it was significantly lower (67%) in winter than summer (Malanga et al., 2007). However, besides the great seasonal fluctuations in environmental parameters, such as productivity, temperature and photoperiod, LR• content in the sea urchin gonads over the year was not significantly changed over the studied period (Malanga et al., 2009).

4.2. Fe role in oxidative deterioration of lipids

Fe, an essential element for the growth and well-being of almost all living organisms (Harrison and Arosio, 1996), is involved in many biological functions and can participate in many e^{-} transfer reactions both in the hydrophilic and the lipophilic medium (Galatro et al., 2007). Fe was recognized as a bioactive element (Bruland et al., 1991) and a deficiency in Fe has been suggested to limit primary productivity in some ocean regions (Martin et al., 1993). Bioassays experiments conducted in high nitrate surface waters in the subarctic North Pacific (Coale, 1991), equatorial Pacific (Price et al., 1991), Southern Ocean (Hebling et al., 1991), and North Atlantic (Martin et al., 1993) provide support for the assertion that the phytoplankton community is affected by Fe availability. There, appears to be general agreement that the final biomass yield is higher in samples enriched with 1–10 nM Fe than in unamended samples, whether biomass is measured by chlorophyll, cell abundance or consumption of macronutrients. However, interpretation of these findings differs widely and it is unclear if addition of Fe would stimulate phytoplankton productivity. On the other hand, in spite of the fact that algal growth is limited by light intensity and available Fe in unpolluted freshwater, it was pointed out that it is critical to avoid Fe overload in water with low organic matter content under aquarium conditions, to prevent Fe-dependent toxicity (Brand et al., 1983). As mentioned above, by using EPR technology, it was shown that LR• content significantly increased in cells grown under Fe overload (Estévez et al., 2001a).

The labile Fe pool (LIP) is defined as a low-molecular-weight pool of weakly chelated Fe that rapidly passes through the cell. It likely consists of both forms of ionic Fe (Fe^{2+} and Fe^{3+}) associated with ligands with low affinity for Fe ions. This Fe is catalytically active in the reactions leading to the generation of ROS, such as $^{\bullet}OH$ that causes lipid peroxidation, DNA strand breaks and degradation of several biomolecules (Harrison and Arosio, 1996; González et al., 2010). Also, ferric complexes, such as ferric-adenine nucleotides and ferric-citrate are effective catalysts for lipid peroxidation (Puntarulo and Cederbaum, 1988).

Dissolved Fe concentrations increased from Antarctic oceanic to Antarctic coastal waters, where high values were measured in near surface waters (8.8–12.6 nM) and in near-bottom waters (18.4–22.6 nM) (Bucciarelli et al., 2001). Moreover, the vulcanic nature of the rocks containing Fe and Cu in King George Island may be a source for a higher Fe content in Potter Cove sediments, 24.2 mg Fe/g DW (Ahn et al., 1996). By contrast, in the Wadden Sea riverine inputs come from salt marshes and are not enriched in Fe. Dorum Wadden Sea sediments contain only 7.5 ± 0.6 mg Fe/g DW. Thus, since there is a potential effect of different Fe concentrations in the environment, the rate of generation of LR• by isolated tissues of animals from these habitats was tested (Estévez et al., 2002). The rate of generation of LR• was measured in DG from *L. elliptica* and *M. arenaria* after *in vivo* incubation at temperatures between 2 and 15 °C. LR• content after 10 min at a fixed temperature was significantly higher in the Antarctic bivalve than in the temperate mud clam (50 ± 5 and 10 ± 2 pmol LR•/mg prot min, respectively). Thus, higher levels of lipid peroxidation seem to correlate with elevated accumulation of transition metals in animal tissues (Estévez et al., 2002). As a matter of fact, Fe toxicity and resulting cellular damage depend on the concentration, bioavailability, and the organism capacity to deal with the metal by regulating/limiting its internal concentrations. The regulation can be physiological, and when this is not possible, some tissues have the ability to detoxify metals, or to sequester them inside special cellular compartments or even in proteins, such as ferritin, which apparently is a less harmful way of storing metals. Marine invertebrates seem as less tolerant than vertebrates to metal accumulation and can be affected at lower metal concentrations (González et al., 2012). The presence of the LIP in the tissues could be involved in the basal stress detected in the lipophilic and hydrophilic fractions (González et al., 2008), and it seems that only the LIP (and not the

total Fe) in the tissues is responsible for triggering lipid damage (González et al., 2012).

