Chapter 10

Soil Remediation Assessment by Detection of Reactive Oxygen Species in Lizard Testis: An Electron Spin Resonance (ESR) Approach

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Abstract

Recent developments in applied research have led to implement novel operative protocols for life-based restoration of contaminated soils, including new monitoring approach. Here, we report the measurements of reactive oxygen species (ROS) content in lizard testis performed in the framework of the project Life Ecoremed. The ROS levels detected by electron spin resonance (ESR) spectroscopy using the spin-trapping technique were analyzed and validated using measurements of total soluble antioxidant capacity and the poly(ADP-ribose) polymerase enzymatic activity, which detect the gonadal antioxidant defense and DNA repair, respectively. The investigations on soil biosentinel *Podarcis sicula* reproductive health gave significant evidence that the ROS level in the testis well correlates with alteration of the antioxidant capacity. In specimens coming from polluted sites, before remediation, a significant increase of ROS content was detected respect to that found in specimens from an unpolluted site. Thereafter, an evident decrease of the ROS levels, corresponding to high levels of total soluble antioxidant capacity and low repair of DNA integrity, has been detected after remediation. Thus, the data relative to all the polluted sites examined support the testis of *Podarcis sicula* as an elective tissue for an innovative and reliable screening method, based on ESR analysis of ROS, in the soil remediation assessment.

Keywords: *Podarcis sicula*, testis, reactive oxygen species, ROS, total soluble antioxidant capacity, poly(ADP-ribosyl)ation, DNA repair, electron spin resonance, ESR, 11 Env/IT/275 Ecoremed, soil remediation assessment

1. Introduction

Soil contamination represents an important source of environmental oxidative stress to terrestrial life and is able to induce the generation of free radicals, including reactive oxygen species, ROS, in living cells (for a review see [1]. Knowledge of the ROS content is therefore vital in order to evaluate environmental risks and, in particular, to study the pollution effects on living vertebrate organisms and their germinal and somatic cells. Some of the works present in the literature in which a quantitative determination of ROS amounts are related to the vertebrate exposure to pollutants are listed in **Table 1**.

ROS inflict, when overexpressed, oxidative damage upon lipids, proteins, DNA and other components of the cell [10]. We began to work on this topic at the laboratory of Comparative Endocrinology lab (EC*lab*) of University Federico II of Naples (Italy) by studying the reproductive health of various animals in presence of metal pollution, in the framework of Life Ecoremed (2011–2017 program). The aim of this research program, which brought together researchers belonging to six faculties (Agriculture, Architecture, Economics, Engineering, Literature and Sciences), was to contribute to the development of a method to implement eco-compatible protocols for agricultural soil remediation in Litorale Domizio Agro Aversano (Campania, Italy); in particular, different biomonitoring systems, including animals, were optimized to evaluate the requalification actions.

Free radicals present in living cells include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{-\bullet}$), nitric oxide ($NO^{\bullet}$), thyl ($RS^{\bullet}$), and peroxyl ($RO_2^{\bullet}$) species. The term ROS identifies the radicals in which oxygen is the main reactive atom, and is often used also for nonradical species, e.g., hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and ozone (O_3), which can easily lead to free radical production in the organism. Because of the short lifetime and typical low concentration, direct detection of ROS is extremely difficult. As a consequence, indirect methods are usually adopted. They are based on the reaction of ROS with a probe molecule so to obtain a more stable, long-lived chemical specie [11]. Most of the indirect methods for ROS analysis rely on ultraviolet/visible (UV/Vis) or fluorescence spectroscopy. Electron Spin Resonance (ESR) spectroscopy, by using

Vertebrate organism	Tissue ROS effect	Reference
Fish	Damage to cellular constituents	[2]
	Oxidative damage	[3]
Lizard	Oxidative damage	[4]
Frog	Hepatocytes and melanomacrophages alteration	[5]
	Oxidative damage	[6]
Frog tadpole	Alteration of mitochondrial efficiency and reduced growth	[7]
Rat	Reduction of spermatozoa motility	[8]
Boar	Reduction of spermatozoa motility	[9]

Table 1. ROS amounts in vertebrate living cells and their effect on the tissues.

appropriate nitrones as spin traps, is a valid alternative, allowing the quantitative detection of products deriving from ROS oxidative attack to lipids [12–14]. The choice of ROS was motivated since they are of crucial importance for living organisms (see **Table 1**). They are involved in metabolic cell processes but their overexpression might damage seriously cell functionality [1].

