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THE SARNO RIVER AND THE BIO-PRESERVATION OF SPECIES OF THE GENUS *Pelophylax*

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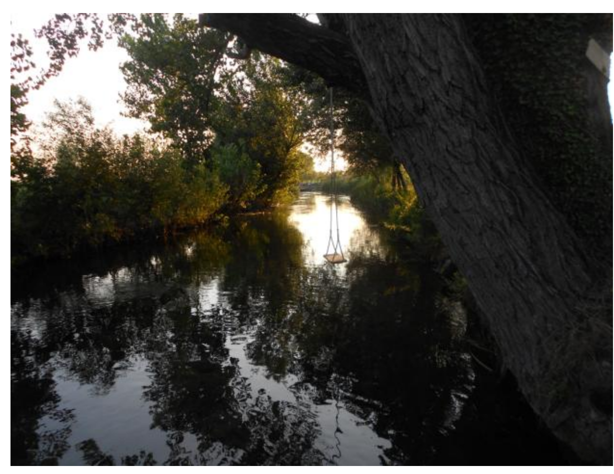
The Sarno River And The Bio-preservation Of Species Of The Genus *Pelophylax*

The frogs are sending an alarm call to all concerned about the future of biodiversity and the need to protect the greatest of all open-access resources: the atmosphere.
(AR Blaustein)



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ABSTRACT

The set of living beings, animals and plants that inhabit our planet in their wide variety, as the result of long and complex evolutionary processes, represent the diversity of life on Earth, defined by the term biodiversity.

During the twentieth century, the world has experienced an unprecedented technological and industrial explosion, that has had several negative consequences, including an increasing risk due to the effects that some compound and industrial discharges, means of threats to biodiversity (Massa, 2005; Agapow et al., 2004). Environmental degradation, the introduction of non-native species, over-exploitation of species, climate change, the presence of environmental contaminants, and the use of fertilizers, pesticides and often unpleasant herbicides exert toxic effects on the endocrine system and have, each, a prominent place among the risk factors for reproductive health and therefore for the maintenance of a specie (Hoffmann et al., 2010; Stork, 2010).

Scientific research, lately, basically it's oriented at the study of the toxic effects that many compounds have on molecules, cells, tissues, organisms and populations belonging to different classes of vertebrates with the aim to identifying, evaluating the damage and even more to predict the risk of species extinction (Mace et al., 2012).

The following study, starting from biosensors potentially at risk of extinction species, such as Amphibians belonging to the genus *Pelophylax,* present along one of the most polluted river in Europe, develops and validates, with non-invasive biomarkers, skin and testis by gonadectomy, an innovative approach in environmental diagnostics for their preservation and the subsequent evaluation of the state of health of watercourses.

In the first part of this research was detected and monitored the state of the health of the Plain of the Sarno river on the base of previous studies, by a synergic analysis of physical, chemical, biological and eco-toxicological components, which permitted to assess the

ecological vulnerability of the river ecosystem, evidenced by the actual subletal stress on the organisms present in the Plain, due to the concentration of pollutants deriving from human and industrial activities.

The study of the area allowed to define two favorite site, with different pollution degree.

In the sites was monitored the presence of different frogs belonging to the genus *Pelophylax*, which were, in first instance, morphologically identified by classic taxonomic keys; since the difficult to specifically identify some specimens each other, due to the phenomenon of hybridization, only specimens of dubious belonging were analyzed by molecular approach, using the barcoding method. This even permitted to widen the genetic database for the specie *Pelophylax bergeri*, chosen as biosentinel specie.

Furthermore, the evaluation of the oxidative and genotoxic damage on skin biopsy and gonadectomy, was carried out to the assessment of innovative non-invasive techniques.

The second part of this research was devoted to develop an effective method for an eco-monitoring based on the analysis of ROS species in frog tissues, by an optimized spin-trapping EPR protocol. Furthermore, the genotoxic damage was detected by mobility shift, by monitoring the fragmentation degree of DNA, extracted by skin biopsy and gonadectomized testis. It's interesting to note that, while the skin resulted to be damaged in both sites of Sarno River, testis seem to have developed a repair mechanism. This carried out at the study of enzymes involved in repair systems. After characterization and purification of the enzyme PARP-2, which is known to be responsible for the repair of DNA damage caused by heavy metals, the attention was paid to the testis. So, thanks to the examination of repair to DNA during spermatogenesis we realized the PARP, implicated in genome surveillance and protection, might represent in frog spermatogenesis an appealing tool for genotoxic risk assessment, useful to define a warning alarm for its survival.

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LIST OF ABBREVIATION

ROS: Reactive Oxygen Species

PARP1: poly(ADP-ribose)polymerases1

PARP2: poly(ADP-ribose)polymerases2

EPR: Eletron Paramagnetic Resonance

EDCs: Endocrine Disruptor Compounds

PCB: polychlorinated biphenyl

PBB: Polybrominated biphenyl

BPA: Bisphenol A

DTT: Dithiothreitol

DES: Diethylstilbestrol

PDCF: polychlorinated dioxines

Cd: Cadmium

As: Arsenic

Pb: Lead

Hg: Mercury

Mn: Manganese

IARC: International Agency for Research on Cancer

MED: Europe en Mediterranèe

IUCN: International Union for Conservation of Nature

BoLD: Barcode of Life Database

MtDNA: Mitochondrial DNA

Cytb: cytochrome b

CO I: cytochrome oxidase subunit I

CO II: cytochrome oxidase subunit II

EC: electrical conductivity

BOD: Biological Oxygen Demand

COD: Chemical Oxygen Demand

$NaHCO_3$: Sodium Carbonate

NaCl: Sodium Chloride

KCl: Potassium Chloride

$Ca(HCO_3)$: Calciu Carbonate

Cr: Chrome

Cu: Copper

ARPAC: Agenzia Regionale Protezione Ambientale Campania

NO_3-: nitrate

NH^{4+}: ammonium

EBI/IBE: Extended Biotic Index

LIM: Livello di Inquinamento da Macrodescrittori

FSH: follicle-stimulating hormone

LH: Luteinizing hormone

GnRH: Gonadotropin-releasing hormone

ESR: Electron Spin Resonance

NMR: Nuclear Magnetic Resonance

ASL: Agenzia Sanitaria Locale

MS222: Tricaine mesylate

PCR: Polymerase Chain Reaction

PBN: α-phenyl-tert-butylnitrone

HPLC: High Performance Liquid Chromatography

GHz: Giha Hertz

MW: Mega Watt

TBE: Tris-Boric Acid-EDTA

EDTA: Ethylenediaminetetraacetic acid

$PhMeSO_4F$: phenylmethylsulfonyl fluoride

$MgCl_2$: Magnesium Chloride

NP-40: nonyl phenoxypolyethoxylethanol

SDS: Sodium Dodecyl Sulphate

EGTA: ethylene glycol tetraacetic acid

NAD: Nicotinamide adenine dinucleotide

TCA: Trichloroacetic acid

PVDF: polyvinylidene difluoride

Rf: Relative mobility

Lc: Length dye

Lf: Length frontgel

SH: Skin Homogenate

SNF: Skin Nuclear Fraction

SCF: Skin Cytoplamic Fraction

SD: standard deviation

DM: Dalton Marker

RTN: Rat Testis Nuclei

ADPR: polyADPribose

E66: Eluted protein 66KDa

E150: Eluted protein 150KDa

SSa: Skin Sarno

SSc: Skin Scafati

SML: Skin Matese Lake

TSa: Testis Sarno

TSc: Testis Scafati

TML: Testis Matese Lake

CHAPTER 1

INTRODUCTION

1.1 THE PLAIN OF THE SARNO RIVER AND ITS ACTIVITIES

The river Sarno, since 1988 has been declared "high risk area of environmental crisis" by the Ministry of the Environment (Ministerial Decree, 1994) being one of the most polluted river in Europe.

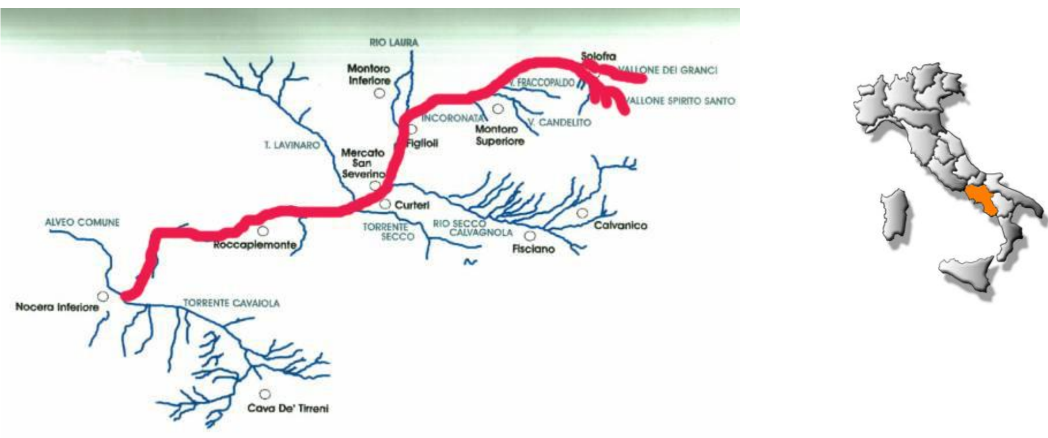

Figure 1.1. Hydrographic network of the River Sarno (Southern Italy).

The basin originates, from east to west, from the Picentini mountains (in the municipality of Solofra) to the Gulf of Naples, where it flows into the Tyrrhenian Sea, between the towns of Torre Annunziata and Castellammare di Stabia, opposite the picturesque "rock of Rovigliano"; while going from south to north, it goes from the Lattari mountains to the mountains of Sarno, for a total extension of 438 km², and affects the provinces of Salerno, Naples and Avellino, and up to 39 municipalities. The hydrographic network consists of three major rivers: the streams Solofrana and Cavaiola, which flow into the artificial

stream Nocerino. The Plain of the Sarno is one of the largest and most important economic areas of Campania. The morphology of the plain, together with the composition of the soil and climate, make it an ideal area for agricultural and industrial development (De Pippo, 2006).

The initial stretch of the river, at one time, was fed by numerous springs, but from the middle of the last century, the main courses were used to power up the Campano Aqueduct. The Sarno river is still powered by waters of three sources: the Outfall, at north-west of the town of Sarno and from which also originated the Canal “Conte di Sarno”. The second source, Palazzo, is located behind the town, and the third, Santa Marina, near the municipality of Lavorate.

These three feed streams, the "Outfall of the Rio", "Water of Palazzo" and "water of Santa Marina", which, after a run respectively of 2.5 km, 2 km and 6.7 km, meet together in a single stream, in the Nocerino riverbed, mark the boundaries of the provinces of Salerno and Naples, and of the towns of Sarno, Striano, Poggiomarino, San Valentino, San Marzano, Scafati, Pompei, Castellammare di Stabia and Torre Annunziata.

In the territory there are food processing industrial centers, metalworking and leather industries, towing to the local economy, but they also bring a high level of environmental pollution in the entire area (Department of Civil Protection: www.protezionecivile.gov.it). The combination of high population density and the presence of highly polluting economic activities has created a situation of extreme precariousness of the environment, which constitutes an obstacle to the development of the area. For these reasons, the Sarno river was, perhaps, the only one among all the rivers of Campania, to be subject of numerous investigations and monitoring campaigns, although sporadic, prompted by the perennial situation of deprivation in which it pours, from the feared danger of health risks for the large population, and numerous species of Fishes, Amphibians, insects and birds present.

The great fertility of the soil have made the agriculture the driving economic activity for the municipalities of the plain, where there are numerous agri-food companies and for the food packaging, which spill their discharges in the watercourse.
The slope of the Solofrana, indeed, is more affected by the presence of numerous tanning industry, which accounts for about 50% of the total national production; such activity, however, is one of the most polluting in the entire basin, due to the use of hazardous substances used for the disposal and processing of materials.

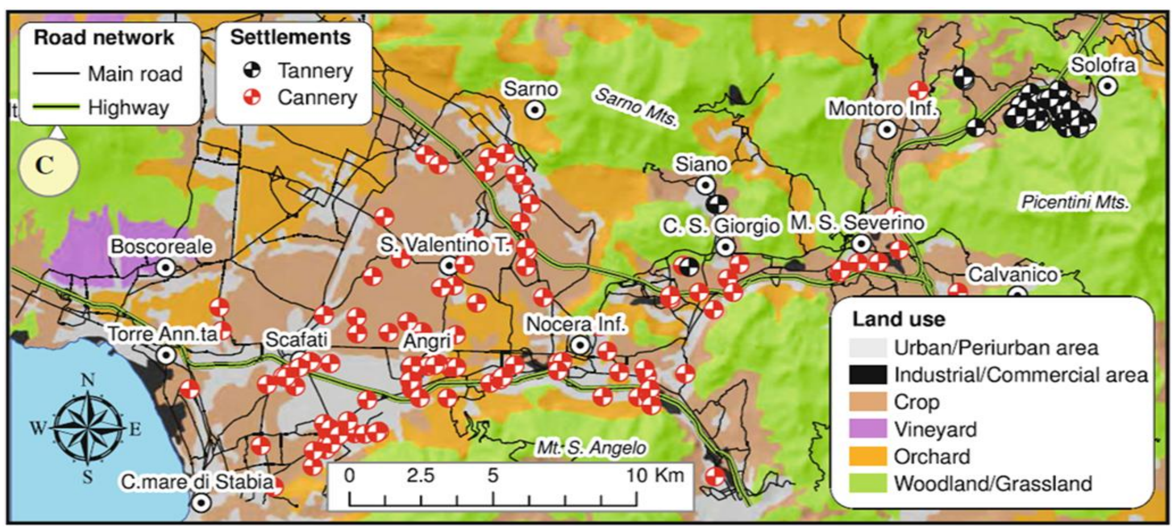

Figure. 1.2. The Sarno river basin: localization of tanneries and cannieries farms. (from Albanese et al., 2013).

On the west side, and specifically in the town of Torre Annunziata, the main industrial activity is the pharmaceutical industry, with the presence of the Company Novartis Pharmaceuticals, specialized in the production and packaging of drug-coated tablets.

1.1a Pollutants and their effects

The pollution of water or soil, if we neglect the natural contamination, can be defined as any alteration in the chemical, physical, biological or microbiological quality that causes an unacceptable depreciation for the purposes of normal use or preservation of the

environment (Bourez et al., 2013). This definition leads to a classification of pollutants in terms of chemical, physical, biological and microbiological components.

Chemical pollution is generated by the organic and inorganic substances released into the water, which may carry out their polluting action both directly and indirectly, through biological processes. Direct actions are carried out through the removal of oxygen from the body of water, caused by the presence of reducing agents (nitrates and/or phosphates), which are decomposed by bacteria that use for their vital processes the dissolved gas. When decomposers bacteria convert these substances, the level of oxygen in the water may decrease significantly until it's exhausted. This leads to an excessive growth of algae and aquatic plants, which deplete the oxygen from the water, and cause the consequent death of other aquatic organisms, such as fish, by eutrophication. An example of indirect action is exerted by heavy metals : low concentrations of inorganic mercury (in the form of salts) can not exert a direct effect, whereas its transformation into methyl derivatives, as a result of anaerobic preocesses, affects the value of water, since such compounds are highly toxic (Mannarino et al., 2013).

Physical pollution can be caused by changes in the volume, flow rate and bed of a body of water, by the increase or decrease of temperature, by the introduction of solid waste (Gou et al., 2014).

Biological pollution is caused by the entry, into water, of compounds that slow or inhibit the growth of those species which, through their metabolism, normally intervene in the processes of self-purification (Diamanti et al., 2009). They are all the acid or basic substances that alter the neutrality of the water, cations of heavy metals, chromates, cyanides and all soluble inorganic pollutants in water.

Microbiological pollution is caused by the introduction of intestinal microorganisms, such as coliform bacteria (*Streptococcus, Staphylococcus*, etc), pathogens (*Salmonella, Shigella,* etc.); viruses responsible for the transmission of hepatitis and polio and, generally, all disease agents (bacteria, viruses, protozoa and parasitic worms) that are found in sewage

systems and untreated waters. Large amounts of these compounds in the water make it non-potable and cause, eventually, death of the aquatic life.

The physico-chemical and biological pollutants that can be found in the environment are often a threat to the health of humans and animals, as they can interfere with the biosynthesis, metabolism and function of sex steroids and/or thyroid, causing adverse effects on the endocrine system and the reproductive system, and for that reason they are called endocrine disrupting chemicals, EDCs (Diamanti et al., 2009).

Generally, the endocrine disrupting chemicals show low water solubility and high lipid solubility and, due to their lipophilic character, they have the ability to diffuse through the cell membranes and thus have the ability to progressively concentrate in the fatty tissues of organisms (Thornton, 2001).

The EDCs are able to reproduce the physiological activity of the hormones, participating in the same chemical reactions and causing the same effects or increasing them by acting as agonists, blocking, with a competitive action, the hormone receptors and thus block the activity of natural hormones; interfere with the synthesis, transport, metabolism and excretion of hormones, altering the physiological concentrations and consequently the corresponding function (Waring & Harris, 2005).

The group of molecules identified as endocrine disruptors, as shown in Table 1, is highly heterogeneous and includes synthetic chemicals such as industrial solvents and their by-products, among which dihydric phenols (PCBs, PBBs, BPA), plasticizers, pesticides (DTT), pharmaceutical compounds or pesticides (DES), heavy metals, but also compounds of natural origin such as phytoestrogens (Kuiper et al., 1998; Dickerson, 2007).

Persistent halogenated contaminants (dioxins, polychlorinated biphenyl-PCB, polychlorinated-PDCF), as it's known, show a correlation between serum levels of PCBs and dioxins compared to an abnormal motility of the spermatozoa, to their number and morphology (Meeker and Hauser, 2010).

Pesticides, phytodrugs (thiocarbamates, organochlorine compounds, imidazoles, triazoles, triazines) determine antiandrogenic action, as evidenced by macroscopic sexual

changes found in animals, such as the emasculation in rats and fish (Hayes et al., 2011) and estrogen production and hermaphroditism in frogs (Guillette, 2000; Hayes et al., 2002; Storrs-Mendez and Semlitsch, 2010). They are certainly the most numerous group of substances, and highly hazardous, since the mechanism of their accumulation in the tissues and in the environment (Baatrup et al., 2001; Bourez et al., 2013).

Industrial substances that exhibit estrogen-like and anti-adrogenic action, are the bis-phenol A (BPA), that has a great interest because of its wide distribution in plastics, showing a particularly estrogenic activity by interacting with membrane receptors even at low doses (Quesada et al., 2002; Zhang et al., 2014).

Phthalates are considered estrogen modulators, since they showed the induction of high levels of luteinizing hormone and increased serological levels of sex hormones associated with cellular hyperplasia (Akingbemi et al., 2004). Phytoestrogens which are able to change the levels of luteinizing hormone (LH) and prolactin (PRL) acts directly on the central nervous system (Romanowicz et al., 2004). They also lead to morphological and histopathological changes in the vagina and the ovaries and uterus (Masutomi et al., 2004).