Total Fe content was analyzed in *M. edulis platensis* and *Navicula* sp. DG samples and algae were mineralized with a muffle. A sequence of temperatures was applied up to 500 °C, with 100 °C increases by 30 min (Du Laing et al., 2003). Fe concentration in the digests was measured spectrophotometrically after reduction with thioglycolic acid followed by the addition of bathophenanthroline (Brumby and Massey, 1967). Total Fe content in the DG of *M. edulis platensis* was 0.60 ± 0.05 nmol/mg FW and in cells of *Navicula* sp. (18 ± 2) μmol/10^7 cells.

The LIP in isolated tissues was determined by a fluorescence technique with the Fe sensor calcein according to Darbari et al. (2003) with modifications by Robello et al. (2007). *M. edulis platensis* DG and sonicated algae were treated as previously described by González et al. (2008). The LIP was 30 ± 2 pmol mg/FW in *M. edulis platensis*, and 290 ± 70 nmol/10^7 cells in *Navicula* sp. Kakhlon and Cabantchik (2002) reported that in mammals LIP represents only a minor fraction of the total cellular Fe (3–5%). The data showed here indicate that not only in vertebrates but also in marine organisms, the reactive Fe is kept as a low percentage of the total Fe to limit dangerous reactions to occur since these percentages were 3.7 and 1.6% for *M. edulis platensis* and *Navicula* sp., respectively.

Alterations in Fe metabolism should be carefully controlled when evaluating cellular oxidative damage, since Fe not only serves as a critical micronutrient, but as a catalyst of ROS generation as well, by the Haber Weiss reaction (Haber and Weiss, 1934). Thus, EPR measurements by themselves could not be sufficient to fully understand, for example the physiological scenario of $LR^{\bullet}$ production. It is required to evaluate other factors, such as the availability of metals, lipid composition, etc. by employing other tools. As it was shown here, other experimental procedures such as spectrophotometic (to evaluate Fe content) and spectrofluorometric (to measure LIP content) techniques and HPLC (to evaluate AH^- content) were combined to get a better picture of the oxidative status in *M. edulis platensis* and *Navicula* sp. Moreover, the access to a complete set of these measurements in other organisms allowed us to visualize the complexity of the relationship between the biological systems and their environments. This global estimation could not be done just from the isolated EPR experiments.

5. Concluding remarks and perspectives

Nowadays it is widely accepted that EPR is the method of choice for studying biologically important paramagnetic species including free radicals. Therefore, these techniques have become increasingly used in biochemical and biophysical research, and most likely their use will increase further as more marine scientists become aware of their capabilities. Spin-trapping followed by EPR analysis has proven to be the most direct and sensitive method not only to detect short-lived ROS generated in low concentrations in biological systems but also it provides a better way of identifying ROS actually present in biological systems. The spin-trapping reagents have extremely high rates of reactivity in biological reactions, and thus have the potential for detection and quantization of radicals that would not be possible by other methods. If it is true that the absence of an EPR signal need not denote absence of meaningful free radicals, it is equally valid to warn that the mere presence of an EPR signal from a biological system, is not sufficient to prove that a significant free radical component or pathway has been uncovered or documented (Malanga and Puntarulo, 2012). Further correlations are needed to substantiate the relevance of an EPR signal to any such conclusion; for example, quantification of an EPR spectrum may distinguish free radicals that are on the main path of a reaction from those that arise from minor side reactions or from adventitious impurities. Even more conclusive, kinetic analysis of the EPR signal intensities can provide especially firm support for the role imputed to the radical. In this regard, the $A^{\bullet}/AH^-$ ratio, but not the $A^{\bullet}$-EPR signal by itself, is a valid marker for oxidative stress in tissues. An increase of $A^{\bullet}/AH^-$ under natural and experimentally induced stress conditions provides an early and simple diagnostic tool in the study of aquatic organisms. EPR measurements with tissue homogenates provide important advantages over the frequently used *in vitro*, because it limits the production of artifacts during time consuming sample preparation. Moreover, the measurement of the $A^{\bullet}$ content is performed at room temperature avoiding more difficult procedures required for low temperature studies. Thus, EPR spectroscopy in conjunction with HPLC detection of the AH^- content can be considered as a powerful tool for detection of the initial stages of oxidative stress in the cellular hydrophilic compartment.