ROS effects were evaluated on testis of lizard, an organism phylogenetic close to mammalian. Details on the ROS protocol detection can be found in Ref. [4]. Adult male lizards *Podarcis sicula* were collected, during spermatogonial recrudescence, in different sites of the "Land of Fires", a contaminated area of the Litorale Domizio Agro Aversano (**C**) in accordance with the Italian Legislative Decree No. 152/2006 concerning the Environment's Code [15]. The polluted sites had been a temporary storage of industrial toxic and solid urban waste dumping. Lizards were collected before remediation and after remediation. Chemical analysis of soil samples allowed the baseline pollution levels to be determined prior to the intervention [15, 16]. As a control (**N**), lizards were also collected in a unpolluted site. This study, performed in the framework of subaction C2c "Biomonitoring of oxidative damage and characterization of reproductive health status of selected vertebrate and invertebrate species" has been conducted through 2013–2016. A selective strategy was carried out to identify and follow the biosentinel at risk in contaminated soil. During the first phase, the definition of reliable and reproducible protocols for species identification of different invertebrate and vertebrate biosentinels was reported. During the second phase, the reproductive health of biosentinels before (2013) and after (2015) re-qualification actions was analyzed by morphological, biochemical and molecular approach. As detailed in previous literature work [4] we monitored all metal polluted sites by measuring ROS, total antioxidant capacity and DNA repair in lizard testis before and after remediation.

2. Results and main findings

2.1. Difficulties in ROS quantitative measurements: choice of the optimal technical approach

ROS are difficult to handle due to the short lifetimes and typically low concentrations in terrestrial systems. Their direct observation is only possible on the sub-millisecond timescale, with the relatively stable H_2O_2 being an exception. Indirect methods involve the reaction of ROS with a probe molecule to yield a more stable, long-lived analyte [11]. Such methods typically involve specific chemical derivatization (the spin trapping procedure) or are based on competitive kinetics. Even with these approaches, products of oxidation, which attack biomolecules rather than ROS themselves, are often monitored. Much of the method development for aqueous ROS analysis has focused on ultraviolet/visible (UV/Vis) light spectroscopic techniques and the use of relatively common and hence lower cost probe molecules. Fluorescence and chemiluminescence spectroscopies have also been applied [17]. These strategies are also compatible with methods such as steady-state kinetic analyses, stopped flow methods, time-resolved laser spectroscopy, flash photolysis and pulse radiolysis. Other analytical techniques for ROS detection, such as electron spin resonance (ESR), nuclear magnetic resonance (NMR), derivatization with attendant mass spectrometric (MS) analysis and liquid scintillation counting can also be