Some heavy metals such as Cd, As, Pb, Hg, Mn are toxic for the reproductive system, showing, in the female population, an increased risk of spontaneous abortion, intrauterine fetal death, preterm delivery (Sharara et al., 1998), while in the male population lead to oligospermia and decreased sperm motility (Pant et al, 2003, Sura et al., 2011). Mercury, lead, manganese and nickel are considered to be embryotoxic and more targeted studies have also highlighted an estrogen-like effect; in addition mercury, lead and manganese ingested with food, are attributed to damage the central nervous system of the fetus and/or mental delay. Particularly important seems to be cadmium, an element added by the IARC in Group I as "carcinogenic to humans" and defined as a new environmental estrogen (Jarup, 2003).

1.2. SPECIES PRESENT ALONG THE PLAIN OF THE SARNO RIVER AND IDENTIFICATION OF THE BIOSENTINEL SPECIE

The ecosystem of the basin of the Sarno river is highly dependent on interactions between the river itself and the surrounding environment, that is the civil sewage, industrial waste and residues of chemicals used in agriculture and from the continuum of environments that alternate from the source to the outfall of the river, since at different sections also correspond different depths, and thus the factors that influence the relationships and exchanges between the different sections. Important morphological elements, in fact, can be found, and they're essential for the development and survival of certain species.

A major area is the area of the riparian area, which provides food and habitat for the development of micro-organisms, micro and macro Invertebrates, Vertebrates, and especially for Reptiles and Amphibians, as it provides wetlands suitable for reproduction and development of the embryo, while the roots and the presence of overhanging branches provide the ideal habitat for the entire life cycle of fishes.

This area, however, is often defined and dismembered by man at the time, more and more parts are removed from the natural environment and used for agricultural or industrial use.

The wetland and coast host a large number of wildlife species, thanks to the richness of the soil. In Table 1 (a and b) are listed the species surveyed along the Sarno River Plain and in the MED program from 2007 to 2013 as a result of project "Characterization of biotic community of Sarno's catchment area and preliminary analysis of high waterway environmental condition, by application of biotic indices" and the taxonomy of both Invertebrates, and Vertebrates.

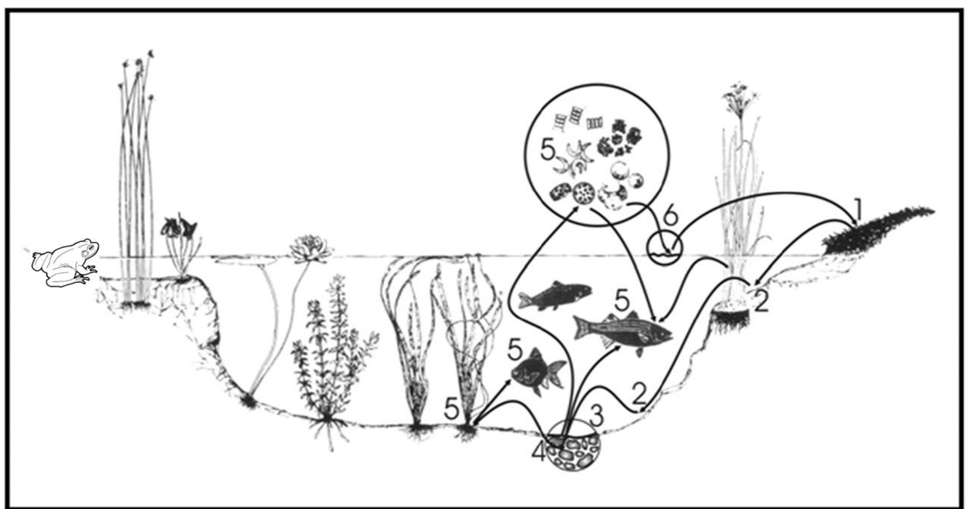

Figure 1.3 Scheme showing connections between various segments of the ecosystem in the presence of xenobiotics. (1) Land, (2) sediment, (3) sediment–water interface, (4) interstitial water, (5) biota, and (6) air–waterinterface. (modified from Torres et al. 2008).

In addition to the community of Mammals, birds, Reptiles, Amphibians and Fishes many macroinvertebrates have been identified in view, mostly insects belonging to the orders Plecoptera, Trichoptera and Effemerottera, also mollusks, annelids and arthropods.

Table 1.1. List of Invertebrates and Vertebrates identified along the Sarno river integrated (*) with their IUCN classification, starting by the MED programme 2007-2013 (http://www.free-med.eu/fichiers/files/repeuplement_fleuve_sarno.pdf)

Phylum	Class	Order	Family	*Genus*	*Species*	Common name
Arthropoda	Insecta	Plecoptera	ND	ND	ND	ND
Arthropoda	Insecta	Trichoptera	ND	ND	ND	ND
Arthropoda	Insecta	Ephemeroptera	ND	ND	ND	ND
Arthropoda	Malacostraca	Decapoda	Astacidae	*Austropotamobius*	*Austropotamobius pallipes*	White-clawed crayfish
Annelida	Clitellata	Hirudinida	Hirudinidae	*Hirudo*	*Hirudo medicinalis*	European medicinal leech

Phylum	Class	Order	Family	*Genus*	*Species*	Common name
Chordata	Actinopterygii	Anguilliformes	Anguillidae	*Anguilla*	*Anguilla anguilla*	European eel
Chordata	Actinopterygii	Clupeiformes	Clupeidae	*Alosa*	*Alosa fallax*	Twait shad
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Rutilus*	*Rutilus rubilio*	South European roach
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Squalius*	*Squalius cephalus*	Chub
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Tinca*	*Tinca tinca*	Doctor fish
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Scardinius*	*Scardinius erythrophthalmus*	Common rudd
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Alburnus*	*Alburnus albidus*	Italian Bleak
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Barbus*	*Barbus plebejus*	Italian Barbel
Chordata	Actinopterygii	Cypriniformes	Cobitidae	*Cobitis*	*Cobitis taenia,*	Spined Loach
Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salmo*	*Salmo trutta ,*	trout
Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salmo*	*Salmo trutta fario*	Brown trout
Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Oncorhynchus*	*Oncorhynchus mykiss*	Rainbow trout
Chordata	Actinopterygii	Gasterosteiformes	Gasterosteidae	*Gasterosteus*	*Gasterosteus aculeatus*	Three-spined stickleback
Chordata	Actinopterygii	Mugiliformes	Mugilidae	*Mugil*	*Mugil cephalus*	Flathead Mullet
Chordata	Actinopterygii	Perciformes	Mugilidae	*Liza*	*Liza ramada*	Thinlip mullet
Chordata	Actinopterygii	Mugiliformes	Mugilidae	*Liza*	*Liza aurata*	Golden grey mullet
Chordata	Actinopterygii	Perciformes	Mugilidae	*Liza*	*Liza saliens*	Leaping mullet
Chordata	Actinopterygii	Atheriniformes	Atherinidae	*Atherina*	*Atherina boyeri*	Big-scale sand smelt
Chordata	Actinopterygii	Perciformes	Moronidae	*Dicentrarchus*	*Dicentrarchus labrax*	European seabass
Chordata	Actinopterygii	Perciformes	Sparidae	*Sparus*	*Sparus aurata*	Gilt head sea bream
Chordata	Actinopterygii	Perciformes	Blenniidae	*Salaria*	*Salaria fluviatilis*	Freshwater blenny

Phylum	Class	Order	Family	*Genus*	*Species*	Common name
Chordata	Petromyzontida	Petromyzontiformes	Petromyzontidae	*Petromyzon*	*Petromyzon marinus*	Sea lamprey
Chordata	Cephalaspidomorphi	Petromyzontiformes	Petromyzontidae	*Lampetra*	*Lampetra fluviatilis*	European river lamprey
Chordata	Cephalaspidomorphi	Petromyzontiformes	Petromyzontidae	*Lampetra*	*Lampetra planeri*	European Brook lamprey

Phylum	Class	Order	Family	*Genus*	*Species*	Common name
Chordata	Amphibia	Anura	Bufonidae	*Bufo*	*Bufo viridis ,*	European green toad
Chordata	Amphibia	Anura	Bufonidae	*Bufo*	*Bufo bufo,*	Common toad
Chordata	Amphibia	Anura	Ranidae	*Pelophylax*	*Pelophylax esculentus**	Edible Frog or Green Frog
Chordata	Amphibia	Caudata	Salamandridae	*Lissotriton*	*Lissotriton italicus*	Italian newt
Chordata	Amphibia	Anura	Ranidae	*Pelophylax*	*Pelophylax bergeri**	Italian Pool Frog

Phylum	Class	Order	Family	*Genus*	*Species*	Common name
Chordata	Reptilia	Squamata	Colubridae	*Natrix*	*Natrix natrix*	Grass snake
Chordata	Reptilia	Squamata	Colubridae	*Natrix*	*Natrix tessellata*	Dice snake
Chordata	Reptilia	Squamata	Colubridae	*Hierophis*	*Hierophis viridiflavus*	Green Whip Snake
Chordata	Reptilia	Squamata	Viperidae	*Vipera*	*Vipera aspis*	Central Italian aspide
Chordata	Reptilia	Squamata	*Lacertidae*	*Podarcis*	*Podarcis sicula**	Lizard
Chordata	Reptilia	Squamata	*Lacertidae*	*Podarcis*	*Podarcis muralis**	lizard

Phylum	Class	Order	Family	*Genus*	*Species*	Common name
Chordata	Aves	Gaviiformes	Gaviidae	*Gavia*	ND	Loon
Chordata	Aves	Podicipediformes	Podicipedidae	ND	ND	Grebe
Chordata	Aves	Pelecaniformes	Pelecanidae	*Pelecanus*	ND	Pelican
Chordata	Aves	Suliformes	Phalacrocoracidae	*Phalacrocorax*	ND	Cormorants
Chordata	Aves	Pelacaniformes	Ardeidae	ND	ND	Herons
Chordata	Aves	Ciconiiformes	Ciconiidae	ND	ND	Storks
Chordata	Aves	Pelecaniformes	Threskiornithidae	ND	ND	Spoonbills
Chordata	Aves	Phoenicopteriformes	Phoenicopteridae	ND	ND	Flamingos
Chordata	Aves	Anseriformes	Anatidae	ND	ND	Ducks, geese and swans
Chordata	Aves	Gruiformes	Gruidae	ND	ND	Cranes
Chordata	Aves	Gruiformes	Rallidae	ND	ND	Rails, coots and gallinules
Chordata	Aves	Charadriiformes	Haemotopodidae	*Haematopus*	ND	Oystercatchers
Chordata	Aves	Charadriiformes	Recurvirostridae	ND	ND	Avocets and Stilts
Chordata	Aves	Charadriiformes	Burhinidae	ND	ND	Stone-curlews
Chordata	Aves	Charadriiformes	Glaereolidae	ND	ND	Pranticole and Courser
Chordata	Aves	Charadriiformes	Charadriidae	ND	ND	Plovers and Lapwing
Chordata	Aves	Charadriiformes	Scolopacidae	ND	ND	Sandpipers, Curlew and Snipe
Chordata	Aves	Charadriiformes	Laridae	ND	ND	Gull
Chordata	Aves	Charadriiformes	Sternidae	ND	ND	Terns
Chordata	Aves	Accipitriformes	Accipitridae	ND	ND	Hawks, Eagles, Kites, Harriers
Chordata	Aves	Strigiformes	Strigidae	ND	ND	True owl
Phylum	**Class**	**Order**	**Family**	***Genus***	***Species***	**Common name**
Chordata	Mammalia	Erinaceomorpha	Erinaceidae	*Erinaceus*	*Erinaceus europaeus*	European hedgehog
Chordata	Mammalia	Soricomorpha	Soricidae	*Sorex*	*Sorex araneus*	Appennine shrew
Chordata	Mammalia	Soricomorpha	Soricidae	*Neomys*	*Neomys fodiens*	Eurasian water shrew
Chordata	Mammalia	Soricomorpha	Talpidae	*Talpa*	*Talpa romana*	Roman mole
Chordata	Mammalia	Rodentia	Sciuridae	*Sciurus*	*Sciurus vulgaris*	Red squirrel

Chordata	Mammalia	Rodentia	Gliridae	*Eliomys*	*Eliomys quercinus*	Garden dormouse
Chordata	Mammalia	Rodentia	Gliridae	*Glis*	*Glis glis*	Edible dormouse
Chordata	Mammalia	Rodentia	Muridae	*Apodemus*	*Apodemus sylvaticus*	Wood mouse
Chordata	Mammalia	Carnivora	Mustelidae	*Mustela*	*Mustela nivalis*	Least weasel
Chordata	Mammalia	Carnivora	Mustelidae	*Meles*	*Meles meles*	European badger
Chordata	Mammalia	Carnivora	Canidae	*Vulpes*	*Vulpes volpe*	Red fox
Chordata	Mammalia	Carnivora	Felidae	*Felis*	*Felis silvestris*	Wildcat

Purple: critically endangered, believed that it may be extirpated from its only known distribution
Deep red: endangered, found in very small numbers
Red: vulnerable, widespread but its range is disjunct and it is uncommon
Brown: near threatned, population size might not be much great, and a decline of >10% over the next three generations
Orange: least concern, wide distribution, presumed large population, and because it is unlikely to be declining fast enough to qualify for listing in a more threatened category
Green: not present in the IUCN red list

Many native species are presents, even mentioned by the residents of areas adjacent to the river, nowadays endangered (as *Austropotamobius pallipes*, Lereboullet, 1858), and this, as we know, is closely linked to water pollution from organic and inorganic pollution of agricultural, civil and industrial origin, as well as the destruction of riparian vegetation due to the overbuilding of the riverbanks, with the consequent reduction of self purification processes. Until the end of the seventies, in fact, the river was inhabited by various species of fish such as eel, shad, the arborella, crabs and shrimp particularly in the loops (Patti, 1999; La Fratta, 2002). In the table are, fortunately, many biomarkers between Reptiles, Birds and Amphibians. Among the latter, different species belonging to the genus *Pelophylax* that are sometimes difficult to frame taxonomically for the presence of hybrids (Lymberakis et al., 2007).

1.2a Barcoding and the importance of the mitochondrial DNA

Today, classical taxonomy still permit to identify species, but communities still going up growing and therefore the process requires a wider approach for unequivocal

confirmations (Kohler, 2007). The building up of genetic databases has an enormous potential for the identification of species, and it's a method that keeps in mind the ecology and biodiversity (Bisby, 2000).

Many studies have been performed on the ethology of the species and are still ongoing biometric analysis to highlight morphological differences (Lodè, 2000; Spigonardi et al., 2003). In order to develop reliable systems for the identification of taxa, different from genetic ones, several methodological approaches have been tempted, taking advantage of the opportunities offered by biological disciplines. The study of acoustic signals showed to be of big interest to specialists in this group of animals, and has already provided some promising results (Wilczynski, 1992; Spigonardi et al., 2006).

Paul Hebert, from the University of Guelph in Canada, in 2003, started to develop the idea of the identification of species through the use of a mitochondrial gene as "universal marker" (Hebert et al. 2003a, b); he gave to this approach the name of "barcoding", as a simulation of the barcode used on commercial products (Brown, 1997). Barcoding is proposed as a diagnostic technique, to implement the identification of a species through the use of fragments of mitochondrial DNA. It offers a wide range of benefits, such as the speed of the system, the possibility of identification of a species at any stage of the cycle, and the construction of specific databases (Savolainen et al., 2005).

DNA barcode sequences are generally organized by submission to databases such as GenBank or the Barcode of Life Database, BoLD (Ratnasingham and Hebert, 2007) a good DNA identifier has to have some characteristics: (i) an archived library of sequences for the various species; (ii) a standardized method to compare the sequences; (iii) a criterion for the delimitation of species; and (iv) species-specific diagnostic criteria for the attribution of a specific nomenclatural epithet (Goldstein and DeSalle, 2010).

The gene chosen for this method, in Animals, is the gene for cytochrome oxidase I, which has high interspecies variability and low intraspecies variability, it encodes the subunit '1 of the enzyme complex involved in electron transport chain in the inner mitochondrial membrane.

The mitochondrial DNA (mtDNA) is a circular DNA molecule with a length of about 15-20kb, involved in the synthesis of mitochondrial proteins, in energy production and in the structure of cell membranes (Brown et al., 1979). It also contains the information for the messenger RNA (mRNA) encoding for proteins of the electron transport chain present at the level of the inner mitochondrial membrane; encodes for ribosomal RNA (rRNA) and transfer RNA (tRNA).

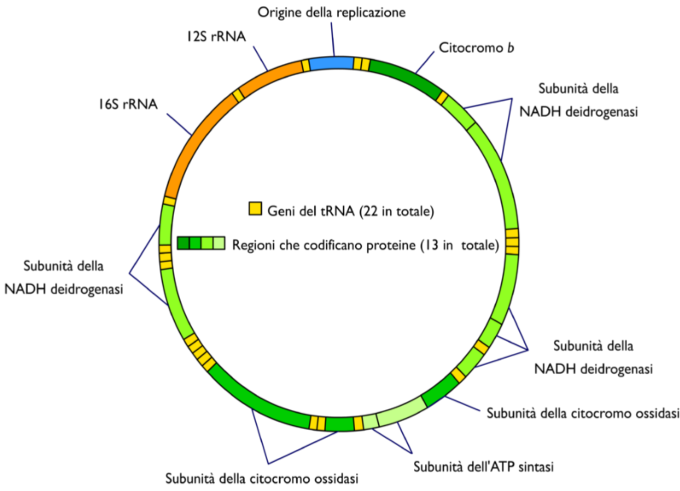

Figure. 1.4. The mitochondrial DNA.

The mitochondrial genome (mtDNA) is transmitted to the progeny in clonal and autonomous way, through the cytoplasm, or through uniparental mode: all mtDNA is inherited from the mother. Numerous studies have examined sequence variations within the mtDNA, revealing that nucleotide sequences of the animal mitochondrial DNA evolve with a higher rate than the coding sequences of the nuclear genes (Vawter & Brown, 1986). Due to its stability, at the intraspecific level, the molecular analysis of mtDNA allows to determine the evolutionary relationships between groups of closely related individuals. The mtDNA accumulate relatively simple mutations: essentially base substitutions and length mutations occur; they accumulate, predominantly, in small non-coding regions. Mitochondrial genes, including cytochrome b (cytb), the 12S and 16S

subunits, and the cytochrome oxidase I and II (CO I, CO II) are linked by the characteristic of being highly conserved traits in Vertebrates (Martin and Palumbi, 1993). The barcoding by the use the cytochrome oxidase I and II (CO I, CO II) has allowed the synthesis of a huge number of universal primers, which made it possible to perform precise correlations for different taxa, especially among groups of individuals whose morphological characters often give few information or may be easily confused (Goldstein and Desalle, 2010).