Regarding EPR detection of $LR^{\bullet}$, both PBN and POBN have the advantage of forming very stable spin adducts not affected by light, heat and oxygen and have been widely used to trap the radicals generated in biological systems. The data presented here shortly summarized the optimization of the assay performed to successfully apply this methodology to marine organisms.

Even more, recently EPR analysis has been applied to toxicological studies of ROS generation in the fish *Carassius auratus* exposed to several compounds (Luo et al., 2006; Yin et al., 2007; Luo et al., 2009) opening the scenario to possible future application of this methodology to evaluate contamination of the environments under risk. Also, mussels or other bivalves are commonly preferred for biomonitoring of aquatic metal pollution (Tanabe and Subramanian, 2003). Even more, algae species and amounts may directly reflect the water quality (Zhou et al., 2008). Heavy metal exposure can cause the disturbance of normal metabolism and biological function, ROS production, reduction of cytochromes, cellular mutation, even death. More importantly, once heavy metal pollutants are accumulated in algae, they enter the food chain and may pose serious threaten to animals and human health through bio-magnification processes. Algae may not only be significant for biomonitoring studies, but could also be a useful phytoremediation technology to restore water quality due to high bioaccumulation abilities (Zhou et al., 2008). Water pollution can be identified by analyzing the species, amounts, physiological and biological responses, and content of residues. In all cases, EPR methods could successfully contribute to improve the quality and accuracy of these studies.

Drawbacks of the approach are mainly the high costs and the difficult access to an EPR spectrometer, not available in laboratories furnished for routine biochemical studies. Probe instability, tissue metabolism, and lack of spin specificity are drawback factors for employing EPR for *in vivo* determination of free radicals. However, the dependability of this technique, mostly by combining it with other biochemical strategies, enhances the value of these procedures as contributors to the knowledge of oxidative condition in aquatic organisms. In summary, EPR spectroscopy has become a routinely used biophysical tool applied to many marine systems. No other technique allows such a powerful combination of flexibility of approach, and sensitivity to concentration to explore the presence of species highly difficult to identify in tissues and fluids.

Acknowledgments

This study was supported by grants from the University of Buenos Aires and National Council for Science and Technology (CONICET). S.P. and G.M. are career investigators from CONICET, P.M.G. is a postdoctoral fellow from CONICET and M.B.A. is a doctoral fellow from CONICET.

References

Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S., 2002. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. J. Exp. Biol. 205, 1831–1841.