quite useful [12]. We used the ESR technique for the assays [18]. This techniques, using appropriate nitrones as spin traps, allows the quantitative detection of products deriving from ROS oxidative attack to lipids [13, 14]. Analysis of the results clarifies regulatory mechanisms that appear as a response to xenobiotic attack, hormone treatment, metals or pesticide exposure, constituting a powerful tool for environmental call [1]. The spin trapping and adduct extraction procedure reported in Ref. [4] are an optimization of a method previously reported in the literature [18]. The analyses were performed on 40 mg of testicular tissue of lizards from the polluted sites (C) pre and post remediation, and of unpolluted site (N). The samples were weighed on an analytical balance and homogenized in physiological saline. The amount of added saline, which was adjusted for each sample, was in all cases lower than 1 mL per gram of tissue. An aqueous solution of the spin-trap N-tert-butyl-α-phenylnitrone, PBN, was prepared (140 mmol/L) and kept in a darkened room. Proper volumes of this solution were added to the homogenized samples at a 1:5 vol/vol ratio. We strongly recommend strict control of the temperature at 4°C and the darkness when working on ROS, in order to limit the risk of side reactions. The samples were allowed to equilibrate for 10 min and then centrifuged for 10 min at 3500 rpm. The supernatant was separated and mixed with HPLC-grade toluene (Sigma) at a 1:1 vol/vol ratio. A small aliquot (10 μL) of the organic phase, which contains the PBN adduct, was transferred in a quartz capillary, which was rapidly vacuum degassed and flame-sealed. The same extraction procedure was used to perform blank experiments. Samples were analyzed by ESR for radical content within 24 h from preparation. Capillaries were inserted in a 3 mm i.d. quartz tube. Measurements were performed at room temperature on a Elexsys E-500 X-band spectrometer (Bruker). Instrumental settings were: microwave frequency, 9.871 GHz; incident microwave power, 6.4 mW; modulation amplitude, 0.1000 mT; modulation frequency, 100 kHz; time constant, 10 ms; scan width, 6.000 mT; magnetic field center, 351.0 mT. The obtained first derivative signals were double integrated to quantitatively determine their intensity [19, 20]. The data were normalized by the weight of the tissue samples subjected to the radical extraction procedure and quantitatively analyzed in terms of relative variations.

2.2. Testis radical content in *Podarcis sicula* before and after remediation

The results of Life Ecoremed research, subaction C2c, show the reliable detection and quantification of radicals deriving from ROS attack to lipids ($LR^{\bullet}$). PBN forms very stable spin adducts with these species, poorly affected by light, heat and oxygen. All the samples analyzed show the typical EPR spectrum of the PBN spin adduct. An example is reported in **Figure 1**.

Two hyperfine coupling constants can be determined from the spectra analysis: a_N (whose mean value was found to be 13.7 ± 0.2 G) and a_H (1.9 ± 0.1 G). The amount of radical species in the samples was estimated by double integration (DI) of the spectra; the obtained values were normalized per weight of the tissue used for the preparation of each sample. The DI values were further normalized by the average value determined for samples coming from the unpolluted site (N), considered as a reference. In this way, it was possible to straight forwardly compare DI values obtained for lizards from different sites. The data indicative of radical content in male gonads of *Podarcis sicula* specimens from unpolluted (N) and polluted sites (C), examined in both pre and post remediation conditions, are shown in **Figure 2**.

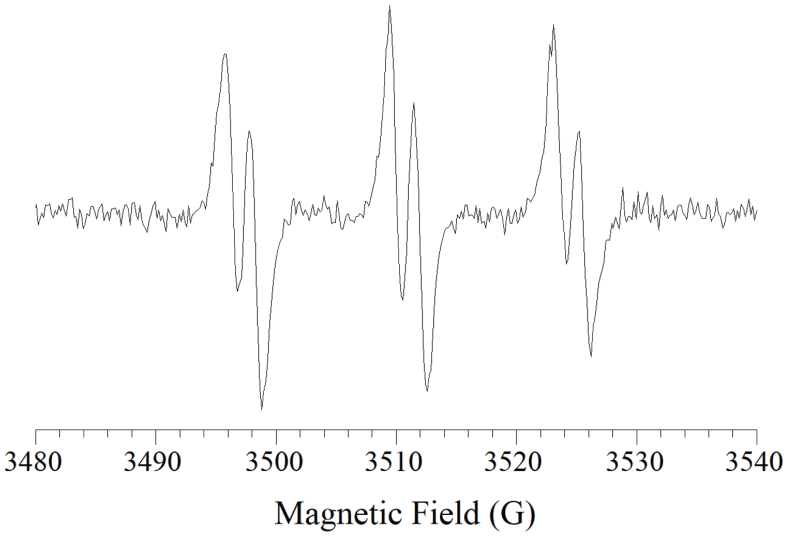

Figure 1. EPR spectrum of PBN adduct of radicals extracted from *Podarcis sicula* testis.