1.3 STATE OF THE HEALTH OF THE SARNO RIVER

The actual condition of the river Sarno is still critical due to continuous spills by many industries in the area, and also because of sewer systems that illegally flow into the river.
Thanks to a group of local authorities, and fans of the territory, the association "Amici del Sarno", in October 2003 was founded, and its purpose is to revive the Sarno river.
In 1973 the special clean-up project of the Gulf of Naples was approved, and in 1992 the project was entrusted to the Ministry of the environment, but it is only in November 2004 that the Commission of Inquiry about the causes of the pollution of the Sarno river began to acquire useful informations in the investigation of the state of pollution of the river.
The source waters maintain a clear color for about 200 meters, but already in the municipality of Striano conditions become "bad." It is then in the municipality of San Marzano, that the waters start to get muddy and the smell becomes nauseating. In the district of “Ciampa di Cavallo” in the Sarno river flowthe riverbed waters of Solofrana and Cavaiola. In this trait of the river grows a plant called "Lemma" (*Lemna minor*), the common "Duckweed", which, is assumed to have a phytopurifying action.
The worst situation is found starting from Scafati train Station, with putrid waters, with a high degree of domestic waste and discharges of all kinds, representing an ideal habitat for rats. The Sarno river, flows through the town of Pompeii, arrives in Torre Annunziata, where it culminates near the village of Rovigliano in bad health conditions. Therefore, the Gulf of Naples receives a pollutant load non easily biodegradable in a short time. In 1993

the entire area of the Sarno river was declared "area of high environmental risk" and the ENEA had the assignment to study for the construction of a mega-depurator. In reality it was then considered the idea of building seven smaller establishments, more manageable, by dividing the entire basin into three main areas : High Sarno, Middle Sarno and the Outfall. For the High Sarno, in 1995 started the construction of water treatment establishment, in MS Severino and Solofra with a discouraging outcome, because of the too high pollutant load resulting from the tanning center of Solofra. The construction of water treatment establishments in the area of the Middle Sarno are still in progress. At the outfall there is a sewage treatment establishment on the left riverside, and there s a project for various sewer establishments.

From the obtained results from the integrated analysis it's clear the disastrous situation of the river, with the confirmation that the part of the river most affected by human impact is the common riverbed because it receives the waters of the tributaries Solofrana and Cavaiola. These factors lead to classify the river Sarno environmental quality as "poor". In Arienzo et al., (2000) pollution levels are studied in a period of about 20 years: 1975-1998. This study shows that the electrical conductivity (EC) has risen from 27 to 34 %, indicating a high concentration of salts, such as sodium carbonate ($NaHCO_3$), sodium chloride (NaCl), potassium chloride (KCl) and calcium carbonate ($Ca(HCO_3)$), attributed to anthropogenic discharges. Another evident datum is the low concentration of dissolved oxygen, which also contributes to a high bacterial respiration. The values of BOD and COD found are well beyond the minimum allowed, especially during the summer, coinciding with the industrial processing of tomatoes. In all sampling sites there is a high density of coliforms and streptococci, values attributed to direct and indirect urban discharges and agricultural discharges. Inversely to the values of BOD and COD, in the summer months there is a reduction of bacterial concentration, probably due to direct sunlight. With regard to heavy metals, the situation appears fairly homogeneous, with high peaks only for the Iron, due to industrial discharges. Ultimately, in the twenty years considered, the

increase of pollution is mainly due to the expansion of industrial, human activities, with a greater input of discharges and waste products into the river (Arienzo et al., 2000).

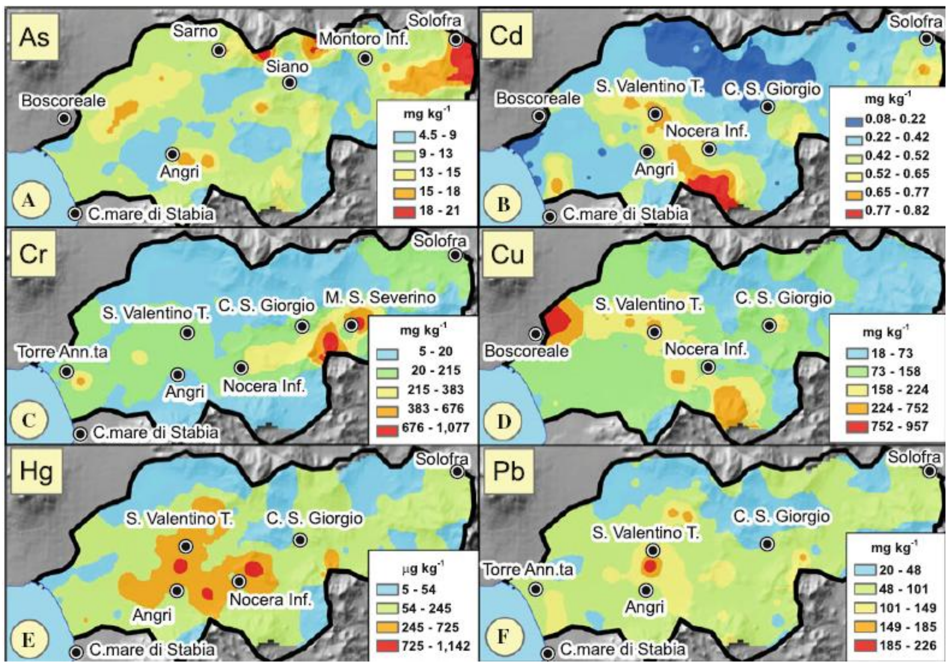

Figure 1.5. Interpolated distribution maps of studied elements (for each map, only place names useful to the description of the metal element distribution patterns are reported) (from Albanese et al., 2013).

In a recent study (Albanese et al., 2013), the concentrations of various metals have allowed to build real maps of distribution of pollutants in sites of the Plain of the Sarno river, shown in figure 1.4 (AF). The values of arsenic (As) were found higher in areas proximal to Solofrana. Although levels can undoubtedly be affected by the volcanic zone, is not to rule out the strong contribution due to the use of fertilizers and pesticides. The highest concentrations of cadmium (Cd) were found in the municipalities of Nocera, and towards the outfall, in the countries of Castellammare di Stabia and Torre Annunziata, associated to vehicular traffic and the use of fertilizers; values of chromium (Cr) appear to be high both in Solofrana, than in other stretches of the river, but the concentrations increase in Solofrana, probably due to the presence of tanneries. The concentrations of

copper (Cu) increases from the source to the outfall, due to the presence of fertilizers, and the values of lead (Pb) and mercury (Hg), despite having always high values are not very concern, since measured concentrations are mainly due to vehicular traffic .

In addition, a situation of serious environmental damage of the river is evidenced, with low values of dissolved oxygen concentration, probably due to excessive organic content, which make it difficult aquatic life because of anoxia, increased also by the content microbiological *E.coli*, present in concentrations similar to those of the waste waters. In recent decades, the activities carried out by local authorities, by region, by Casmez and by AgenSud, by the Ministry of Environment and the Ministry of Infrastructure, by the Department of Civil Protection, and by Authority of the basin of the Sarno are trying to assess this area severely compromised, through an investment policy to overcome the socio-economic and environmental emergency of the area of the Sarno river (personal communications).

Since 2001, the ARPAC is responsible for the oversight and technical control on the sources of physical, chemical and biological pollution of water, air and soil quality and hygiene of the environment, with continuous monitoring, which allowed to identify the classes of environmental quality for each station, showing a general trend from “poor” to “very poor” all along the river. The analysis consider both the ecological status, as the set of aquatic ecosystems and the physico-chemical state of the sediments, on the other the chemical status, defined by the presence of organic and inorganic micropollutants. As provided for the national law (No. 152/99, um 152/ 06), concerning the defense of waters against pollution, the parameters for analysis of water quality considered are: 1) the amount of dissolved oxygen (O_2% saturated), 2) the biological oxygen demand in 5 days (BOD5), or the amount of oxygen required for the biochemical oxidation of the substances contained in the water, in particular organic compounds, whose carbon atoms are used by microorganisms for the growth, respiration and reproduction; oxidized nitrogen compounds, used as an energy source by specific bacteria, inorganic substances , such as iron (II) sulfides and sulfites; 3) the chemical oxygen demand (COD) or the

measurement of the oxygen needed to oxidize the substances present in the water. This analysis is carried out mainly in cases of suspected toxic spills; 4) the amount of nitrogen (N) in the form of ammonium ion (NH_4^+), toxic to aquatic life, linked to the presence of organic materials in water deficient of oxygen, or the presence of a landfill; 5) nitrate ion (NO_3^-): elevated levels are a marker for potential organic pollution normally associated with the use of fertilizers in agriculture, 6) total phosphorus (P), which evaluates the trophic level of the waterways: in high quantities indicates civil and agricultural pollution due to the use of fertilizers; 7). enumeration of *Escherichia coli*, which provides guidance on pollution of faecal origin; 8) finally, the Extended Biotic Index (EBI), is able to assess the level of the quality of the river according to the degree of agitation of the communities of aquatic organisms.

The class of IBE quality of water along the Sarno river today is equal to V and this marks the presence of the condition of "exceptionally polluted environment or altered."

1.3a Bioindicators in the environmental monitoring

Monitoring of a water course, in Italy, takes into account seven macro-descriptors parameters as provided by Legislative Decree 152/06 Consolidated Law for the protection of the water: O_2 (oxygen) BOD5 (biochemical oxygen demand), COD (chemical oxygen demand), N-NH_4^+ (ammonium nitrogen), N-NO_3-(nitrate nitrogen), total P (total phosphorus), and fecal coliforms.

Table 1.2 Changeover of the Levels of pollution by Macrodescriptors (LIM) in Quality Levels.

PARAMETER	LEVEL 1	LEVEL 2	LEVEL 3	LEVEL 4	LEVEL 5>
100-OD (%sat.)	≤|10|	≤|20|	≤|30|	≤|50|	>|50|
BOD5 (O2 mg/L)	<2,5	≤4	≤8	≤15	>15
COD (O2 mg/L)	<5	≤10	≤15	≤25	>25
NH4 (N mg/L)	<0,03	≤0,1	≤0,5	≤1,5	>1,5
NO3 (N mg/L)	<0,30	≤1,5	≤5	≤10	>10
Total Phosphorus (P mg/L)	<0,07	≤0,15	≤0,30	≤0,6	>0,6
Escherichia coli (UFC/100 ml)	<100	≤1.000	≤5.000	≤20.000	>20.000
Score attributed to each analyzed parameter (75th percentile of the surveyed period)	80	40	20	10	5
Level of Pollution by Macrodescriptors	480-560	240-475	120-235	60-115	<60

Adding the scores obtained by the seven chemical and microbiological parameters the Level of Pollution from Macro-descriptors (LIM) is obtained. The result is expressed as a scale with quality levels decreasing from one to five (Table 1.2).

All monitoring methods for assessing the structure of an ecosystem, however, comprehend sampling of specific groups of biota (Ghetti, 1997), to which is applied a series of biotic indices, which summarize the characteristics of the community, so they can be easily readable (Table 1.3).

Table1.3. Principal indexes used in the monitoring of watercourses.

Indexes for bioindicator species	Based on the presence or abundance of individuals of selected species
Indexes of richness	Based on the number of taxa present
Indexes of abundance	Based on the size of the populations
Indexes of uniformity	Based on the proportional composition of the community
Indexes of diversity	From the combination of the wealth of information about the index and the index of uniformity
Biotic indexes	From the combination of the index information of the presence and abundance of individuals and in synergy with the diversity index

Through the analysis of the macrobenthic communities and, in particular, by evaluating the presence / absence of most demanding taxa and the total wealth of the population is possible to calculate the Extended Biotic Index (EBI), especially suited to detect in time the effects of the various factors of stress (physical, chemical, biological) on the river ecosystem .

The I.B.E. derived from Extended Biotic Index (EBI developed in England by Woodiwiss in 1978) and has been introduced and adapted to the rivers from Ghetti in 1997.

The Legislative Decree n. 152/99 considers the I.B.E. the only method to bioindicate, whose application is mandatory for the classification of water bodies, this method is applied together with the level of pollution expressed by Macrodescriptors (LIM) for the definition of the Ecological Status of watercourses, and expresses the state of quality of the stretch of waterway analyzed, by integrating the effects of different causes, chemical,

physical and biological which alter the environment, but provides only a qualitative method. It allows to classify waters according to a scale of values of the biological quality (Siligardi et al., 2003) in a range from 1 (extreme pollution index) to 12 (index of unpolluted waters), and divided into 5 classes of quality: each class is assigned a specific color that highlights the quality of waters.

Classes of Quality	EBI values	Judgment	Reference color
Class I	10-11-12	Environment not significantlaltered y	
Class II	8-9	Environment with moderate symptoms of alteration	
Class III	6-7	Altered environment	
Class IV	4-5	Much altered environment	
Class V	1-2-3	Highly altered environment	

Figure 1.6 Changeover of the values of Extended Biotic Index (EBI) in Quality Classes and related judgment.

The current monitoring techniques use, with the classical methods, responses by animal and plant species, in the biomonitoring. These methods provide an evaluation of the possible synergistic effects of pollutants over long-term exposure to even low concentrations. Biomonitoring uses bioaccumulators organisms, able to survive in the presence of pollutants, accumulating them in the tissues, and organisms biosentinels that, instead, undergo noticeable changes in the physiology, morphology or in the spatial

distribution under the influence of the substances present in 'environment (Burger, 2006).

Biomonitoring offers numerous advantages: short times and low costs; guarantees good adaptability, as it is possible to employ different species for various pollutants, and provides retroactive information, because the symptoms can be found at a certain distance by the pollution site, extension of the measurement points and of controlled areas, with the possibility of including areas in which it could be particularly costly to install electronic equipment. A good biomarker should have the following characteristics: it must be stationary, readily available and identifiable, must be able to react to all the changes to the environment in the area to be examined and have no particular sensitivity to a single source of pollution, should not be too sensitive to pollutants, otherwise it would die too soon, should have a long life cycle, high resistance to environmental stresses and show continuous growth (Fossi, 2000).

Depending on the area to monitor the most suitable and representative bioindicator should be selected, the one that, better than others, elects its own niche in the ecology environmental compartment where maximum is the probability of accumulation of the pollutant. In addition, its mobility is also important, in order to provide as much information on the area in which the organism lives, a sessile or sedentary organism, in fact, can provide only limited information. Finally, it should be considered that there are different ways of recruitment of polluting compounds; contaminants have a different bioavailability depending on the environmental compartment in which they are located ; organisms are generally not exposed to a single pollutant but to a mixture of many contaminants, such substances may generate different biochemical and toxicological interactions between them; there is a very long latency period before they manifest alterations at the level of population and community (Guerriero and Ciarcia, 2006; Zhou et al., 2008).

1.4 SPECIES AT RISK OF EXTINCTION

As mentioned in § 1.3, until the end of the seventies the river was inhabited by various species of Fishes such as eel, shad, the arborella, crabs and shrimp that favored habitat of the loops with river vegetation (Patti, 1999; La Fratta, 2002). In table 1.2 are listed, to date, several bioindicator species, as Reptiles, Birds and Amphibians. Among the latter, several species belonging to the genus *Pelophylax*, cause concern, due to the small number of species, limited distribution area and the gradual fragmentation and reduction of habitats.

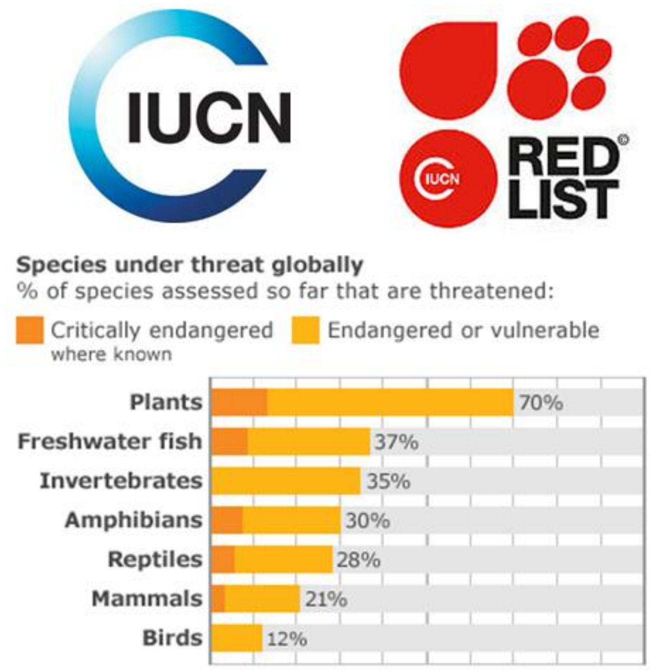

Figure 1.7. Percentage of threatened specie according the IUCN Red List.

The causes that led to this decline are manifold and related, like other Amphibians in the world, to destruction, alteration and fragmentation of habitats, environmental

degradation, especially the introduction of non-native (Kats & Ferrer, 2003), over-exploitation for the purposes of human consumption, the homeopathic treatment, ultraviolet radiation (Blaunstein et al., 1994), to the climate change (Blaustein et al., 2001); to the presence of environmental contaminants, and the use of fertilizers, pesticides and herbicides (Berril et al., 1998; Lips 1998; Kloas, 2009).

The isolation of populations can lead, as known, to an increase of homozygosity and to a decrease of the genetic variability, and this, obviously, affects the reproductive success, the vitality of individuals and their resistance to pathogens (Pearman & Garner, 2005).

Frogs are protected by many international and national legal rules, even for their inclusion in the IUCN Red List of Endangered Species, but there still is much to learn about their preservation, the preservation of their ecosystems and about the application of strategies to preserve the biodiversity (Mace et al., 2012), as the protection of rare species, endemic species, and species or habitats without utilitarian value.

1.4a Issues and measure of the bio-preservation

In the last decades new conservation movement emerged in relation to a new need to preserve the world biodiversity and ensure the survival of threatened life forms (Leopold, 1949; Ladle & Whittaker, 2011). In the last years much has been done in this direction, from local and regional scale activities, following international interest such as the establishment of protected areas and special recovery planning for species. The problem of biopreservation was first raised in the late ‘70s, with studies about the Italian herpetofauna (Pozzi, 1976; Bruno, 1977). Corbett (1989) reported that in Italy only 35 population were present, even if, lately these information revealed to be too pessimistic, due to a lack of data (Barbieri & Mazzotti, 2006). However, since the progressive reduction of elective habitat and the condition of endemicity of the species, numerous monitoring and conservation projects were promoted since the late ‘80s.

The international and national legislation requires special areas of attention, as shown below.

International Regulations

1. Berne Convention, 19.IX.1979, ratified by Italy with Law no. 503 of 5.VIII.1981; Annex II: strictly protected fauna species 2. Habitats Directive, the EEC Council 92/43 21.V.1992; Annex II: Animal and plant species of Community interest whose conservation requires the designation of special areas of preservation; Annex IV: Animal and plant species of Community interest in need of strict protection.

National regulations

1. Regulations implementing Directive 92/43 EEC on the preservation of natural habitats and wild fauna and flora; DPR n. 357 8.IX.1997; Appendix B: Animal and plant species of community interest whose conservation requires the designation of special areas of preservation; Annex D: Animal and plant species of Community interest in need of strict protection [ratification of the Habitats Directive.