Ahn, In-Y, Lee, S.H., Kim, K.T., Shim, J.H., 1996. Baseline heavy metal concentrations in the Antarctic clam, *Laternula elliptica* in Maxwell Bay, King George Island, Antarctica. Mar. Pollut. Bull. 32, 592–598.
Ahola, T., Fellman, V., Kjellmer, I., Raivio, K.O., Lapatto, R., 2004. Plasma 8-isoprostane is increased in preterm infants who develop bronchopulmonary dysplasia or periventricular leukomalacia. Pediatr. Res. 56, 88–93.
Barbehenn, V.R., Poopat, U., Spencer, B., 2003. Semiquinone and ascorbyl radicals in the gut fluids of caterpillars measured with EPR spectrometry. Insect Biochem. Mol. Biol. 33, 125–130.
Borbat, P.P., Costa-Filho, A.J., Earle, K.A., Moscicki, J.K., Freed, J.H., 2001. Electron spin resonance in studies of membranes and proteins. Science 291, 266–269.
Borg, D.C., 1976. Applications of electron spin resonance in biology. In: Pryor, W.A. (Ed.), Free Radicals in Biology, vol. 1. Academic Press, New York, pp. 69–147.
Brand, L.E., Sunda, W.G., Guillard, R.R.L., 1983. Limitation of phytoplankton reproductive rates by zinc, manganese and iron. Limnol. Oceanogr. 28, 1182–1198.
Brockington, S., Peck, L.S., 2001. Seasonality of respiration and ammonium excretion in the Antarctic echinoid *Sterechinus neumayeri*. Mar. Ecol. Prog. Ser. 219, 159–168.
Bruland, K.W., Donat, J.R., Hutchins, D.A., 1991. Interactive influences of bioactive metals on biological production in oceanic waters. Limnol. Oceanogr. 36, 1555–1577.
Brumby, P.E., Massey, V., 1967. Determination of nonheme iron, total iron and copper. Methods Enzymol. 10, 464–472.
Bucciarelli, E., Blain, S., Treguer, P., 2001. Iron and manganese in the wake of the Kerguelen Islands (Southern Ocean). Mar. Chem. 73, 21–36.
Buettner, G.R., 1987. Spin trapping: ESR parameters of spin adducts. Free Radic. Biol. Med. 3, 259–303.
Buettner, G.R., 1993. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and acerbate. Arch. Biochem. Biophys. 300, 535–543.
Buettner, G.R., Jurkiewicz, B.A., 1993. Ascorbate free radical as a marker of oxidative stress: an EPR study. Free Radic. Biol. Med. 14, 49–55.
Buettner, G.R., Kiminyo, K.P., 1992. Optimal EPR detection of weak nitroxide spin adduct and ascorbyl free radical signals. J. Biochem. Biophys. Methods 24, 147–151.
Coale, K.H., 1991. Effects of iron, manganese, copper and zinc enrichments on productivity and biomass in the subArctic Pacific. Limnol. Oceanogr. 36, 1851–1864.
Darbari, D., Loyevsky, M., Gordeuk, V., Kark, J.A., Castro, O., Rana, S., Apprey, V., Kurantsin-Mills, J., 2003. Fluorescence measurements of the labile iron pool of sickle erythrocytes. Blood 102, 357–364.
Detcho, A., Stoyanovsky, D.A., Melnikov, Z., Cederbaum, A.I., 1999. ESR and HPLC-EC Analysis of the interaction of hydroxyl radical with DMSO: rapid reduction and quantification of POBN and PBN nitroxides. Anal. Chem. 71, 715–721.
Du Laing, G., Tack, F.M.G., Verloo, M.G., 2003. Performance of selected destruction methods for the determination of heavy metals in reed plants (*Phragmites australis*). Anal. Chim. Acta 497, 191–198.
Eisenberg, D., Crothers, D., 1979. Biochemical spectroscopy. In: Eisenberg, D., Crothers, D. (Eds.), Physical Chemistry with Applications to the Life Sciences. The Benjamin/Cummings Publishing Company, Inc., California, pp. 562–645 (Chapter 13).
Esteves, J.L., Solís, M., Sastre, V., Santinelli, N., Gil, M., Commendatore, M., González Raies, C., 1996. Evaluación de la contaminación urbana de La Bahía de San Antonio (Provincia del Río Negro). Informe técnico 20 (1), 25.
Estévez, M.S., Malanga, G., Puntarulo, S., 2001a. Iron-dependent oxidative stress in *Chlorella vulgaris*. Plant Sci. 161, 9–17.
Estévez, M.S., Malanga, G., Puntarulo, S., 2001b. UV-B effect on Antarctic *Chlorella* sp. cells. J. Photochem. Photobiol. B 62 (1–2), 19–25.
Estévez, M.S., Abele, D., Puntarulo, S., 2002. Lipid radical generation in polar (*Laternula elliptica*) and temperate (*Mya arenaria*) bivalves. Comp. Biochem. Physiol. B 132, 729–737.
Galatro, A., Rousseau, I., Puntarulo, S., 2007. Ferritin role in iron toxicity in animals and plants. Res. Trends Curr. Topics Toxicol. 4, 65–76.