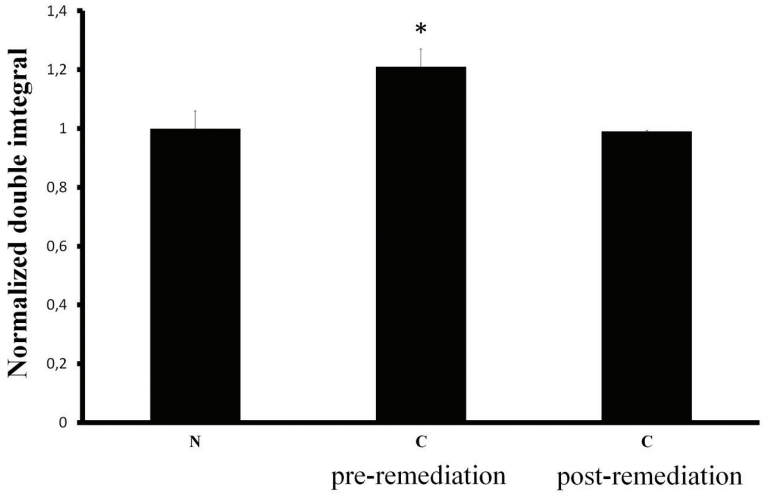

Figure 2. Radical content in *Podarcis sicula* testis collected from C, polluted sites of the Land of Fires, before and after remediation, and N, unpolluted site. The values are expressed as double integral of the ESR spectrum of PBN spin adduct, normalized by the sample weight and expressed relatively to the mean value in the unpolluted site. Bars represent the mean value ± standard deviation. *The significance differences of C pre remediation values compared with the N and C post remediation ($P < 0.001$).

In samples collected before the remediation the EPR measurements detect a concentration significantly higher than that observed after the remediation ($P < 0.001$), which is similar to that observed in the unpolluted site.

The sensitive alteration of ROS was confirmed by the results of total soluble antioxidant capacity and DNA repair [4].

2.3. Total soluble antioxidant capacity in lizard testis

All living organisms and their cells have developed antioxidative defense systems to protect themselves against ROS. At the cellular level, the oxidative stress is strictly associated to the changes in the prooxidant/antioxidant balance due to an overproduction of free radicals and/or to a reduction of the antioxidant defense system. These systems include enzymatic and nonenzymatic antioxidants that are usually effective in blocking the harmful effects of free radical [21]. Nonenzymatic antioxidant compounds [i.e. Vitamin C, Vitamin E, polyphenols], which react directly with oxidizing agents and disarm them, are named "scavengers". For example, vitamin C can directly scavenge $O_2^{-}\cdot$ and ·OH by forming the semidehydroascorbate free radical that is subsequently reduced by GSH, see for review [22]. The accurate assessment of oxidative stress in biological systems is a problem for all investigators working on the role of free radical damage in disease. Numerous biotechnological applications for potential recovery have been described to measure various free radical damage product or antioxidant status in soil and marine polluted areas as genes encoding stress related proteins and/or ultrastructural alterations [1, 23–29].

In the present research we used the method described in [30], and modified in [4], to determine total soluble antioxidant capacity in *Podarcis sicula* testis in recrudescence phase collected from the polluted sites (C) pre and post remediation and from the unpolluted site (N). The assay provides for the formation of phosphate/Mo(V) complex at acidic pH, after reduction of Mo(VI) to Mo(V). All samples (0.5 g), reduced to fine powder, were extracted with water (1 ml/g) for 1 h at room temperature in the dark. The extracts were subsequently centrifuged at 10,000× g for 20 min. This procedure was repeated twice and the two supernatants were combined and kept at 4°C. For the spectrophotometric determinations, aliquots of samples (0.1 ml) were mixed with the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 min, after which the absorbance at 695 nm was measured. A blank solution containing 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample was analyzed under the same conditions of the samples. Total soluble antioxidant capacity was expressed as equivalents (mmoles g-1 fresh weight) of ascorbic acid. For statistical treatment of the data, the Mann-Whitney U test was used.