The projects implemented or initiated so far may be grouped, as usual, into four main types of intervention, a) monitoring of populations, b) habitat management of breeding sites or the surrounding environment, c) safe of eggs and adults, d) translocations.

The conservation measures are often species-specific and follow technical documents called “action plans”. Among these plans there are benefits and challenges binging to: reintroduction; re-stocking; monitoring the impact of predation and / or host organisms or symbionts; improvement of water quality; reforestation in relation to the type of the original forest; monitoring the status of the populations; monitoring of the habitat; monitoring of chemical and biological quality of water; environmental Education and local disclosure; environmental Education and high broad disclosure.

1.4b Non-invasive biomarkers in the environmental monitoring

The conservation of ecosystem starts, as it is known, from the environmental monitoring choosing valid bioindicators and biomarkers. Over the past two decades , always more application are finding, in a wide range of environmental conditions, the biomarkers, ie biochemical cellular, physiological or behavioral changes measured in an organism, or in tissue, in a biological fluid, which provide the evidence of exposure and/or effect to one or more pollutanst. The role of biomarkers in ecotoxicological investigations is therefore not to give "quantitative” information on the levels of exposure of an organism to a given contaminant, but to provide guidance on its "health" as a signal of potential changes at the highest levels ecological (Fossi, 2000), in function of response to different level in categories such as: DNA damage; responses of proteins, metabolic products, changes in the immune system; histopathological changes; non-specific biomarkers and physiological, behavioral biomarkers.

The use of biomarkers, both in environmental chemistry and toxicology, or in the conventional use of bioindicators in biomonitoring programs, has numerous advantages. They provide: an "integrated" response to the total exposure of the bioindicator species, or biosentinel, considering the summation of both the various ways of assumptions that expositions over time within a certain spatial "range"; as an "integrated" signal of toxicological and pharmacokinetic interactions of the mixture of compounds to which the organism is subjected; an immediate response to exposure at toxic compounds (hours-days); these data allow to predict the long-term negative effect; information on the inter- and intra-specific susceptibility to a contaminant and / or to a mixture of contaminants; ecological evidence of the effect in the long term of a contaminant depending whether or not the organism is exposed to a level of contamination that exceeds its ability to detoxify and repair (levels of homeostasis).

The application of this methodological approach in biomonitoring programs should however consider the presence of certain "noise" factors that can alter the signal supplied

by the biomarkers. For example, certain multienzimatic reactions (the system of mono-oxygenase at mixed function) undergo changes in function of hormonal status, age and sex of the organism and species (Ziolkowska et al., 2013). The knowledge, however, of the reproductive cycles of the biosentinel species, and its physiological characteristics allow, with an adequate sampling program, to remove all or part of such factors.

Currently technologies and advanced techniques now provide alternative methods very effective and reliable, such as microdosing and the use of cultured cells or tissues, and the use of feathers, hair, blood as non-invasive methods. Many regulatory processes that occur at genomic, transcriptional, post-transcriptional levels, translational and post-translational mediate the cellular stress responses can be considered as markers (Silins & Högberg, 2011) of exposure, effect and susceptibility. Biomarkers of exposure include measurements of various compound, DNA or protein adducts, and reflect internal doses. Biomarkers of effects may be represent by an altered expression of some metabolic enzymes. Biomarkers of susceptibility may be indicated as the ability of an individual to respond to a specific exposures.

1.4c DNA damage and reproduction: theoretical and applicative concepts

The reproductive processes usually occur by stages, rigidly fixed, or present a more or less flexibility rate. The performance of these processes is related to changes in endogenous factors (amino acids, neuropeptides, gonadotropins, hormones, steroid receptors, anti-oxidants) and exogenous (photoperiod, temperature and ethological and ecological factors). The complicated interactions that occur between endogenous and exogenous factors and the production of gametes have so far been studied and explored in a diverse number of species (Beckett and Arthur, 2005; Stradaioli et al, 2009). In Amphibians, as in other Vertebrates, the development and the gonadal activity, and reproductive processes generally are under the control of the hypothalamic-pituitary axis. This system is basically similar among the different classes of vertebrates although the structure and functional activity, may be specie-specific, as it’s been gradually demonstrated in last studies.

Among endogenous factors GnRH (gonadotropin-releasing hormone for), a decapeptide secreted mainly in the hypothalamus, plays a leading role in the control of reproduction. GnRH, for which has been identified and characterized several molecular forms, similar in the various Vertebrates, regulates the synthesis and/or the release of pituitary gonadotropins (FSH and LH) which, in turn, control the activity of gonadal steroidogenesis and gametogenesis. The sex steroid hormones (estrogen, androgen and progesterone) in sync with the pituitary gonadotropins promote the maturation of gametes and at the same time are able to modulate reproductive behavior and the activity of the hypothalamic-pituitary axis, due to the presence of specific nuclear receptors and antioxidants involved in the early stages of formation and activation of gametes (Guerriero, 2009; Zhang et al., 2009).

Into target cells, the hormone-receptor complex exerts its function of regulator of the transcription of specific genes, while the antioxidants neutralize or limit excessive production of reactive oxygen species (ROS) that may block the meiotic stages of reproductive events (Parminder and Bansal, 2003).

The importance of integration between these structures is particularly evident in the species in breeding season, in which environmental stimuli are integrated at the level of the central nervous system with the internal hormonal system and with endogenous antioxidants, to trigger the reproductive behavior (Guerriero and Ciarcia, 2001).

In recent decades many studies aimed to identify the promoting factors the different phases of reproduction of organisms in the seasonal cycle (Chieffi and Varriale, 2004; Guerriero and Ciarcia, 2006). In some *Pelophylax* species research has been carried out about variations of sex steroids in plasma and tissues, on the GnRH neural system, and about the hypothalamic receptor of estrogen and progesterone. However, still few informations are available about the mechanisms that regulates, in target organs, the interactions between steroids, their receptors and oxidants.

Increased numbers of agricultural and industrial chemicals are entering the aquatic environment and being taken up into tissues of aquatic organisms. Transition metals,

polycyclic aromatic hydrocarbons, organochlorine and organophosphate pesticides, polychlorinated biphenyls, dioxins, and other xenobiotics play important roles in the mechanistic aspects of oxidative damage.

Such a diverse array of pollutants stimulate a variety of toxicity mechanisms, such as oxidative damage to membrane lipids, DNA, and proteins and changes to antioxidant enzymes. Although there are considerable gaps in our knowledge of cellular damage, response mechanisms, repair processes, and disease etiology in biological systems, free radical reactions, the generation of free radicals as well as Reactive Oxygen Species (ROS) results responsible for a variety of oxidative damages leading to adverse health effects, diseases and infertility (Athanasios et al., 2006, Diamanti-Kandarakis et al., 2010; Poljˇsak and Dahmane, 2011; Bindoli and Rigobello, 2013).

DNA damage can be evaluated by a variety of methods, such as the detection of single strand breaks, by Comet assay, or the micronucleus test, useful to detect chromosome aberration which can occur during the phase M or S of the meiosis. Many compounds may lead to these aberrations, such as Reactive Oxygen Species (ROS) (Mouchet, 2005).

The stability of the genetic information is important for normal function and reproduction of all living organisms. In heterogametic species, intact gametes ensure faithful transmission of genetic information to the next generation. However, gametogenesis tolerates a limited number of mutations, since many DNA repair systems are active in all somatic cell types, and may show an activity during gametogenesis too.

A lot of genes seem to take part in these repair mechanisms, and they seem to be enhanced right during gametogenesis. it may occur that the damage persist in the gametogenesis, or occurs in spermatozoa, but it is subsequently repaired during the process of fertilization (Brandriff and Pedersen, 1981; Ashwood-Smith and Edwards, 1996), with the formation of pronuclei, and pronuclear fusion, before formation of the zygote. Indeed, ROS are largely known for their toxic effects on spermatozoa and have been linked to male infertility (de Lamirande et al., 1997; Shen & Ong, 2000). Excessive ROS production has been associated with sperm morphological defects (Aziz et al., 2004),

inhibition of sperm motility (Armstrong et al., 1999; Parinaud, 1997), fragmentation of sperm DNA (Donnelly et al., 1999), and premature capacitation (Villegas et al., 2003).

Methods for ROS detection can be broadly classified as either direct or indirect. Due to the short lifetimes and typically low concentrations of ROS in aquatic 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 (Christiaan et al., 2013). Such methods typically involve specific chemical derivatization (e.g., trapping a radical with a nitroxide or other spin trap) or are based on competitive kinetics. Much of the method development for aqueous ROS analysis has focused on ultraviolet (UV)/ visible (Vis) light spectroscopic techniques and the use of relatively common and hence lower cost probe molecules. Spectroscopic detection strategies (including absorbance (UV/Vis)), fluorescence (FL) and chemiluminescence (CL) share a common approach with several other techniques for measuring rates of ROS formation and decay in laboratory experiments. 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 paramagnetic (spin) resonance (ESR), nuclear magnetic resonance (NMR), derivatization with attendant mass spectrometric (MS) analysis and liquid scintillation counting can also be quite useful. In particular, the ESR phenomenon was first discovered in 1945, and ESR-based methods have since become widely used because the process can detect free radicals without interference from the sample properties. Therefore, if the ESR spectrum can be observed using an ESR instrument, we can identify the free radical present in a sample (Kohono, 2010). The ESR spin-trapping method is a well known, useful tool to detect short-lived radicals such as ROS. The spin-trapping method was originally proposed in the late 1960s. Fundamentally, spin-trapping reagents react with short lived radicals, which are subsequentely changed to long-lived radicals called spin-adducts. Desiderable characteristics of spin-trapping reagents include high solubility and stability

in water, and the ability to interact with free radicals. Furthermore, high reactivity is required to observe the formation of free radicals. It is also desirable that the resulting spin adduct is stable for a relatively long time and that the spectrum can be easily analyzed.

The interaction of ROS with DNA occurs in the first place by structural alterations to the double helix, which may manifest as adducts, chemical alteration of the nitrogen bases, cross-linking and breakage at the level of single-and double-strand (strand breaks). Most of the assays aim to measure the stability of DNA. These structural alterations to the double helix, usually, are promptly corrected by repair mechanisms, without harmful consequences for the organism.

However, lesions that are not repaired or are processed improperly, can lead to the establishment of abnormal genetic material such as chromosomal aberrations, gene mutations and other long-term effects such as cancer in vertebrates, including humans.

Specific biomarkers of oxidative damage are used to investigate modifications and hydroxylations that occur in DNA bases, and for damage to the deoxyribose-phosphate backbone and DNA-protein cross-links. These processes can occur although under physiological conditions, exposure to some xenobiotics may cause an increased at which oxidative damage can be determined by various assays (Morais et al., 2012).

A lot of genes and related protein seem to take part in these repair mechanisms, and they seem to be enhanced right during gametogenesis. it may occur that the damage persist in the gametogenesis, or occurs in spermatozoa, but it is subsequently repaired during the process of fertilization (Ashwood-Smith and Edwards, 1996; Kourtis & Tavernarakis, 2011), with the formation of pronuclei, and pronuclear fusion, before formation of the zygote.

Many regulatory processes that occur at genomic, transcriptional, post-transcriptional levels, translational and post-translational mediate the cellular stress responses and can be considered as markers.

These responses require a complex network of sensors and effectors from multiple signaling pathways, able to ensure genomic integrity as the abundant and ubiquitous nuclear poly(ADP-ribose)polymerases1 (PARP1) (Luo & Kraus, 2012). Since 1986, PARP activities were detected in different classes of Vertebrates (Avians, Reptiles, Amphibians and Fishes), Arthropods, Mollusks, while no evidence was in non-vertebrate organisms (Scovassi et al., 1986). Moreover, a more recent and extensive phylogenetic analyses of the known PARPs allowed to identify in silico 236 PARP-like enzymes from 77 species across five of the six eukaryotic supergroups. The broad distribution of PARP genes indicates that the ancestor of all extant eukaryotes, including invertebrates, encoded proteins of this type and that the ancestral PARP proteins had different functions and activities (Citarelli et al., 2010).

Among PARPs, both PARP1 (113kDa) and its cognate poly(ADP-ribose)polymerases2 (PARP2) of 62kDa are the sole enzymes whose catalytic activity is immediately stimulated by DNA strand-breaks (Amé et al., 2004). PARP1 catalyzes the covalent attachment of ADP-ribose units from donor NAD^+ molecules on itself and other acceptor proteins (histones, DNA repair proteins, transcription factors, and chromatin modulators), altering in a reversible manner their structure, function, and localization (Krishnakumar & Kraus, 2010; Hassa & Hottiger, 2008; Kraus, 2008). PARP2 is capable of auto-poly(adpribosyl)ation, is involved in DNA repair (Schreiber et al., 2002), but it can not modify histones, which are prototypical PARP-1 substrates (Amè et al., 2004). Both PARP1 and PARP2 are involved in the regulation of several cellular functions related to the maintenance of genomic integrity (DNA repair, gene amplification, apoptosis) and to the expression and propagation of the genetic information (DNA transcription and replication, differentiation, neoplastic transformation (Michels et al., 2013; Hassa and Hottiger, 2008; Kim et al., 2005).

The exposure to different kinds of environmental stresses, such as drought and heavy metals seems to induce the gene of poly (ADP-ribose) polymerase family, member 2 (Amor et al., 1998). Besides the nuclear PARPs, in the cytoplasm of different eucaryotic organisms like Protozoa, Molluscs, Cephalopods, Echinoderms, Fishes, Amphibians, Avians and Mammalian, a vault poly(ADPribose) polymerase (VPARP) of 193kD was found.

PARP family member	Alternative name	Transferase name*	Subclass	Size (aa)[‡]	Subcellular localization	Triad motif	Enzymatic activity[§]	Key functional motifs and domains[‖]
PARP1		ARTD1	DNA-dependent	1,014	Nuclear	H-Y-E	P and B	WGR, zinc-fingers and BRCT
PARP2		ARTD2	DNA-dependent	570	Nuclear	H-Y-E	P and B	WGR
PARP3		ARTD3	DNA-dependent	540	Nuclear	H-Y-E	M (P predicted)	WGR
PARP4	vPARP	ARTD4		1,724	Cytosolic (vault particle)	H-Y-E	P (predicted)	BRCT
PARP5A	Tankyrase 1	ARTD5	Tankyrase	1,327	Nuclear and cytosolic	H-Y-E	P and O	Ankyrin repeat
PARP5B	Tankyrase 2 and PARP6[¶]	ARTD6	Tankyrase	1,166	Nuclear and cytosolic	H-Y-E	P and O	Ankyrin repeat
PARP6[¶]		ARTD17		322	ND	H-Y-Y	M (predicted)	
PARP7	TIPARP and RM1	ARTD14	CCCH PARP	657	ND	H-Y-I	M (predicted)	Zinc-fingers and WWE
PARP8		ARTD16		854	ND	H-Y-I	M (predicted)	
PARP9	BAL1	ARTD9	macroPARP	854	ND	Q-Y-T	M (predicted)	Macrodomain
PARP10		ARTD10		1,025	Nuclear and cytosolic	H-Y-I	M	
PARP11		ARTD11		331	ND	H-Y-I	M (predicted)	WWE
PARP12	ZC3HDC1	ARTD12	CCCH PARP	701	Cytosolic (stress granules)	H-Y-I	M (predicted)	Zinc-fingers and WWE
PARP13	ZC3HAV1 and ZAP1	ARTD13	CCCH PARP	902	Cytosolic (stress granules)	H-Y-V	M (predicted)	Zinc-fingers and WWE
PARP14	BAL2 and COAST6	ARTD8	macroPARP	1,801	Cytosolic (stress granules)	H-Y-L	M	Macrodomain and WWE
PARP15	BAL3	ARTD7	macroPARP	444	Cytosolic (stress granules)	H-Y-L	M (predicted)	Macrodomain
PARP16		ARTD15		630	ND	H-Y-I	M (predicted)	

Figure 1.8. The PARPs family members, their subcellular localization and the enzymatic activity: mono-(M); oligo-(O) or poly(ADP-ribosyl)ation (P); or brancing (B) (from Kotz, Nature 2010).

This enzyme is one of three protein components of the vaults, ribonucleoprotein particles, containing a small untranslated RNA molecules of 88-141 bases (De Lisa et al., 2012). Regulation of the poly(ADPribose) turnover, variations of PARP amount, as well as changes of PARP transcription level, involves germinal cell differentiation too, being implicated in DNA replication, repair, and transcription (Baarends et al., 2001; Aoufouchi & Shall, 1997). During meiosis recombination events play an important role in creating genetic diversity among individuals within a population. Recombination involves the

induction of double strand breaks (DSBs), followed by crossing over between homologues and ligation of DNA molecules. A functional relationship between poly(ADP-ribosyl)ation and spermatogenesis was widely demonstrated (Celik-Ozenci & Tasatargil, 2013). Studies related to PARP expression in testicular tissue have been performed mainly in rodents and most of these studies found high PARP activity in spermatogonia and pachytene nuclei of spermatocytes (Tramontano et al., 2005; Di Meglio et al., 2003, Faraone-Mennella et al., 1998) and on tetraploid spermatocytes which undergoes meiotic division (Quesada et al., 1996,2000).

In human, during spermatogenesis, alterations in sperm DNA topology occur but the persistence of DNA strand breaks during different phases of spermatogenesis can contribute to DNA damage detected in mature spermatozoa and/or in the infertility (Baarends et al., 2001; Laberge & Boisonneault, 2005). DNA repair mechanisms are involved not only in the repair of different types of DNA damage in developing germ line cells, but also take part in the meiotic recombination process (Guerriero & Ciarcia, 2006). Based on the degree and type of the stress stimulus, PARP directs cells to specific fates (such as, DNA repair vs. cell death).

Furthermore, the DNA repair mechanisms should tolerate mutations occurring during gametogenesis, to a limited extent (Guerriero et al., 2003; Baarends et al., 2001). Excessive activation of PARP contribute to the pathogenesis of several diseases associated with oxidative stress, which has been known to play a fundamental role in the etiology of male infertility (Celik-Ozenci & Tasatargil, 2013).

CHAPTER 2

STRATEGIES OF PRESERVATION OF SPECIES BELONGING TO THE GENUS *Pelophylax*

2.1 GENERAL OBJECTIVES: DEVELOPMENT AND VALIDATION OF NEW PRESERVATION STRATEGIES

Habitat destruction or fragmentation, climate change, predators or competitors, diseases and the presence of chemical pollutants represent the main causes of species' extinction risk (Kryston et al., 2011). Constant exposure to indiscriminate use of herbicides, accidental spills or contaminants discharges into natural waterways have, however, the major adverse effects on population due to the accumulation of endocrine chemicals in reproductive tissues (Guillette& Edwards, 2008). Their cytotoxic effects are exerted through multiple mechanisms, but most of them are involved by reactive oxygen species (ROS) and nitrogen (RNS) that potentially damage lipids, proteins and DNA, impair cell functions and reproduction (Bobe and Labbé, 2010; Rothfuss et al., 2010; Guerriero and Ciarcia 2006; Abele and Puntarulo, 2004). Conservation biology studies, which include population analysis, demographic surveys and reproductive biology allow to assess the conservation status of the species exposed to the potential effects of endocrine disruptors (http://portal.isiknowledge.com). The possibility of long-term survival populations, results facilitated by the identification of strategies for their management and protection, to avoid severe genetic damage, threatening the survival of the organisms, and their reproductive capacity (Wilson & Roberts, 2011). To gain new insight into the environmental impact of endocrine disrupting chemical on specie conservation, aim of the present research was to validate, in more representative frog species in the

studied area and, both during the reproductive and non reproductive phase, biomarkers for oxidative, genotoxic and endocrine damages-.