Galleano, M., Aimo, L., Puntarulo, S., 2002. Ascorbyl radical/ascorbate ratio in plasma from iron overloaded rats as oxidative stress indicator. Toxicol. Lett. 133, 193–201.
González, P.M., Puntarulo, S., 2011. Iron and nitrosative metabolism in the Antarctic mollusc *Laternula elliptica*. Comp. Biochem. Physiol. C 153, 243–250.
González, P.M., Abele, D., Puntarulo, S., 2008. Iron and radical content in *Mya arenaria*. Possible sources of NO generation. Aquat. Toxicol. 89, 122–128.
González, P.M., Abele, D., Puntarulo, S., 2010. Exposure to excess of iron in vivo affects oxidative status in the bivalve *Mya arenaria*. Comp. Biochem. Physiol. C 152, 167–174.
González, P.M., Piloni, N.E., Puntarulo, S., 2012. Iron overload and lipid peroxidation in biological systems. In: Catalá, A. (Ed.), Lipid Peroxidation. InTech, Rijeka, Croatia, pp. 89–108 (Chapter 4).
Guillard, R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrates Animals. Plenum Publishing, New York, pp. 29–60.
Haber, F., Weiss, J.J., 1934. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. Lond. A 147, 332–351.
Halliwell, B., Gutteridge, J.M.C., 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219, 1–14.
Halliwell, B., Gutteridge, J.M.C., 1989. Oxygen is poisonous — an introduction to oxygen toxicity and free radicals. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free radicals in Biology and Medicine, 1. Oxford University Press, London, pp. 1–21.
Han, D., Hanawa, N., Saberi, B., Kaplowitz, N., 2006. Mechanisms of liver injury. III. Role of glutathione redox status in liver injury. Am. J. Physiol. Gastrointest. Liver Physiol. 291, 1–7.
Harrison, P.M., Arosio, P., 1996. The ferritins: molecular properties, iron storage function and cellular regulation. Biochim. Biophys. Acta 1275, 161–203.
Hebling, E.W., Villafañe, V., Holm-Hansen, O., 1991. Effect of iron on productivity and size distribution of Antarctic phytoplankton. Limnol. Oceanogr. 36, 1879–1885.
Hernando, M., Malanga, G., Puntarulo, S., Ferreyra, G., 2008. Comparative strategies against UV-induced damage in an Antarctic diatom (*Thalassiosira* sp.) and a Sub-Antarctic phytoflagellate (*Asteromonas* sp.). In: Wiencke, C., Ferreyra, G.A., Abele, D., Marenssi, S. (Eds.), Reports on polar and marine research. : The Antarctic Ecosystem of Potter Cove King-George Island (Isla 25 de Mayo), 571. Alfred Wegener Institut fur Polar und Meeresforchung, Bremerhaven, pp. 243–254.
Hubel, C.A., Kagan, V.E., Kosin, E.R., McLaughlin, M., Roberts, J.M., 1997. Increased ascorbate radical formation and ascorbate depletion in plasma from women with preeclampsia: implications for oxidative stress. Free Radic. Biol. Med. 23, 597–609.
Hummel, S.G., Fischer, A.J., Martin, S.M., Schafer, F.Q., Buettner, G.R., 2006. Nitric oxide as a cellular antioxidant: A little goes a long way. Free Radic. Biol. Med. 40, 501–506.
Joseph, J.D., 1982. Lipid composition of marine and estuarine invertebrates. Part II. Mollusca. Prog. Lipid Res. 21, 109–153.
Jurkiewicz, B.A., Buettner, G.R., 1994. Ultraviolet light-induced free radical formation in skin: an electron paramagnetic resonance study. Photochem. Photobiol. 59, 1–4.
Kakhlon, O., Cabantchik, Z.I., 2002. The labile iron pool: characterization, measurement, and participation in cellular processes. Free Radic. Biol. Med. 33, 1037–1046.
Kirchin, M.A., Wiseman, A., Livingstone, D.R., 1992. Seasonal and sex variation in the mixed function oxygenase system of digestive gland microsomes of the common mussel, *Mytilus edulis* (L.). Comp. Biochem. Physiol. C 101, 81–91.
Kotake, Y., Tanigawa, T., Tanigawa, M., Ueno, I., Randel Allen, D., Lai, C.-S., 1996. Continuous monitoring of cellular nitric oxide generation by spin trapping with an iron–dithiocarbamate complex. Biochim. Biophys. Acta 1289, 362–368.
Kutnink, M.A., Hawkes, W.C., Schaus, E.E., Omaye, S.T., 1987. An internal standard method for the unattended high-performance liquid chromatographic analysis of ascorbic acid in blood components. Anal. Biochem. 166, 424–430.
Lattuca, M.E., Malanga, G., Aguilar, M.B., Hurtado, C., Pérez, A.F., Calvo, J., Puntarulo, S., 2009. Main features of the oxidative metabolism in gills and liver of de *Odontesthes nigricans* Richardson (Pisces, Atherinopsidae). Comp. Biochem. Physiol. B 154, 406–411.