The results show that, in *Podarcis sicula* testis collected from polluted sites (C) before remediation, total soluble antioxidant capacity is significantly lower than that measured in the sample from unpolluted site (N). Total antioxidant capacity detected in samples collected from polluted site after remediation, instead, is comparable to that of unpolluted site (N) (**Figure 3**).

Thereafter in *Podarcis sicula* testis, collected from polluted sites, the decrease of total soluble antioxidant capacity results correlate to the oxidative stress insurgence, responsible in turn for oxidative DNA damages and PARP activation.

2.4. Repair of DNA damage in lizard testis

The cells are continually exposed to different signals of extrinsic and intrinsic stresses, (i.e. genotoxic, oncogenic, inflammatory, metabolic stresses) [31]; their propagation involves the

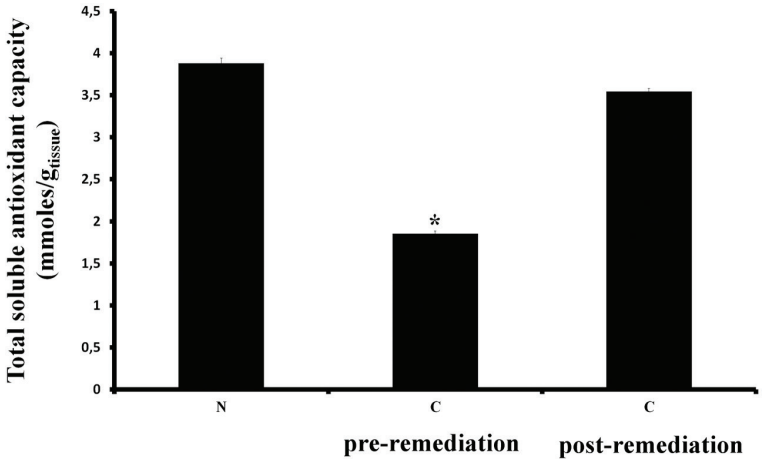

Figure 3. Total soluble antioxidant capacity in the testis of the lizard, *Podarcis sicula*; *N*: unpolluted site, **C**: polluted sites. *The significance differences of C pre remediation values compared with the N and C post remediation ($P < 0.05$).

cross-talk among multiple signaling pathways that lead to defined outcomes. Cell responses to stress determine a series of regulatory processes occurring at genomic, transcriptional, post-transcriptional, translational and post-translational levels. These need a complex network of sensors and effectors from multiple signaling pathways that includes poly(ADP-ribosyl)ation reactions [32]. Poly(ADP-ribosyl)ation is one of the post-translational protein modifications able to influence specific nuclear proteins. Modification of protein with ADP-ribose polymers (pADPr) is a reversible process where pADPr synthesis from NAD+ is catalyzed by pADPr polymerases (PARPs). Following DNA damage, PARPs modify histones, non-histonic and enzymatic proteins by means of long and branched ADP-ribose polymers. These polyanions reversibly alter the functions of accepting proteins, inducing conformational changes, and a net negative charge that, in the case of histones, destabilizes DNA interaction. Poly(ADP-ribosyl)ation is involved in the regulation of numerous cellular functions related to the maintenance of the genomic integrity and the expression and propagation of gene information [33]. It is also implicated in response to abiotic and biotic stresses [34, 35], in stress tolerance [36, 37] and in developmental processes [33, 38]. On the basis of these knowledge, we decided to assay PARP activity in nuclei of *Podarcis sicula* testis in the recrudescence phase collected from polluted sites before remediation, to indirectly verify whether and how the exposure to pollution induce DNA damage and, whether, the remediation was a positive effect on the restore of genomic integrity [4]. The isolation of nuclei was performed at 0–4°C. Briefly, all operations were carried out on ice or at 4°C starting from 500 mg of testicular tissues of lizards from polluted sites (C), before and after remediation, and unpolluted site (N). Tissues were harvested, cut and resuspended in 10 mM TrisHCl (pH 7.0), 1 mM EDTA, 1 mM EGTA, 1 mM $PhMeSO_2F$, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 0.5% Triton X-100 (1/4, w/v) (buffer A). The samples were homogenized for 30–40 s at low speed by an Ultra Turrax T8 (IKA-WERKE) and the homogenates filtered through three layers of cheesecloth. The filtrate centrifuged at 1500 × g for 30 min at 4°C and the pellet (containing nuclei) suspended in buffer A was centrifuged as above for three times. The pellets (nuclear fractions) washed with