2.2 SPECIFIC OBJECTIVES: MONITORING OF THE STATE OF THE HEALTH OF THE SARNO RIVER WATERCOURSE BY THE MOST REPRESENTATIVE BIO-SENTINEL SPECIES

The exposure to high concentrations of pollutants can cause the accumulation of their residues in sediment, water, soil, at the level of tissues and organs, producing irreparable genotoxic damage to genomic materials, such as DNA mutations, with tragic consequences for the survival of the species and the whole ecosystem (Guerriero and Ciarcia, 2006; Diamanti-Kandarakis et al., 2009, 2010).

It is well documented that pollutants, UV radiations and pathogens have negative effects on the survival of Amphibians, which are important components of the ecosystem, for their role of prey, predators and herbivors (Blaustein and Kiesecker, 1994; Blaustein and Kiesecker, 2002). Frogs, in fact, are directly exposed to toxic substances both in water and soil, because of their high permeable skin (Blaustein and Kiesecker, 2003).

In this optic the aim of the present research was to study how Reactive Oxygen Species (ROS) can affect the reproductive health of the biosentinel *Pelophylax bergeri*; how the synergy between the several pollutants in the environment lead to genotoxicity, and the activation of events of poly(ADPribosyl)ation, implicated in the genome surveillance and, indirectly in specie preservation, to define a warning alarm for health of environment and survival of some species.

Moreover the investigations were conducted by skin biopsy, and gonadectomy, in the full respect of bio-preservation, avoiding the sacrifice of a specie, yet endangered by human and industrial activities.

The same effect were studied in the same tissues and organs of frogs of the same specie sampled in a low-polluted area, the Matese Lake, chosen as control.

CHAPTER 3

MATERIALS AND METHODS

3.1. SITES AND ANIMALS

3.1.a. Sampling points and sample monitoring and collection

The first step consisted in identifying breeding sites for Amphibian species, making a census for view of the species present in every biotope and evaluating the semi-quantitative abundance in numbers and the state of preservation, according to the "Habitat Directive", after veryifing the physico-chemical and the biological state of the watercourses environments, based on the analysis of the macroinvertebrate community index (EBI); of the ecological status related to the systematic detection of the levels of micropollutants, kindly provided by the Agency for Environmental Protection (ARPAC). Only preliminary techniques prescribed by the aforementioned directive were actually applied, since this wanted to be the very first survey (sampling and transects by nets for periodical and standard collections).

For the state of preservation IUCN categories were used, as indicated in the *CKmap* database, and reported below:

Table 3.1. IUCN categories utilized for frog classification.

EX	(Regionally extinct)
EN	(Endangered)
VU	(Vulnerable)
NT	(Nearly threatened)
LC	(Least concern)
DD	(Data deficient)
NA	Not applicable (alien species)

Adult male frogs were collected, with the help of the Association "Amici del Sarno"; along the Sarno River (**Fig 3.1**) exposed to a wide variety of ECDs, originating from discharged industrial, agricultural and municipal effluents, bounded by transects and from the

unpolluted area, Matese Lake. Sampling campaigns occurred in the period from April 2011 to April 2013. Only *Pelophylax* specimens non-morphologically identified were used for the molecular investigation by skin biopsy.

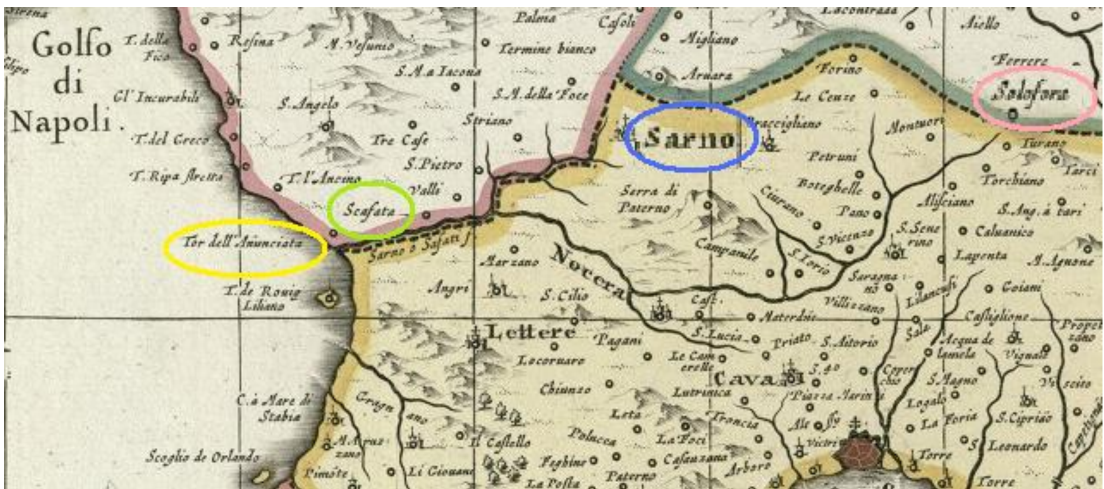

Figure 3.1. The area of the plain of Sarno River.

The methods of capture, dissection and the captive rearing conditions (**Figure 3.1.1**) were in accordance with Italian law (D. L.vo 116/92) and authorized by the appropriate Italian government administrative office (A.S.L. 44 Veterinary Service, Prot. Vet. 22/95).

Figure 3.1.1. Frogs sampled and stored in terraquarium.

3.1.b. Biopsy and gonadectomy in species of the genus *Pelophylax*

Frogs were put into a cylinder containing a piece of absorbent cotton saturated with anhydrous ether and, after an exposure of about 1–2 min, a surgical knife (scalpel) was used to shave off the superficial epithelium (0.56mm^2) from the dorsal skin for a single specimen. Monsel's solution (Xenex sln) was used to protect the wound from infections.
The minimum amount was estimated by comparing different and scalar quantities of skin fragment weight per DNA quality.
From the total weight skin, aliquoted by five sacrificed frogs, different amount of skin tissue were measured (**Fig 3.1.2**), and the DNA was extracted from all the amounts. The DNA quality was estimated electrophoretically and by measuring the absorbance at 260/280 nm, and the ratio value.

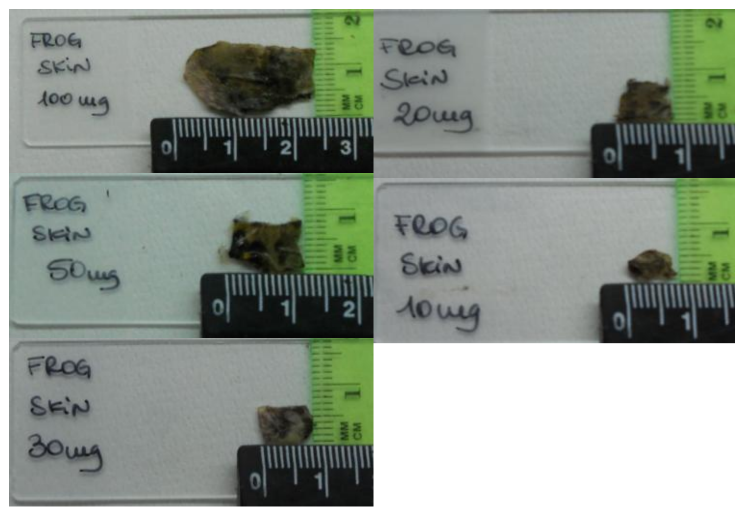

Figure 3.1.2. Technical proves of skin biopsies.

Frogs had been anesthetized by immersion in MS222 for both skin biopsy and corresponding testis gonadectomy purpose. Only one gonad was removed using a surgical knife.

Monsel's solution (Xenex sln) was used to protect the wound from infections. After the surgery animals were fed on meat worms ad libitum and followed in the stabularium for 10 days before their release into the wild.

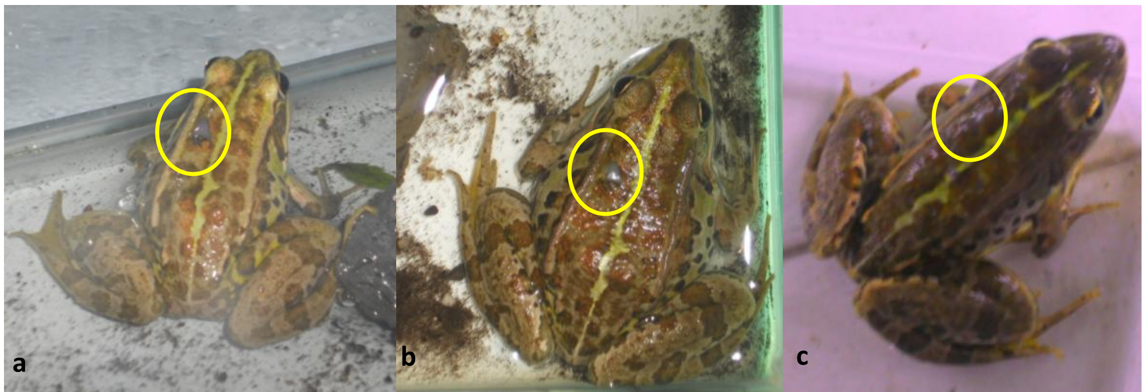

Figure 3.1.3. Frog specimen after biopsy; a: 1 day; b; 3 days; c: 7 days.

Figure 3.1.4. Gonadectomy of only one testis on frogs' specimens.

All the experiments were approved from Federico II University Institutions.

Pool of skin biopsy, and corresponding gonadectomized *Pelophylax bergeri* frogs (n=5/selected month for each identified specie and for three years) were examined during the main phase of the reproductive cycle: in April and May, during active spermatogenesis and breeding season, and in October and November, during spermatogenetic regression.

3.2. IDENTIFICATION OF AMPHIBIANS OF THE GENUS *Pelophylax* PRESENT IN THE SAMPLING POINTS AND CHOICE OF THE BIO-SENTINEL ORGANISM

3.2.a. DNA extraction from skin biopsy in *Pelophylax* species

The DNA extraction from the frog tissue was performed as reported in Guerriero et al., (2010) with a phenol/chloroform standard method by using autoclaved glassware and equipment.

About 30mg samples of ground freeze dried tissues were mixed in a DNA extraction buffer (50 mM NaCl, 10 mM EDTA and 10 mMTris base) and the cells were lysed by adding 2% sodium dodecyl sulfate. The RNA was removed by adding RNAse (10 mg/mL) followed by incubation at 37°C for 30min. Proteinase K was added (0,5 mg/mL) to remove protein and the samples were incubated for 1h 37°C in a shaking water bath. The extracts were further purified by extracting twice with phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and by centrifuging at 10,000g for 15 min at 4°C. The upper aqueous layer was transferred into a new micro-centrifuge tube and the DNA was precipitate by adding 1/10th volume of 3 M sodium acetate at pH 5.2 and two volumes of 100% chilled ethanol to each sample and mixed centrifuged at 15,000g for 30 min at 4°C. The pellet was washed with 70% ethanol, air dried, and finally re-suspended in 50 µl sterilized deionized water. Optical density (OD) of each sample was measured at 260 and 280 nm respectively, by UV-spectrophotometer (Eppendorf BioPhotometer, D-5000-3000), and the purity of DNA was measured by the OD260/OD280 ratio (ideal ratio = 1.7–2.0), and the quality by electrophoresis on a 0,8% agarose gel and visualized under UV light.

3.2.b. PCR amplification and sequencing of cytb, 16S and CO I mt-rRNA gene fragments in *Pelophylax* species

PCR amplification was performed as previously published in Guerriero et al., (2010) using the following primers: USP 5’-CCATCCAACATCTCAGCATGATGAAA-3’ and LSP 3’-GCCCCTCAGAATGATATTTGTCCTCA -5’ to amplify a 389 bp fragment of the cytb rRNA gene (Palumbi, 1991) and COI_UP (5’-ACTTCAGGGTGACCGAAGAATCAGAA-3’) and COI_DW (5’-ATCTTTGGTGCATGAGCAGGAATAGT-3’) for the COI gene fragment (Ward et al., 2005).

A parallele amplification for the 16s rRNA mitochondrial gene (16 sar: 5'-CGCCTGTTTATCAAAAACAT-3', and 16Sbr 5'-CCGGTCTGAACTCAGATCACGT-3') has been set up to compare known sequences each other.

PCR reaction was performed in a Techgene Thermal Cycler (Thecne Ltd., Cambridge, UK). Thirty-five cycles of amplification were carried out in a reaction buffer containing 10 ng of genomic DNA, 50 mM KCl, 10 mM Tris/HCl, pH 9.0; 10 mM NaCl; 0.01 mM EDTA; 2.5 mM of each dNTP; 1 µM of forward and reverse primer; 0.5 unit of Taq DNA polymerase (Invitrogen, Milan, Italy). Cycling conditions were 5 min at 94°C for the initial denaturation, followed by 35 cycles: denaturation at 94°C for 45 s, annealing at 55°C for 50 s, and extension at 72°C. for 90 s, and a final extension at 72°C for 7 minutes.

The thermal profile to amplify the COI gene fragment consisted of 35 cycles: 94 °C for 50 s, 54 °C 50 s and 72 °C for 1 min with an initial denaturation of 5 minutes at 94°C and a final extension of 7 minutes at 72°C.

Cytb and COI amplicons were separated by 2% agarose gel electrophoresis and bromide staining, and visualized under UV light. A 100 bp ladder (Invitrogen, Milan, Italy, or Fermentas, M-Medical srl, Milan, Italy) was used to estimate the fragment size of the amplicons generated. Amplified DNA was desalted with Microcon 100 spin columns (Millipore-Amicon, Belford, MA, USA) according to the manufacturer's instructions. Purified amplicons were directly sequenced using Big Dye TM Terminator Cycle Sequencing Chemistry (Applied Biosystems, Foster City, CA, USA) in an automatic capillarity sequencer (ABI 310 Genetic Analizer; Applied Biosystems). Primers for sequencing were the same used for PCR amplification.

Obtained sequences were checked with BioEdit sequence alignment editor (version 7.0.5 Hall, 1999), compared against references sequences using the GenBank National Centre for Biotechnology Information Database (http://www. ncbi.nlm.nih.gov/), and aligning sequences by Clustal W (http://www.ebi.ac.uk/).

3.2.c. Evaluation of the abundances indexes basing on the size of population of frogs of the genus *Pelophylax*

After the observations and collecting data throughout the basin of the River Sarno, an evaluation of the number of population according to the occurred sightings. The detection method adopted for such research is the direct capture of individuals in water and in the ground (Heyer et al., 1994); this technique is based on the principle that for the same unit of effort of capture, the number of individuals captured should be proportional to the number of individuals present in the whole population. The unit of the effort can be represented by the number of passages of landing net, in a delimited area with a known volume of water. In addition samples were made by direct observation, using the methodology of the transepts, which consists in counting the observed specimens within a delimitated area. In order to have a valid estimation of the population and obtain valuable informations on the biological and ecological dynamics, the monitoring comprehended both the breeding and non-breeding season. We proceeded then to the percentage compilation of survey forms.

The same procedure was adopted for sampling in the Matese Lake.

3.3. OXIDATIVE AND GENOTOXIC DAMAGE IN THE BIOSENTINEL ORGANISM *Pelophylax bergeri*

3.3.a.1 Electron spin resonance spectroscopic detection of oxygen-centred radicals: *Pelophylax* sample extraction procedure

The method used for the extraction procedure was modified starting from Ashton et al., (1998) for the electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise.

A 140 mmol/l solution of the spin trap α-phenyl-tert-butylnitrone (PBN) (Sigma, Dorset, UK) in de-ionized water was freshly prepared in a darkened room. Skin from biopsy and corresponding testis from gonadectomy of adult *Pelophylax bergeri* were weighed and homogenated in 0.5 ml of physiological saline; then 1/5 of the spin-trap solution was added in the dark at 4°C, and shaken for 10 minutes. Samples were centrifuged for 10 min

at 3500 rpm. The surnatant containing the PBN adduct was immediately transferred in a new tube and placed on ice in the dark prior to organic extraction of the PBN adduct. An equal volume of HPLC-grade toluene was added to the serum/adduct and vortex mixed for 30 s.

3.3.a.2 ESR Spectroscopy

A volume of the spin trap α-phenyl-tert-butylnitrone (PBN) adduct was extracted and placed in a precision-bore quartz ESR-sample tube and analyzed. Room-temperature ESR analysis was carried out on a Bruker Elexys E-500 X-band spectrometer with 100 kHz frequency modulation using the following operating conditions: Microwave frequency, 9.871 GHz; incident microwave power, 6,4 MW; scan width, 6.000 mT; modulation amplitude, 0.1000 mT; magnetic field centre, 351.0 mT; time constant, 0.10 or 0.30 s. The signal or the relative concentration of ESR adduct was obtained by calculating the double integral of each spectrum using as external standard an ethanolic solution.

3.3.a.3 Double integral calculation

The calculation of the double integral is carried out to obtain a more precise determination of the magnitude of the recorded signal and, consequently, the relative concentration of adduct. To calculate it it's used a program on the computer associated with the instrument, while viewing and recording the spectra. The method begins by starting from a known spectrum and proceed to the correction of the base line of the spectrum. The first integration allows to obtain the spectrum in absorption among the field and no longer in derived, as it is recorded. Another correction of the base line, in the same way as before, permit to calculate the second integral, which allows to calculate the area under the peaks of the spectrum.

3.3.b. Agarose gel electrophoresis and DNA staining in *Pelophylax bergeri* samples

The purified DNA extracts from skin biopsy and corresponding testis of adult *Pelophylax bergeri* together with a marker were loaded on a non-denaturing 0,8% agarose gel in TBE buffer (45 mM boric acid, 2.5 mM EDTA, 135 mMTris base, pH 8). Bromophenol blue was added to each sample before its loading to mark the migration. Gel electrophoresis was

carried out at 70 V for around 1h using a horizontal mini gel electrophoresis system (VWR, UK). The migrating DNA was observed by staining the gel for about 40 min in the dark in 200 ml TBE buffer and 20 µl SYBR Green I. The stained gels were captured by using Gene Snap Version 4.01.00 of Syngene. The migration distance of the DNA molecules from the top of the gel was used as a measure of DNA damage and was performed as previously published in Chaudhry & Jabeen (2011). The migration of the DNA is inversely proportional to the length of the DNA molecules and it was used to estimate the distribution of different strand lengths (fragments) together with the relative mobility (Rf) value. Where Rf means the distance from the origin to the centre of each fragmented DNA strand on the gel divided by the total distance travelled by the tracking dye from the top to the bottom of that gel. It is recognized that the highly fragmented, low molecular weight DNA strands will migrate farther than not damaged high molecular weight DNA strands. The gel photographs were used to estimate the Rf values of DNA fragments in frog testis samples. The DNA fragments and their Rf values were then compared with DNA ladder to determine the effect of River water environment on the DNA of captured frogs.