Luo, Y., Su, Y., Lin, R., Shi, H., Wang, X., 2006. 2-Chlorophenol induced ROS generation in fish *Carassius auratus* based on the EPR method. Chemosphere 65, 1064–1073.
Luo, Y., Wang, X., Ji, L., Su, Y., 2009. EPR detection of hydroxyl radical generation and its interaction with antioxidant system in *Carassius auratus* exposed to pentachlorophenol. J. Hazard. Mater. 171, 1096–1102.
Malanga, G., 2001. Respuesta oxidante a la radiación ultravioleta en microalgas. Ph.D. thesis, Univerity of Buenos Aires, Argentina, pp. 57.
Malanga, G., Puntarulo, S., 2012. The use of electron paramagnetic resonance (EPR) in the study of oxidative damage to lipids in aquatic systems. In: Abele, D., Zenteno-Savín, T., Vázquez-Medina, J.P. (Eds.), Oxidative Stress in Aquatic Ecosystems. Willey-Blackwell, West Sussex, pp. 448–457 (Chapter 35).
Malanga, G., Juarez, A.B., Albergheria, J.S., Veléz, C.G., Puntarulo, S., 2001. Efecto de la radiacion UVB sobre el contenido de ascorbato y radical ascorbilo en algas verdes. In: Alveal, K., Antezana, T. (Eds.), Sustentabilidad de la biodiversidad, un problema actual, bases científico-técnicas, teorizaciones y proyecciones, Universidad de Concepción- Chile, pp. 389–398.
Malanga, G., Estévez, M.S., Calvo, J., Puntarulo, S., 2004. Oxidative stress in limpets exposed to different environmental conditions in the Beagle Channel. Aquat. Toxicol. 69, 299–309.
Malanga, G., Estévez, M.S., Calvo, J., Abele, D., Puntarulo, S., 2007. The effect of seasonality on oxidative metabolism in *Nacella (Patinigera) magellanica*. Comp. Biochem. Physiol. A 146, 551–558.
Malanga, G., Pérez, A., Calvo, J., Puntarulo, S., 2009. The effect of seasonality on oxidative metabolism in the sea urchin *Loxechinus albus*. Mar. Biol. 156, 763–770.
Malanga, G., Aguiar, M.B., Puntarulo, S., 2012. The ascorbyl/ascorbate ratio as index of oxidative stress in aquatic organisms. In: Abele, D., Zenteno-Savín, T., Vázquez-Medina, J.P. (Eds.), Oxidative stress in Aquatic Ecosystems. Willey-Blackwell, West Sussex, pp. 458–464.
Martell, A.E., 1982. Chelates of ascorbic acid formation and catalytic properties. In: Sieb, P.A., Tolbert, B.M. (Eds.), Ascorbic acid: Chemistry, Metabolism and Uses. American Chemical Society, Washington DC, pp. 153–178.
Martin, J.H., Fitzwater, S.E., Gordon, R.M., Hunter, C.N., Tanner, S.J., 1993. Iron, primary productivity and carbon nitrogen flux studies during the JGOFS North Atlantic bloom experiment. Deep-Sea Res. 40, 115–134.
Pietri, S., Culcasi, M., Stella, L., Cozzone, P.J., 1990. Ascorbyl free radical as a reliable indicador of free-radical-mediated myocardial ischemic and post-ischemic injury a real-time continuous-flow ESR study. Eur. J. Biochem. 193, 845–854.
Pietri, S., Séguin, J.R., D'Arbigny, P., Culcasi, M., 1994. Ascorbyl free radical: a noninvasive marker of oxidative stress in human open-heart surgery. Free Radic. Biol. Med. 16, 523–528.
Pollard, R.T., Salter, I., Sanders, R.J., Lucas, M.I., Moore, C.M., Mills, R.A., Statham, P.J., Allen, J.T., Baker, A.R., Bakker, D.C.E., Charette, M.A., Fielding, S., Fones, G.R., French, M., Hickman, A.E., Holland, R.J., Hughes, J.A., Jickells, T.D., Lampitt, R.S., Morris, P.J., Nédélec, F.H., Nielsdóttir, M., Planquette, H., Popova, E.E., Poulton, A.J., Read, J.F., Seeyave, S., Smith, T., Stinchcombe, M., Taylor, S., Thomalla, S., Venables, H.J., Williamson, R., Zubkov, M.V., 2009. Southern Ocean deep-water carbon export enhanced by natural iron fertilization. Nature 457, 577–580.
Price, N.M., Andersen, L.F., Morel, F.M.M., 1991. Iron and nitrogen nutrition of equatorial Pacific phytoplankton. Deep-Sea Res. 37, 295–315.
Puntarulo, S., Cederbaum, A.I., 1988. Comparison of the ability of the ferric complexes to catalyze microsomal chemiluminescence, lipid peroxidation and hydroxyl radical generation. Arch. Biochem. Biophys. 264, 482–491.
Robello, E., Galatro, A., Puntarulo, S., 2007. Iron role in oxidative metabolism of soybean axes upon growth effect of iron overload. Plant Sci. 172, 939–947.
Roginsky, V.A., Stegmann, H.B., 1994. Ascorbyl radical as natural indicator of oxidative stress: quantitative regularities. Free Radic. Biol. Med. 17, 93–103.