buffer A without Triton X-100 (buffer B) were suspended in a small volume of buffer B containing 2% glycerol. The enzymatic activity was performed as described essentially in [4, 39] for testis nuclear fractions of lizards from polluted sites (C), before and after remediation, and unpolluted site (N). The reaction mixture (final volume 50 μl) contained 0.5 M Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 10 mM DTT, 0.4 mM [^{32}P]NAD+ (10,000 cpm/nmole) and a defined amount (20 μg proteins) of whole nuclear fraction from examined lizard testis. After incubation for 20 min at 25°C, the transfer onto ice and addition of 20% (w/v) trichloroacetic acid (final concentration) determine the stopping reaction. The mixture filtered through Millipore filters (HAWPP0001, 0.45 μm) was washed with 7% trichloroacetic acid. The activity was detected as acid-insoluble radioactivity by liquid scintillation in a Beckman counter (model LS 1701). One PARP unit is defined as the amount of enzyme required to convert 1 nmol of NAD+/min under standard conditions. The highest PARP activity is measured in nuclei of *Podarcis sicula* testis collected from polluted sites (C) before remediation. In these, indeed, the poly(ADP-ribosyl)ation levels are more than 100 times higher than those measured in nuclei of samples collected from both the polluted sites after remediation and control site, in which a basal PARP activity is detected. The Spearman test confirmed an indirect correlation ($\rho = -1$) between the PARP activity and the antioxidant capacity for all the examined samples. In fact, in samples before remediation, where the antioxidant capacity is lower than control, PARP activity is significantly higher and vice versa in unpolluted site, N, and C post-remediation samples (**Figure 4**).

These interesting evidences suggest that in *Podarcis sicula* testis collected from polluted sites pre-remediation, the exposure to polluted compounds might have caused the significant decrease of total soluble antioxidant capacity caused by ROS increase and consequent insurgence of oxidative stress. In turn, this is responsible for oxidative DNA damage that have induced activation of PARP, biosensor of DNA damage, being involved in DNA repair. In

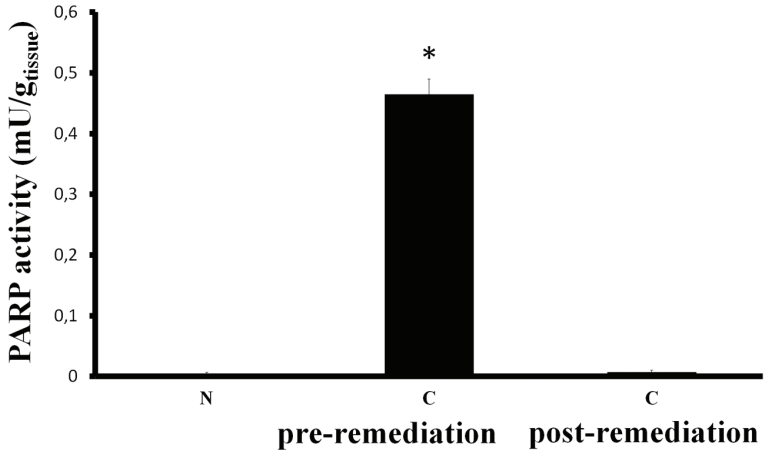

Figure 4. PARP activity in nuclei testis of the lizard, *Podarcis sicula*; **N**: unpolluted site, **C**: polluted sites. *The significance differences of C pre remediation values compared with the N and C post remediation ($P < 0.05$).

testis collected post remediation, DNA repair seems to be occurred, as the total antioxidant capacity and PARP activity levels return to values measured in sample of unpolluted site.