3.3.c. Purification, characterization and validation of oxidative stress response poly(ADP-ribose)polymerase enzyme in *Pelophylax bergeri*

3.3.c.1. Homogenates preparation, nuclear and cytoplasmic fractions isolation

Modified method of Adamietz and Böttcher, (1994) was used to isolate nuclei from a pool of skin biopsies and from a corresponding pool of testis after gonadectomy of adult frogs, *Pelophylax bergeri.* All operations were carries out on ice. The tissues were resuspended and homogenized at low speed in 10 mM TrisHCl pH 7.4, 10mM NaCl, 0.5 mM $PhMeSO_2F$, 3mM $MgCl_2$, 0.5% NP-40 and 5-10µg/ml inhibitor cocktail (buffer A, 1:4 w/v). After centrifugation at 2000g for 15 minutes at 4°C in Thermo Scientific SL 16R centrifuge, the supernatant containing the cytoplasmic fraction was frozen at -20° C and the pellet was resuspended in buffer A (1:2 weight / volume). Buffer B (buffer A, containing 0.88 M

sucrose,) was added v/v. Subsequently, the suspension was layered on a cushion of buffer B 81.0.5, v/v) and centrifuged at 3000g for 10 minutes at 4°C. Pellet was suspended in buffer DBT (buffer A/0.3M sucrose/1mM EGTA/1% Triton X-100, 1:1 w/v) and centrifuged as above. Pellet, washed once in buffer DB (buffer A/0.3M sucrose/1mM EGTA/1%, 1:1 w/v), was centrifuged at 750g for 10 minutes at 4 ° C. Nuclear pellet was resuspended in buffer DB, containing 20% glycerol and stored at -80°C.

3.3.c. 2. Enzymatic activity assay

Assay conditions were the same described for eukaryotic ADP-ribosylating system (Althaus and Richter, 1987; Ueda and Hayaishi, 1985; Faraone Mennella et al., 1989). PARP activity in homogenate, nuclear and cytoplasmic fractions (400µg of proteins) was assayed for 15 minutes at 30°C in the presence of 0.51mM [^{32}P]NAD$^+$ (10.000 cpm/nmole). The final specific radioactivity (10,000 cpm/nmole) and concentration (0.51mM) were reached by mixing labeled and unlabeled 0.51mM NAD$^+$. The reaction mixture (mixture A) was composed of 100mM Tris-HCl buffer, pH 7.5; 50mM MgCl2 and 10mM DTT (final volume 50µl). In a parallel experiment, enzyme assay was carried out adding to reaction mixture DNA (1mg/ml) and DNAse (0.5mg/ml). The reaction was stopped with ice-cold 30% trichloroacetic acid (TCA) and the radioactivity present in the acid insoluble material, collected on HAWP filter (0.45µm, Millipore), determined on a Beckman LS 1701 liquid scintillation spectrometer. One PARP unit is defined as the amount of enzyme required to convert 1nmol of NAD$^+$/min. under standard conditions. Finally, PARP activity was also determined in a pool of skin biopsies and corresponding pool of testis after gonadectomy, in frogs from sites with different degree of pollution.

3.3.c. 3 SDS-PAGE and anti-PARP immunoblotting

SDS-PAGE of rat testis nuclear fraction (used as control), homogenate, nuclear and cytoplasmic fraction from a pool of skin biopsies and corresponding pool of testis after gonadectomy was performed according to (Arena et al. 2011). Proteins (20 µg) were analyzed on 12% polyacrylamide slab gels in presence of 0.1% SDS and electrotransferred onto PVDF membrane (0.2 µm BioRad) at 200 V for 2 hours at 4°C in the same buffer used

for the electrophoretic run. Gel was stained in 0.1% Coomassie G in 10% acetic acid/30% methanol.

Immuno-blot experiment procedures and buffers were according to (Arena et al., 2011). Polyvinylidene fluoride (PVDF) sheets were treated for 1.5 hour with the blocking solution (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 % (v/v) Tween 20, TBST) and 3 % (w/v) gelatine. Incubation with commercial rabbit anti-PARP (Santa Cruz; rabbit anti-human PARP, H-250, 1:1000, v/v) was performed for 2 hours at room temperature in the same solution supplemented with 0.3 % gelatine. The blot was then washed several times with buffer TBST and antibody binding was detected by using horseradish peroxidase (HRP)-conjugated goat anti-rabbit. HRP reaction was revealed by using a kit for chemiluminescence (Super Signal West Dura Extended Substrate, 34075, PIERCE) and reading by Chemidoc XRS (BioRad, Milan, Italy). The same filter, after stripping procedure was incubated with anti-PARP1 (N20) (Santa Cruz 1:1000) polyclonal primary antibodies and The horseradish peroxidase-conjugated goat antirabbit as secondary antibody (Pierce, 1:2000). Filter stripping was carried out as described in (De Maio et al., 2013). This procedure was used to remove the anti-PARP (H-250, Santa Cruz) antibody from PVDF membrane, to allow the sequential incubation first with anti-PARP (N20) (Santa Cruz). Stripping buffer contained 62.5 mM Tris-HCl (pH 6.8), 2% SDS, a final concentration of 0.1 M 2-mecaptoethanol. The filter was washed in TBST (1 x 5 min) and incubated in stripping buffer for 30 min at 50-60°C (in a heating oven). Washes in TBST (2 x 5 min) followed and membrane was blocked for 1 h in TBST 3% gelatine.

3.3.c. 4 ADP(ribosyl)ated proteins

Nuclear and cytoplasmic fractions (20µg of proteins) from a pool of frog skin biopsies were incubated under standard conditions with ^{32}[P]NAD^{+} (50,000cpm/nmole). The reaction was stopped by transferring the mixture on dry ice and liophylizing. The dried samples were suspended in electophoretic buffer and loaded on polyacrylamide (12%) gel. Images of stained gels and autoradiographic patterns of labeled proteins were acquired by a Phosphor imager (mod. Fx, Biorad).

3.3.c. 5 PARP electrolution

The bands recognized by anti-PARP (H-250) antibodies were electroluted from SDS–PAGE. After electrophoresis of nuclear fractions (400µg of proteins), the gel was stained for 15 min with G Coomassie Blue. As soon as the proteins of interest became visible, the bands was cut out with a razor blade. The gel pieces were washed and placed into dialysis tube (14.000c.o.), containing 1-mL of electrolution buffer (0.01M Tris-acetate pH 8.6. Finally tubes were placed in the electrolution apparatus containing 0.01M Tris-acetate pH 8.6 in central lodging and 0.04M Tris-acetate in the central lodging. The electroeluiction was carried out to 8mA overnight. The electroluted proteins concentration was determined by Bradford method (Bradford, 1976).

3.3.c. 6 PARP activity in electroluted proteins

PARP activity of two electroluted proteins was measured in standard assay conditions and in presence of DNA (1mg/ml) and DNAse (0,5mg/ml) in the reaction mixture.

3.3.c. 7 SDS-PAGE and Western blotting of electroluted proteins

The electroluted proteins extracted from a pool of skin biopsies and a corresponding pool of testis after gonadectomy were analyzed by SDS-PAGE and identified Western Blotting with anti-PARP (H-250, Santa Cruz antibodies) as already described. The same filter after stripping was also incubated with polyclonal anti-polyADPribose (Alexis, 1:1000) as primary antibody. The horseradish peroxidase-conjugated goat antirabbit as secondary antibodies (Pierce, 1:2000) were used.

3.3.c. 8 Synthesis of poly(ADPribose)

The electroeluted enzymes (1µg), extracted from a pool of frog skin biopsies were incubated in the presence of 0.50 mM [^{32}P]NAD^{+} (100,000 c.p.m/ nmol) under standard conditions (final volume 500 µl). Reactions were stopped by transfer onto ice and the addition of 20% (w/v) trichloroacetic acid (final concentration). After washing with ethanol, the precipitates were resuspended in 10 mM Tris-NaOH pH 12 buffer, containing 1 mM EDTA, and incubated for 6h at 60°C. Pure protein-free ^{32}[P] poly(ADP-ribose) was

extracted three times with alcohol/chloroform (1:49, v/v) and separated by 10% polyacrilamide gel electrophoresis, according to Panzeter and Althaus (1990). After drying, the gel associated radioactivity, was detected by Phosphor-imager (mod. Fx, Biorad).

3.4. ENDOCRINE DAMAGE IN THE BIOSENTINEL ORGANISM *Pelophylax bergeri*

3.4.1. Seasonal Poly-ADP-ribose polymerase testis evaluations

Adult frogs representative from Sarno River and available in Matese Lake identified as *Pelophylax bergeri* (n=5/selected month for three years), were examined during the main phase of the reproductive cycle: in April and May, during active spermatogenesis and breeding season and in October and November, during spermatogenetic regression. Pool of testis, taken after gonadectomy, were combined and homogenized for biochemical procedures followed by nuclei isolation and Poly-ADP-ribose polymerase standard assay and protein analysis and Western Blotting, as reported in §§3.4.c. 3.

3.5. STATISTICAL ANALYSIS

The results are expressed as a mean ± standard deviation. Data were analyzed by ANOVA and considered statistically significant at $p < 0.05$ (*) and $p < 0.001$ (**).

CHAPTER 4

RESULTS

4.1. SITES AND ANIMALS

4.1.a. Sampling points and sample monitoring and collection

Surveys and transects were made with seasonal standard sampling by nets in the chosen breeding site, as reported in **§§ 3.1.a**, basing on the level of pollution by macrodescriptors (LIM), which results by adding the scores obtained from chemical and microbiological macrodescriptors parameters, considering the 75th percentile of the range of measures that allow the definition of quality levels decreasing from 1 to 5; density of population; prevailing economy activities (**Fig. 4.1.1**).

The best sites resulted to be, along the Sarno river (see **fig 4.1**):

Sarno: 40,8088N; 14,6079E (class 5, heavily polluted watercourse, industrial pollution);

Scafati: 40.7460N; 14.5229E (class 5, heavily polluted watercourse, agrarian pollution).

A monitoring per view allowed to confirm the presence of species reported by the European Commission in the MED program, but also to integrate it for some Amphibians and Reptiles species (see **table 1§1.2**).

	Sarno (SA)	Scafati (SA)	Matese Lake (CB)
Density of population *ISTAT 2001*	31 463	60.254	
Prevailing economy activities	Industrial-Agrarian	Industrial	Tourism and handicrafts
Industries	Agrarian Mechanical Canning Agri-food Textiles	Canning Agri-food Textiles Mechanical Oil mill	Non present
Hydrogeographic basin	Sarno	Sarno	Matese lake
Biological Index (*IBE*) *ARPAC 2009*	Class V: environment highly degraded	Class V: environment highly degraded	
Physic-Chemical state *LIM* *ARPAC 2009*	Class V: heavily polluted watercourse	Class V: heavily polluted watercourse	
Ecological state: micropollutants *Government Commission for the Emergency of Sarno River*	Vanadium, beryllium, tin, PAHs, PCBs, heavy hydrocarbons, zinc, total chrome, lead, copper	Vanadium, beryllium, tin, PAHs, PCBs, heavy hydrocarbons, zinc, total chrome, lead, copper, mercury	

Figure. 4.1.1. Characterization of chosen sites and their pollution degree.

And the unpolluted sampling point chosen for the Matese Lake is at 41.4079N,14.4042E.

From a preliminary evaluation, in areas adjacent to those indicated by coordinates, was detected the absence of the biotic component, due to the heavy pollution of the riverbed and to the artificialisation of the basin, or sometimes were found malformed specimens, with damages at the skeletal and gonads (see **Fig. 4.1.2**)

Figure. 4.1.2. Specimens with malformation at the skeletal and gonads.

Morphologically identified species, tank to the classical taxonomic keys, all belong to the genus *Pelophylax,* and in particular the species are: *lesson, ridibundus, esculenta* and *bergeri.* (see **table 4.1.**).

Table 4.1.1. Taxonomic characteristics, IUCN classification of specimens identified.

Specie	Taxonomic keys	IUCN classification	Specimen
Pelophylax esculenta	smooth skin, clear vocal sacs, green back with dark stripes, white belly without spots	**LC**	
Pelophylax lessonae	little wrinkled skin, grey vocal sacs, dark back with shades of brown and black spots, grey belly with spots	**LC**	
Pelophylax bergeri	smooth or slightly wrinkled skin, white vocal sacs, green back or light brown with light stripes and spots, white belly without spots	**LC**	
Pelophylax ridibundus	slightly wrinkled skin, grey vocal sacs, brown back or dark green, grey belly with small dark spots	**LC**	

Samples whose identification was difficult were identified by the barcoding molecular investigation, using a non-invasive biomarker: the skin biopsy, obtained as reported in §3.1.b.

4. 1.b. Biopsy and gonadectomy

In **table 4.1.2** are reported from several and scalar quantities (from the canonic 100mg to 10mg) to establish the minimum skin weight amount, medium values of absorbencies. A good yield from the DNA extracted resulted with 100mg, as with 10mg, but the amount of 30 mg was chosen to allow further evaluation on the examined tissues.

Table 4.1.2. Typical average values of absorbancies and DNA concentrations for frog skin tissue amounts.

Sample	A 260 nm	A 280 nm	[DNA] (ng /µL)
Skin biopsy 100 mg	2,887	1,166	184,344
Skin biopsy 50 mg	2,847	1,186	162,361
Skin biopsy 30 mg	2,818	1,137	140,898
Skin biopsy 20 mg	2,814	1,108	130,688
Skin biopsy 10 mg	2,767	1,013	120,366

The skin biopsy and gonadectomy realized (30mg) permitted to:

1. extract the DNA with a good yeld;

2. identify the species by PCR-barcoding;

3. evaluate the oxidative, genotoxic and endocrine damage.

The *Pelophylax* gonadectomized were put in relaying for two weeks before reimmission in the site of collection and no mortality was detected, as for biopsy, following indication reported in §§ 3.1b for non reproductive frog whereas.

4.2. IDENTIFICATION OF AMPHIBIANS OF THE GENUS *Pelophylax* PRESENT IN THE SAMPLING POINTS AND CHOICE OF THE BIOSENTINEL ORGANISM

4.2.a. DNA extraction from skin biopsy in *Pelophylax* species

DNA extracted from all examined fresh frog skin biopsies resulted an efficient target as a PCR template, despite small quantities. From a quantity of 30mg skin, in fact, we successfully obtained a concentration of 140ng/μL, which represents a good amount for an efficient amplification reaction.

4.2.b. PCR amplification and sequencing of cytb, 16S and CO I mt-rRNA gene fragments in *Pelophylax* species

The described set of primers (**§ 3.2b**) successfully amplified the mitochondrial region fragments examined (**data not shown**). The PCR products isolated from gel, were sequenced and aligned, using FASTA tool (http://www.ebi.ac.uk/Tools/sss/fasta/), with GenBank sequences data for 12S rRNA and CO I of *Pelophylax bergeri*, (Günther, 1986) (AJ222650), 16s rRNA for *ridibundus* (Pallas, 1771) (FJ595209) and *lessonae* (Camerano, 1882) (AY322276), confirmed the morphological identification.

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4.2.c. Evaluation of the abundances indexes basing on the size of population of frogs of the genus *Pelophylax*

In **table 4.2** shows the evaluation of the number of samples per specie, belonging to the genus *Pelophylax* morphologically identified in the preliminary phase of the research in selected sites, as indicated in § 4.1.a.

The specie *bergeri,* in both phases of the reproductive cycle examined resulted to be the most representative.

Table 4.2. Semiquantitative abundance index of species belonging to the genus *Pelophylax*, in reproductive (April/May: A/M) and non reproductive (October/November: O/N) phase.

2011	Sarno (40,8088N; 14,6079E)		Scafati (40.7460N; 14.5229E)		Matese Lake (41.4079N; 14.4042E)	
	A/M	O/N	A/M	O/N	A/M	O/N
P. bergeri	68	43	70	38	61	43
P. lessonae	17	12	14	8	22	13
P. esculenta	18	11	17	10	16	18
P. ridibunda	15	10	11	9	11	9

4.3. OXIDATIVE AND GENOTOXIC DAMAGE IN THE BIOSENTINEL ORGANISM *Pelophylax*

Samples of skin and testis examined show an EPR signal characterized by three arrows relatively narrows, as shown in Figure **4.3.1**, typical of radicals. EPR signals were registered, for instrumental reasons, as derivative.

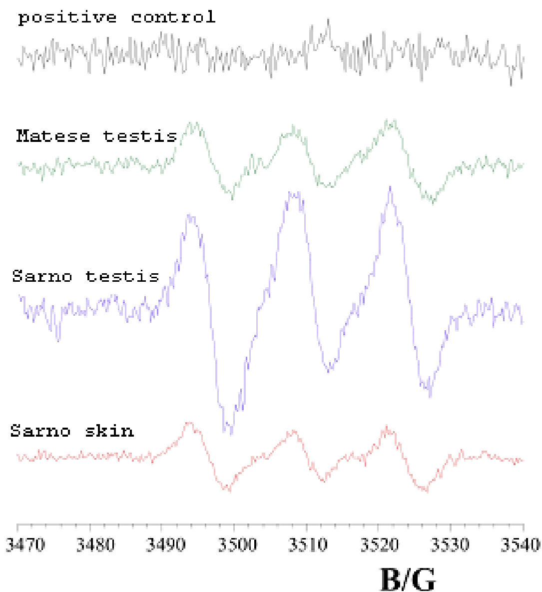

Figure 4.3.1. EPR spectra obtained in skin biopsy and testis.

In **Figure 4.3.2** is shown the first integration, that returns the absorption spectrum of the incident radiation.

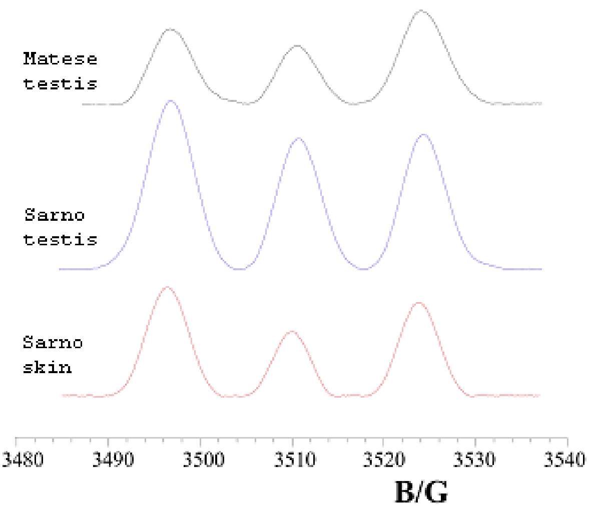

Figura 4.3.2. Integration of the spectra obtained.