Simontacchi, M., Buet, A., Puntarulo, S., 2011. The use of electron paramagnetic resonance (EPR) in the study of oxidative damage to lipids in plants. In: Catalá, A. (Ed.), Lipid peroxidation: Biological implications. Res. Signpost Transworld Res. Network, Kerala, pp. 141–160.

Sun, Y., Yin, Y., Zhang, J., Yu, H., Wang, X., Wu, J., Xue, Y., 2008. Hydroxyl radical generation and oxidative stress in *Carassius auratus* liver, exposed to pyrene. Ecotoxicol. Environ. Saf. 71, 446–453.

Sureda, A., Box, A., Tejada, S., Blanco, A., Caixach, J., Deudero, S., 2011. Biochemical responses of *Mytilus galloprovincialis* as biomarkers of acute environmental pollution caused by the Don Pedro oil spill (Eivissa Island, Spain). Aquat. Toxicol. 101, 540–549.

Swartz, H.M., Swartz, S.M., 2006. Biochemical and biophysical applications of electron spin resonance. In: Glick, D. (Ed.), Methods of Biochemical Analysis, 29. John Wiley and Sons Inc., pp. 210–257.

Tanabe, S., Subramanian, A., 2003. Biomarkers and analytical methods for the analysis of POPs in developing countries. STAP/GEF and Ministry of Environment, Government of Japan (Sponsored) STAP Workshop on the use of bioindicators, p. 1.

Tarpey, M.M., Wink, D.A., Grisham, M.B., 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R431–R444.

Vergely, C., Maupoil, V., Clermont, G., Bril, A., Rochette, L., 2003. Identification and quantification of free radicals during myocardial ischemia and reperfusion using electron paramagnetic resonance spectroscopy. Arch. Biochem. Biophys. 420, 209–216.

Yin, Y., Jia, H., Sun, Y., Yu, H., Wang, X., Wu, J., Xue, Y., 2007. Bioaccumulation and ROS generation in liver of *Carassius auratus*, exposed to phenanthrene. Comp. Biochem. Physiol. C 145, 288–293.

Zhou, Q., Zhang, J., Fu, J., Shi, J., Jiang, G., 2008. Biomonitoring: an appealing tool for assessment of metal pollution in the aquatic ecosystem. Anal. Chim. Acta 606, 135–150.