3. General discussion and conclusions

The purpose of the ROS assays performed on animal organism living in metal-contaminated sites is primarily the evaluation of the environmental risk, assessing its variation in case of remediation. The soil, as known, is a key link in the energy flow and nutrient cycling that characterize the ecosystem earth. According to scientific literature, soil biodiversity i.e. can provide useful insights in a number of issues: environmental impact studies and assessments, remediation programs, sustainable land planning and management, food safety and production sustainability, environmental monitoring and assessment programs, desertification or climate change prevention, adaptation and mitigation projects. Numerous studies indicate that soil contamination impacts on the conservation and sustainable exploitation of resources as well as on human reproductive health [40, 41]. The production, in a large scale, of a variety of chemical compounds is causing the deterioration of environmental quality and human health [42]. The accumulation of municipal waste, the use of fertilizers, motor traffic, incinerators, the thermo-electric power plants and many industrial processes have led to a progressive accumulation of xenobiotics [28]. This affects on the regulation of the natural cycle of water, air, organic and mineral substances, finally resulting in a biodiversity impairment. The mechanical and chemico-physical decontamination processes are expensive and tend to remove the biological activity of soil, modifying its intrinsic characteristics. For this reason, researchers have developed many alternative technologies based on biological processes [43].

A reliable monitoring of contaminated soil or marine remediation requires the species identification [39, 44] and their suitable indicators (https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=285170, [22]). Ecoremed, the project of remediation of the Land of Fires, adopted an ecofriendly approach in which many bioindicators with the inclusion of the health evaluation of vertebrate biosentinels have been applied. In our subaction research, we obtained realistic results in monitoring metal-contaminated ecosystems, analyzing the relation between the reproductive health of selected biosentinels and the soil contamination. We first confirmed the possibility to define a protocol to measure the reactive oxygen species (ROS), the total antioxidant capacity and DNA repair in the testis of the *Podarcis sicula*, useful biosentinel [45], collected in contaminated (C) and in non-contaminated soil (N). Once polluted sites had been restored (a process which took 2 years) all parameters were re-evaluated and their variations examined [4]. Interestingly, results converged in demonstrating that lizard testis of the Land of Fires, before remediation, showed free radicals overexpression.

Based on these evidences, in the framework of the Life Ecoremed Project, the present chapter proposes an optimized monitoring strategy for soil decontamination based on ROS detection in vertebrate biosentinels. The presented data are not restricted to specific sites, since we explored various sites localized in the Land of Fires in which the specified biosentinel was found to flourish. We supplemented our review of published studies with a complete data collection about ROS, total soluble antioxidant capacity and poly(ADP-ribosyl)ation on

lizard testis, providing information about protocols used for their determination and brief comments of results obtained. Specific purpose of our studies was to evaluate the risks in recrudescence phase for lizard reproduction health related to the presence of the ubiquitous contaminants in soil compartment. We surveyed each sites for many markers and we realized how important the ROS detection for remediation assessment was and can be.

Other procedures for ROS testing could significantly affect the results, potentially leading to oxidative damage artifacts. As an example, we showed that the lighting conditions maintained during the spin trap experiments favored a realistic quantitative detection. The average hyperfine coupling constants detected in our research, result compatible with trapping of both carbon- and oxygen-centered radicals [46, 47]. It is now well-assessed that spin trapping investigations are not able to discriminate among the different products of lipid oxidation ($ROO^{\bullet}$, $RO^{\bullet}$ and $R^{\bullet}$), since they generate spin adducts with similar coupling constants. Nevertheless, we are interested in their total concentration, whose altered values can be seen as a finger-print of oxidative stress conditions. The comparison between values obtained for lizard testis from different sites and their variations before and after remediation supports the reliability of our ROS content estimates.