The second integration (see **Figure 4.3.3)** gives the value subtended to it, proportional to the concentration of radicals in the sample. The calculation of the double integral is carried out to obtain a more precise determination of adducts. To calculate it was used a

program on the computer associated with the instrument, while viewing and recording the spectra. The method begins by a known spectrum and proceeds in the correction of the base line. The first integration allows to obtain the spectrum in absorption among the field, and no longer in derived, as it’s recorded. The second base line correction permits to calculate the second integral, obtaining a number value corresponding at the area under the peaks of the spectrum. The values of double integrals obtained, reported in **Table 4.3** show that the concentration in the testis is higher than that measured on the skin.

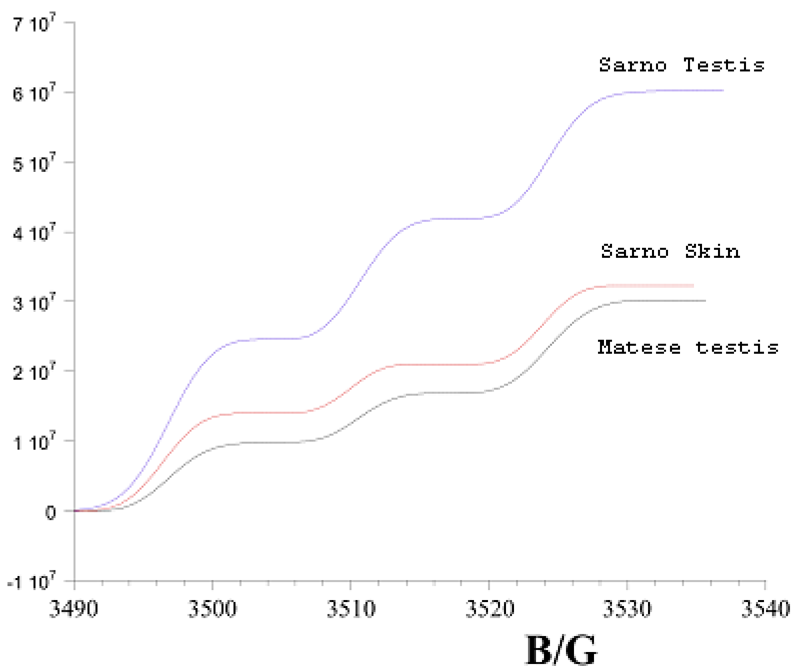

Figure 4.3.3. Double integration of spectra

Skin and corresponding samples from the testis of frogs from Sarno river average concentrations of ROS are greater than those seen in frogs from Matese lake.

The accuracy of a single measurement was estimated of about 1-2%. The error obtained by preparing samples in triplicate is approximately 10%.

Table 4.3. Values of double integrals of the EPR spectra of the PBN adduct obtained with the tissues indicted. Values are reported in arbitrary units and enable a comparative evaluation. They are expressed as a mean ±SD.

Sampling period	Tissue	Sarno river frogs	Matese Lake frogs
October 2011	testis	6.5±0.8	**2.4±0.3**
	skin	3.6±0.3	**1.8±0.2**
October 2012	testis	7.8±1.8	**3.0±0.3**
	skin	3.8±0.5	**1.9±0.2**
October 2013	testis	5.1±0.9	**2.6±0.4**
	skin	3.7±0.3	**1.4±0.2**

4.3.a. DNA stability by mobility shift in *Pelophylax bergeri*

DNA profiles of skin and testis from *Pelophylax bergeri* are shown on the UV gel photographs. The DNA extracted from Matese Lake frogs skin biopsy and corresponding testis from gonadectomy resulted without damage, like frogs' testis from Sarno River, whereas Sarno skin biopsy frogs DNA appear very damaged (**Fig. 4.3.4 and 4.3.5**). Apoptotic DNA band occurs only in the skin of reproductive phase, as in lane 3.

Testis DNA pattern show a high molecular weight, of about 23Kb, with a Rf value of 0,3.

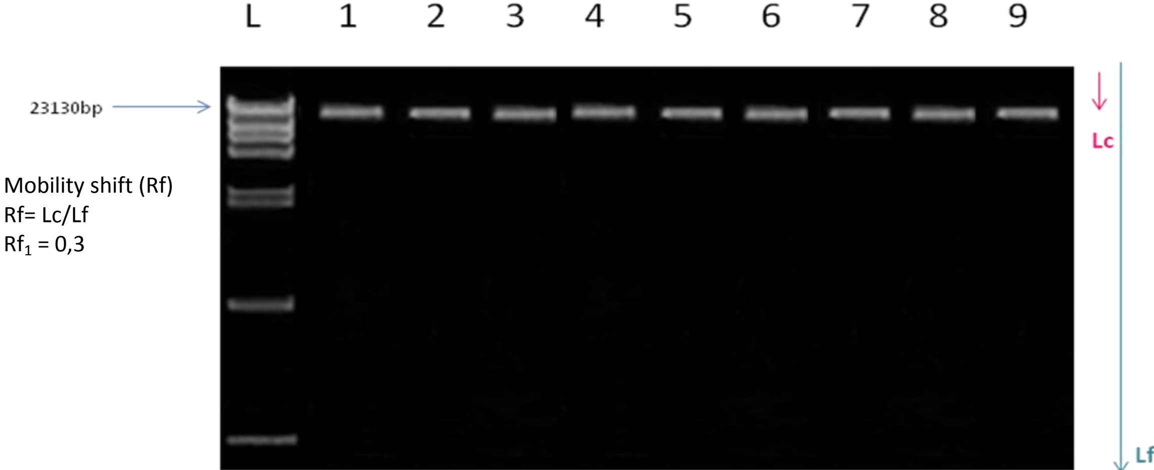

Figure 4.3.4. DNA profile in *Pelophylax bergeri* testis. L λ/Hind III DNA ladder; standard positive control (1); frog nuclear fraction a. from Sarno river in reproductive phase: April (2), May (3) and in spermatogenetic regression: October (4) and November (5); b. from Matese Lake in reproductive phase: April (6), May (7) and in spermatogenetic regression: October (8) and November (9).

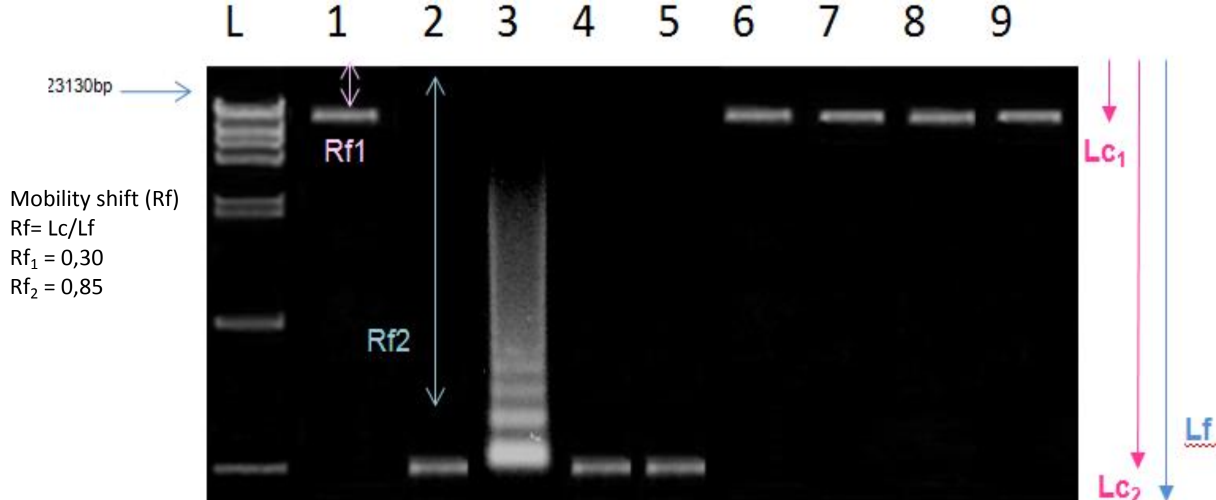

Figure 4.3.5. DNA profile in *Pelophylax bergeri* skin.L λ/Hind III DNA ladder; standard positive control (1); frog nuclear fraction a. from Sarno river in reproductive phase: April (2), May (3) and in spermatogenetic regression: October (4) and November (5); b. from Matese Lake in reproductive phase: April (6), May (7) and in spermatogenetic regression: October (8) and November (9).

Skin DNA pattern, instead show, in specimens from Sarno, a low molecular weight, with a Rf value of 0,85.

4.3.b. Purification, characterization and validation of oxidative stress response poly(ADP-ribose)polymerase enzyme in *Pelophylax bergeri*

4.3.b.1 Localization and identification of PARP enzyme in *P. bergeri* skin by PARP activity and immunochemical analysis

To localize and identify possible PARP enzymes in skin *P. bergeri*, PARP activity in standard assay conditions and immunochemical analysis were carried out on homogenate, nuclear and cytoplasmic fractions.

Table 4.3.1. PARP activity in *Pelophylax bergeri* skin in standard assay conditions, in absence and presence of salmon testis DNA (1mg/ml) and DNase (0.5 mg/ml). The values were the means of four different experiments in duplicate. SH: Skin homogenate; SNF: Skin nuclear fraction; SCF: Skin cytoplasmic fraction. Data are expressed in ± SD.

		pmoles mg^{-1}
SH		6.2 ± 0.05
SH	+ DNA	43.4 ± 0.03
SNF		4.1 ± 0.04
SNF	+ DNA	43.2 ± 0.05
SCF		2.2 ± 0.05
SCF	+ DNA	2.12 ± 0.03

The results showed that the PARP activity measured in homogenate obtained from pool of skin is distributed between nucleus (67%) and the cytoplasm (about 30%). Moreover,

the addition of DNA (1mg/ml) and DNase (0,5mg/ml) produced a PARP activity increase of about seven times in both skin homogenate and nuclear fraction. No increase of PARP activity was observed in cytoplasm (**Table 4.3.1**).

Subsequently, to identify the PARP enzymes, homogenate, nuclear and cytoplasmic fraction from pool of skin of *P. bergeri* together nuclear fraction from rat testis (control) were analyzed by polyacrylamide gel in SDS (12%) (**Figure 4.3.6A**) and subsequently to western blotting with both anti-PARP(H-250) (**Figure 4.3.6B**), and anti-PARP(N-20) antibodies (**Figure 4.3.6C**).

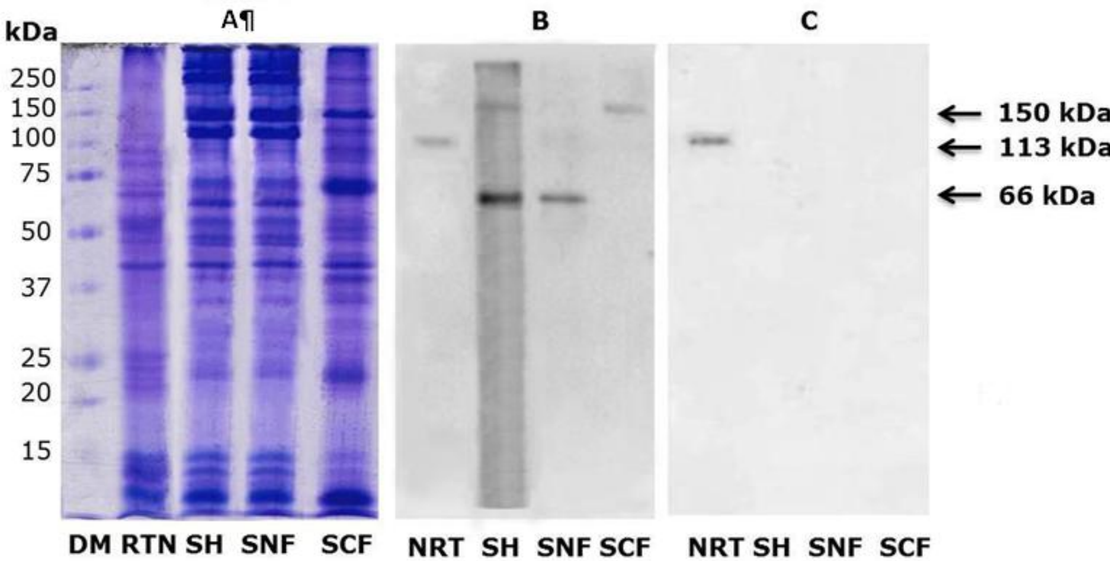

Figure 4.3.6. SDS-PAGE (12%) (A); Immunoblotting with anti PARP antibody (Santa Cruz,; rabbit anti-human PARP, H-250, 1:1000,v/v) (B); Immunoblotting with anti PARP1 (N20) antibody (Santa Cruz, 1:1000,v/v) (C). RTN. Rat testis nuclei; SH. Skin homogenate; SNF. Skin nuclear fraction SCF. Skin cytoplasmic fraction.

The first antibody is able to recognized PARP catalytic domain, while the second cross-reacts with the “zinc finger” N terminal domain, characteristic of the only PARP1. The immunoblotting with anti-PARP(H-250) showed an immunoreactive signal of 113kDa, corresponding to molecular weight of eukaryotic PARP1, in control fraction (**Figure 4.3.6B**).

In pool skin homogenate, the same antibody recognized two proteins with different molecular weight. The first of 66kDa is also present in nuclear fraction, whereas the second with an higher molecular weight (150kDa) is evidenced in cytoplasm too (**Figure 4.3.1B**). Finally, the incubation with anti-PARP(N-20) antibody confirmed the presence of the PARP-1 only in nuclei of rat's testis (**Figure 4.3.1C**).

4.3.b.2 Temperature, pH and NAD-dependence of ^{32}[P]NAD in *P. bergeri* skin nuclear fraction

To identify the standard assay conditions, homogenate was incubated in different experimental conditions: 1) different temperatures and in presence of ^{32}PNAD (0.5mM) at pH 7.5; 2) different pH and in presence of ^{32}PNAD(0.5mM) at 30°C in reaction mixtures with; 3) different ^{32}PNAD concentrations at 30°C and pH 7.5.

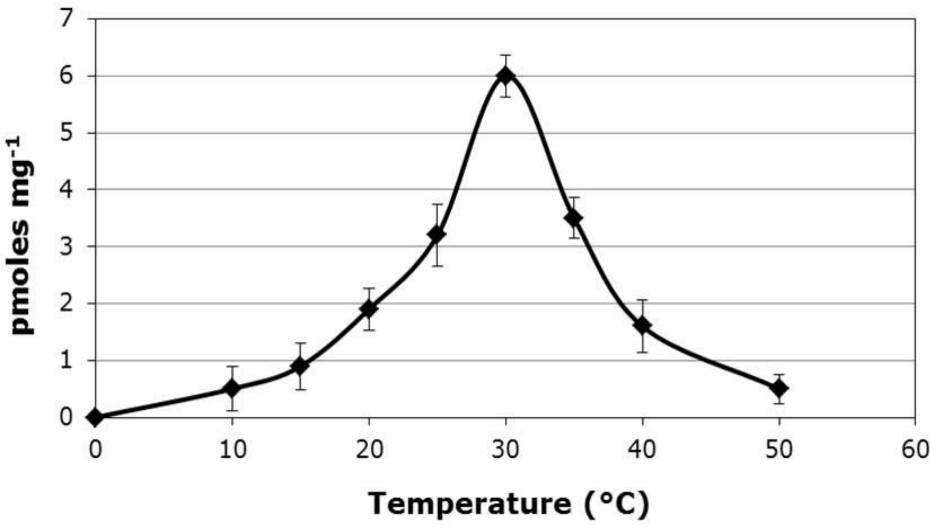

Figure 4.3.7. Temperature–dependent ^{32}P labelling. Homogenate (200 µg) was incubated under standard conditions at different temperature. The value were the means of five different experiments in duplicate, carried out with two different preparation of nuclear fraction from *P. bergeri*. Data are expressed in ± SD.

In the trichloroacetic acid-insoluble fraction of pool of skin homogenate, the uptake of ^{32}P from [adenylate-^{32}P]NAD reached a maximum at 30°C (**Figure 4.3.7**).

The frog PARP exhibited an optimum of pH between pH 7.5 and 8.0 (**Figure 4.3.8**).

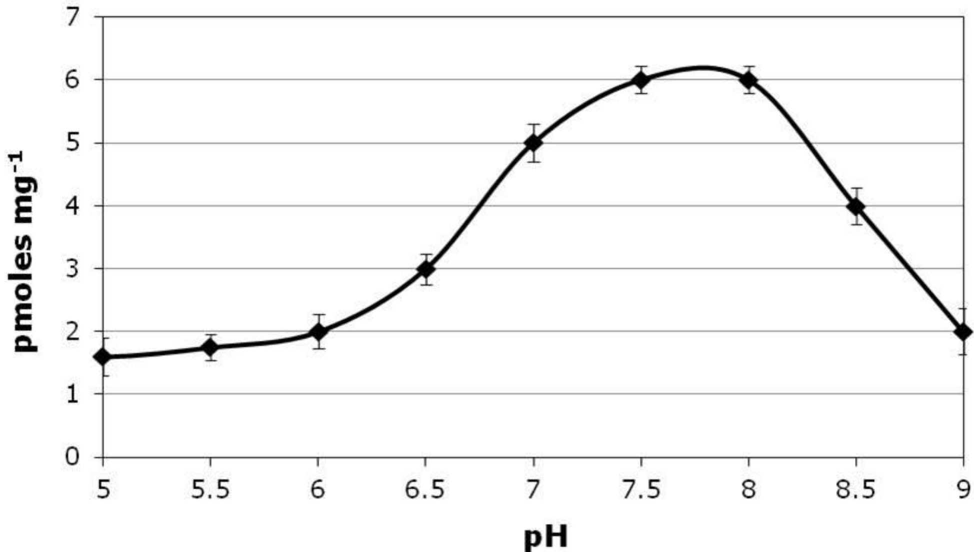

Figure 4.3.8. pH dependence of the enzymic activity. Homogenate (200 μg) was incubated in 0.1 M buffer (sodium acetate, pH 5.0-5.5; potassium phosphate, pH 5.5-7.5; Tris/HCl, pH 7.5-9.0) under standard conditions. The value were the means of four different experiments in duplicate, carried out with two different preparation of nuclear fraction from *P. bergeri.* Data are expressed in ± SD.

The dependence of the ^{32}P labelling of the skin nuclear fraction on adenylate-$^{32}PNAD$ concentration under standard condition showed that the saturation of the labelling levels was reached at substrate concentration of 0.5mM (**Figure 4.3.9**).