The choice of testis was primarily related to the main goal of our study, which was the monitoring of the remediation effects on the reproductive health of biosentinels. The testis is perfectly suited for ESR investigation because it presents abundance of highly unsaturated fatty acids, high rates of cell division and variety of enzymes, thus resulting very vulnerable to ROS overexpression. Lizards from an unpolluted site, collected in the reproductive stage, show the lipid radical amount normally involved in the metabolic cell testis event/reproductive stage through the spermatogonial recrudescence. All metabolic process implies, in normal condition, high rates of mitochondrial oxygen consumption and reactive oxygen species (ROS) generation. As known, ROS overexpression can be harmful, depending on the nature and the concentration as well as the location and length of exposure [1, 10]. The level of lipid radical concentration, in samples from pre remediation soil, is significantly higher with respect to those detected in samples from the unpolluted site, showing a clear correlation with the oxidative stress from the environment. At metabolic concentrations, the ROS are involved in cell physiological processes [2–9] such as control of cell proliferation, playing an important role as messenger in signal transduction pathways; in gonads they may be beneficial or indispensable for gametogenesis processes. The amount of lipid radicals after remediation soil reduces, becoming more similar to that in unpolluted site demonstrating the efficiency of the remediation. High doses and/or inadequate removal of ROS caused by several mechanisms, i.e. bioactivation of xenobiotics, inflammation, increased cellular metabolism, activation of oxidases and oxygenases and loss of antioxidant capacity, cause severe metabolic malfunctions as alterations in gene expression and protein [22, 33, 48, 49]; consequently, we projected to monitor the total antioxidant capacity. As known, to address the risks very common in polluted areas, testis has developed a sophisticated array of antioxidant systems comprising both enzymes and free radical scavengers [23, 50] that can be both diagnostic and prognostic tools [1, 4]. As mentioned earlier, the use of these additional detections was useful to confirm the high levels of ROS detected when the total capacity decreases.

We realized that the increase of ROS and the consequent reduction of antioxidant capacity in *Podarcis sicula* nuclear testis is one of the causes responsible for DNA damage and not all cells in the body have the same degree of genotoxic damage. We also looked into the possibility of extending our research to take into account the DNA repair using PARP activity. Conversely,

the use of the DNA repair by PARP activity contributes to explain why the species where numerous also if their DNA testis were seriously damaged. As known, PARP represents the first molecular response to genomic material damage and is correlated to DNA repair [33, 39]. Regarding this marker, in samples analyzed after remediation, we observe the return of PARP activity and antioxidant capacity values to those measured in the respective controls, suggesting that DNA repair successfully has occurred in all lizard testis examined.

Currently, many bioassay endpoint metrics are species-specific. The approach reported here was applied to other species of lizard of the same genus *Podarcis* as *Podarcis sicula* and *Podarcis muralis* with similar gonadosomatic index (GSI = [testis weight/body weight] × 100), as *Podarcis muralis*, revealing correlation between examined biomarkers (data not shown). Although the hypothesis of effect is generally accepted, we showed these results are in agreement with that found during the investigation of soil remediation of the Life Ecoremed project. In this framework, we think that biomarkers reported here support the value of ROS detection by ESR. Furthermore, the current applied researches demonstrate that antioxidant capacity and DNA repair were sensitive in this species.

Data suggest that, in the testis of *Podarcis sicula*, ROS detection is highly sensitive and involved in the high oxidative insult of each site examined. The analyses done when gonads do not release sperms during spermatogonial recrudescence, let us to eliminate the oxidative stress related to this natural event. Additional studies are ongoing for the evaluation on spermatozoa, mature germinal cell, of lizard separated from testis by centrifugal elutriation. The preliminary investigations related to contaminated soil gave significant evidence of deviations from reactive oxygen species levels in spermatozoa lizard of unpolluted site too.

In conclusion, in the framework of a European remediation project (11 Env/IT/275 Ecoremed) we developed a bioassay to assess the ROS content in a vertebrate biosentinel. ESR measurements of ROS in the biosentinel *Podarcis sicula* testis form the basis of a rapid and reliable assay for decontamination bioassessment and represent a promising tool to be included in the new toxicological screening.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

All authors contributed to conception and design of the experiments. All the authors have given their approval to the final version of the manuscript.

Compliance with ethical standards

The studies were conducted in strict accordance with European (Directive 2010/63) and Italian (Decreto Legislativo n°116/1992) legislation on the care and use of animals for scientific purpose.

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