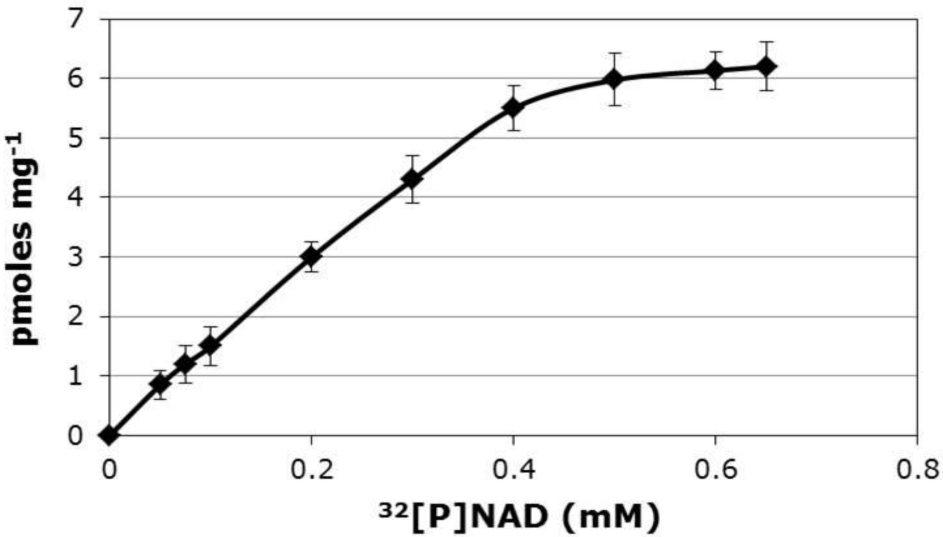

Figure 4.3.9. NAD-dependence ^{32}P uptake into skin homogenate (200 μg). The fraction was assayed under standard conditions, in presence of increasing amount of ^{32}[P]NAD. The experiments were carried out in duplicate using two different preparation of nuclear fraction from *P. bergeri.* Data are expressed in ± SD.

4.3.b.3 Time courses of ADPribosylation of proteins in *P. bergeri* skin

The incorporation of ADPribose into frog pool of skin homogenate proteins showed a linear increase of ^{32}P uptake upon to 15 minutes. At longer times the curve tends to plateau (**Figure 4.3.10**).

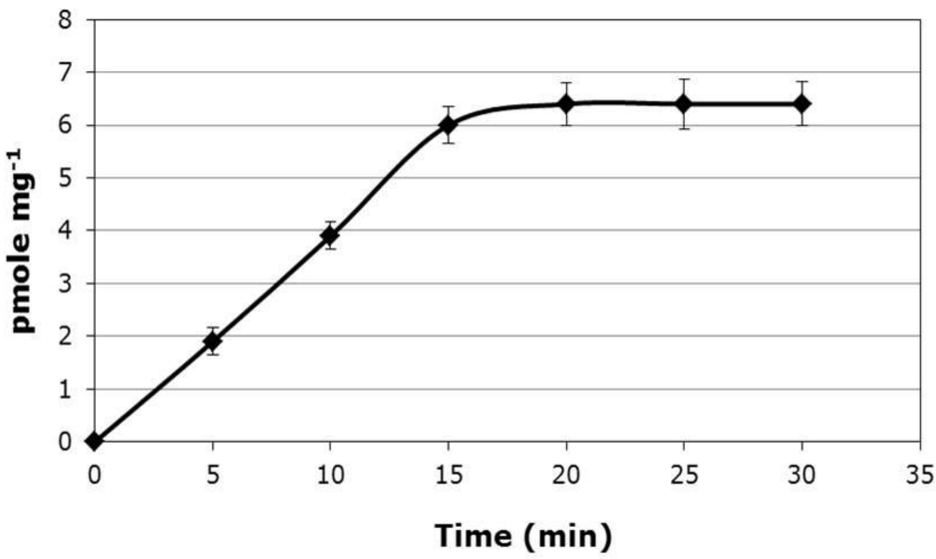

Figure 4.3.10. Time course of incorporation of ADP-ribose into proteins of skin homogenate. The nuclear fraction (200 µg) was incubated under standard conditions for different time. The value were the means of five different experiments in duplicate, carried out with two different preparation of nuclear fraction from *P. bergeri*. Data are expressed in ± SD.

4.3.b.4 Covalent protein acceptors in *P. bergeri* skin

The autoradiographic analysis of both nuclear and cytoplasmic fractions from pool of skin nuclei incubated with [^{32}P] NAD+ and electrophoresed (**Figure 4.3.11**) revealed two strong signals corresponding to two ADPribosylated proteins of 66kDa and 150kDa, respectively. Besides 66kDa signal, another radioactive band was also observed corresponding to a modified protein of about 25 kDa in nuclei (**Figure 4.3.11**).

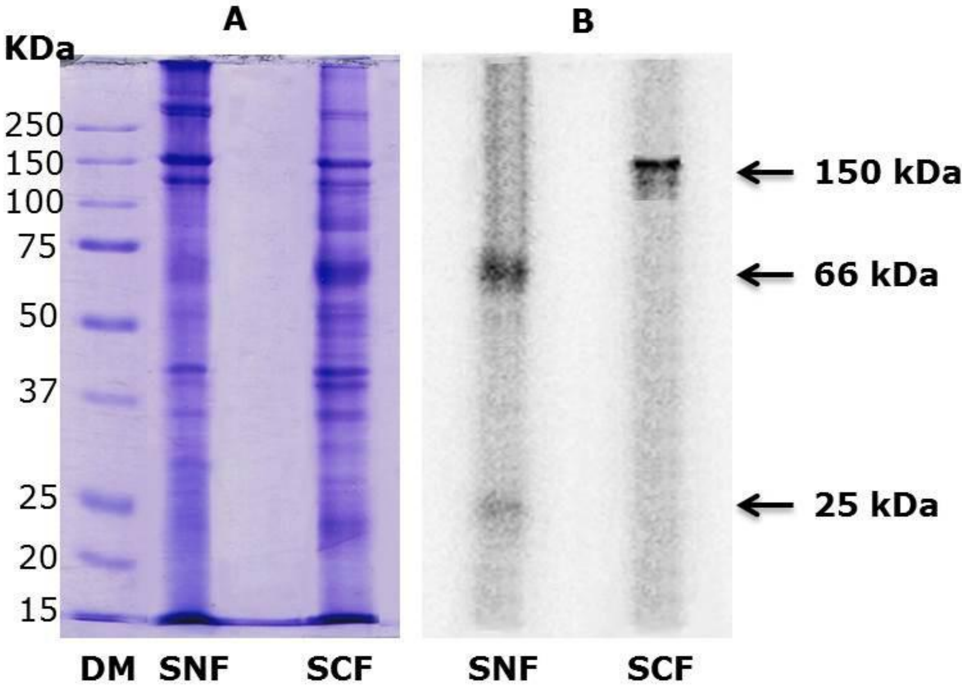

Figure 4.3.11. ADPR covalent protein acceptors: SDS-PAGE of Nuclear and Citoplasmic fractions (20 µg of proteins) from skin frog incubated under standard conditions with ^{32}[P]NAD (50.000 cpm/nmole) (A); Autoradiography of SDS-PAGE (B).

4.3.b.5 Enzyme activity of electroluted bands in *P. bergeri* skin

The bands (66kDa and 150kDa) recognized by anti-PARP (H-250) were purified by electrolution and incubated in standard assay conditions in presence of salmon's testis'

DNA (1mg/ml) and DNAse (0.5mg/ml), to verify whether the DNA influences the activity of both proteins or only one.

The results showed that the addition of DNA to mixture reaction increases the enzymatic activity of the protein of 66kDa of about 60 times compared to that measured in the absence of DNA. On the other hand, no significant PARP activity increase was observed, when the same experiment was carried out incubating the protein of 150kDa (**Table 4.3.2**).

Table 4.3.2. PARP activity of 66kDa and 150kDa proteins, in standard assay conditions, in absence and presence of salmon testis DNA (1mg/ml and DNase (0.5 mg/ml). The value were the means of four different experiments in duplicate. E_{66}: Eluted protien of 66kSa; E_{150}: Eluted protien of 150kDa. Data are expressed in ± SD.

	nmoles mg^{-1}
E_{66}	8.0 ± 0.04
E_{66} + DNA	486 ± 0.03
E_{150}	0.8 ± 0.04
E_{150} + DNA	0.78 ± 0.05

4.3.b.6 Identification of electroluted bands and isolation of their reaction products in *P. bergeri* skin

SDS-PAGE (**Figure 4.3.12A**) and Western blotting with anti-PARP (H-250) antibody (Figure 4.3.12B) allowed to confirm that the proteins electroluted from homogenate

corresponded to two immunopositive bands of 66kDa and 150kDa showed in nuclear and cytoplasmic fractions, respectively (**Figure 4.3.12B**).

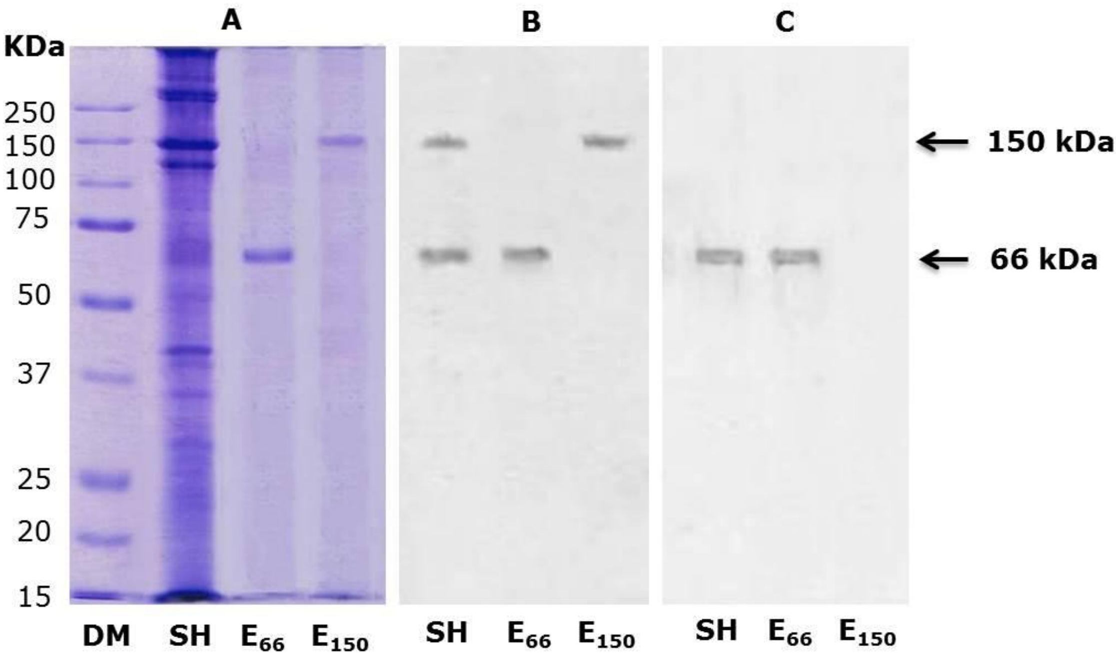

Figure 4.3.12. SDS-PAGE (12%) (A); Immunoblotting with anti PARP antibody (Santa Cruz,; rabbit anti-human PARP, H-250, 1:1000,v/v) (B); Immunoblotting anti-polyADPribose (Alexis, 1:1000, v/v) (C); Activity blot (D) SH: Skin homogenate E: Electroluted protein.

The signal of 66kDa was also observed when the same filter was incubated with anti-poly(ADPR) antibody, able to recognize olygomers bound to protein acceptors consisting of at least 5 ADPR units. The non-significant shift in molecular weight between the band of 66kDa, shown by anti-PARP, and the one recognized by anti- poly(ADPR) antibodies suggested that this protein might be automodified with short ADPR oligomers. No immunopositive signal is observable when the 150kDa band was analyzed (**Figure 4.3.12C**). Probably, this protein may be modified by an oligomer shorter than five units or a single ADPR unit.

Subsequent isolation of the reaction products and their analysis onto high–resolution sequence gel allowed to identify the length of ADPR chains bound to both proteins.

PAGE of ^{32}P-labelled products showed that the 66kDa protein synthesizes oligomers of about 5-6 ADPR units (Figure 4.3.13, line 3), whereas single ADPR molecules were produced by 150kDa enzyme (**Figure 4.3.13, line 2**). ^{32}[P]NAD (200cpm) was used as control, as it migrates as a ADPR tetramer (Figure 4.3.13, line1). Results were analyzed in triplicate.

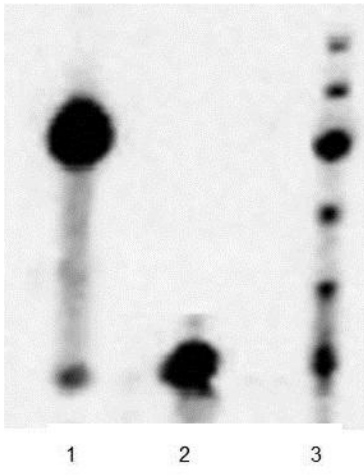

Figure 4.3.13. Separation of protein-free ^{32}[P]-labelled-polyADPR from electroluted PARP. 1: ^{32}[P]NAD (200 cpm); 2:ADPR from electroeluted enzyme (150kDa); 3:ADPR oligomer from electroeluted enzyme (66kDa).

4.3.b.7 PARP activity in different *Pelophylax bergeri* tissues, collected in different sites

PARP activity was also measured in pool of skin and corresponding testis (from one testis gonadectomy) homogenates of *P. bergeri*, collected from Matese Lake (control site), Sarno and Scafati (polluted sites). The higher PARP activity was measured in both tissues of frogs collected from Sarno river, while the lowest was detected in the tissues of frogs from Matese lake, to confirm that it is a good control site (**Figure 4.3.14**).

Therefore, PARP activity was statistically significant different in frog tissues sampled in Sarno (*p< 0,05, Testis SSa vs Skin SSa; TSc and TML vs SSc and SML respectively) and in tissues in differen sites (**p< 0,001, see Skin SML vs Skin SSa and SSc; Testis TML vs Testis TSa ans TSc).

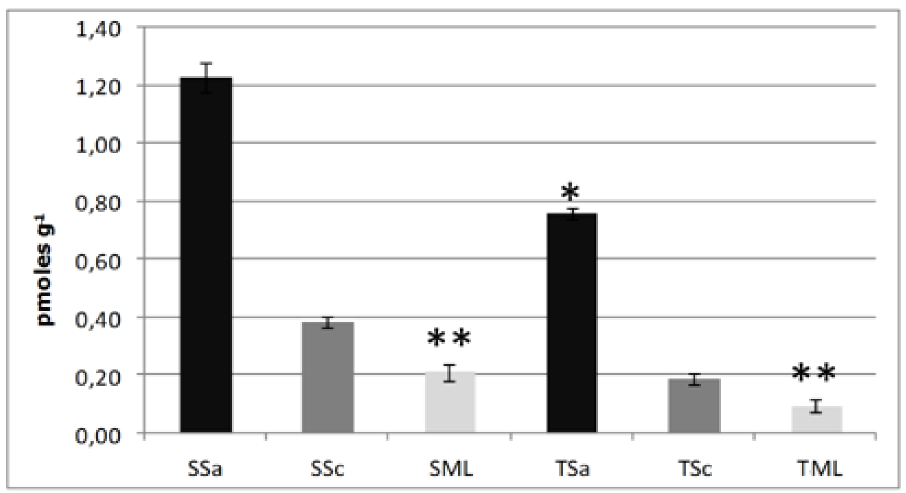

Figure 4.3.14. PARP activity in skin (S) and testis (T) frogs collected in Matese Lake (ML), Sarno river (Sa) and Scafati (Sc) during the reproductive phase. The results (n= 5 pool/selected month for three years) were analysed in triplicate and expressed as mean ±SD. * p< 0.05. Testis, Ssa vs Skin, Ssa; **p< 0.001, see Skin SML vs Skin Ssa and SSc; Testis TML vs Testis TSa and TSc.

4.4. ENDOCRINE DAMAGE IN THE BIOSENTINEL ORGANISM *Pelophylax bergeri*

4.4.1. Seasonal Poly-ADP-ribose polymerase testis evaluations in *Pelophylax bergeri*

PARP activity was measured in pool of testis nuclear fractions from *Pelophylax bergeri*, collected from Sarno river and Matese lake in the main phases of the reproductive cycle. In frog testis of *Pelophylax bergeri*, no significant difference of PARP activity were detected inter testis of the same specie ($p < 0.05$) and intra testis of different specie (p <

0.05) in the same reproductive phase examined (data not shown). The highest PARP frog testis activity is measured when the spermatogenesis is active (**Figure 4.4**)

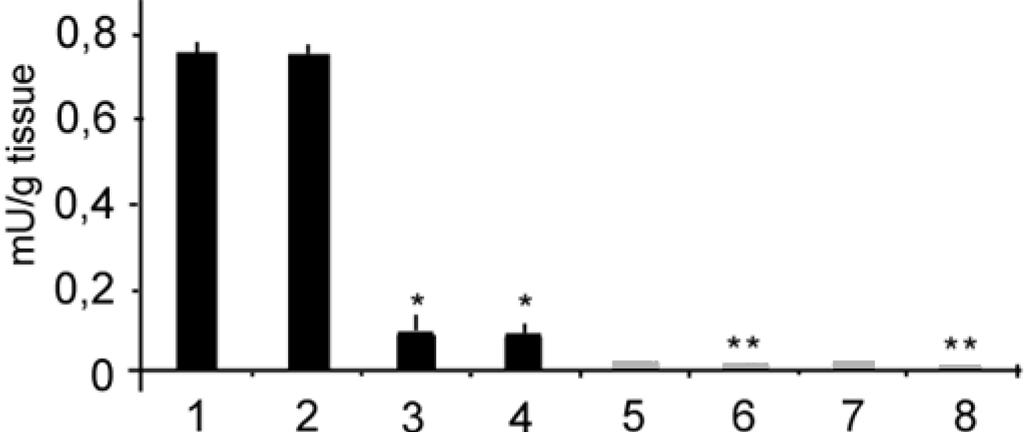

Figure 4.4. PARP activity in *Pelophylax bergeri* testis. Frog nuclear fraction a. from Sarno River in reproductive phase: April (1), May (2) and in spermatogenetic regression: October (3) and November (4); b. from Matese Lake in reproductive phase: April (5), May (6) and in spermatogenetic regression: October (7) and November (8). The results (n= 5/selected month for three years) were analysed in triplicate and expressed as mean ±SD. *p< 0.05; see 4 vs 1-2; **p< 0.001; see 5-8 vs 1-4.

Therefore, there was a statistically significant difference of PARP frog testis activity between Sarno River different phases samples (*p< 0.05, 3-4 vs 1-2) and two samples sites (**p< 0.001, see 5-8 vs 1-4).

4.4.2. PARP expression and identification in *P. bergeri* testis

Electrophoretic analysis of nuclear fractions from examined pool of testis from frogs *Pelophylax bergeri* collected in April and May months of reproductive cycle have not shown remarkable qualitative and quantitative differences in protein patterns (**Figure 4.4.1A**).

Immunoblotting of all examined samples, performed with antibodies either against PARP C-terminal catalytic domain, showed a single immunoreactive band, corresponding to a protein with molecular weight of 66kDa (**Figure 4.4.1B**). This molecular weight corresponds to mammalian PARP2 molecular weight.

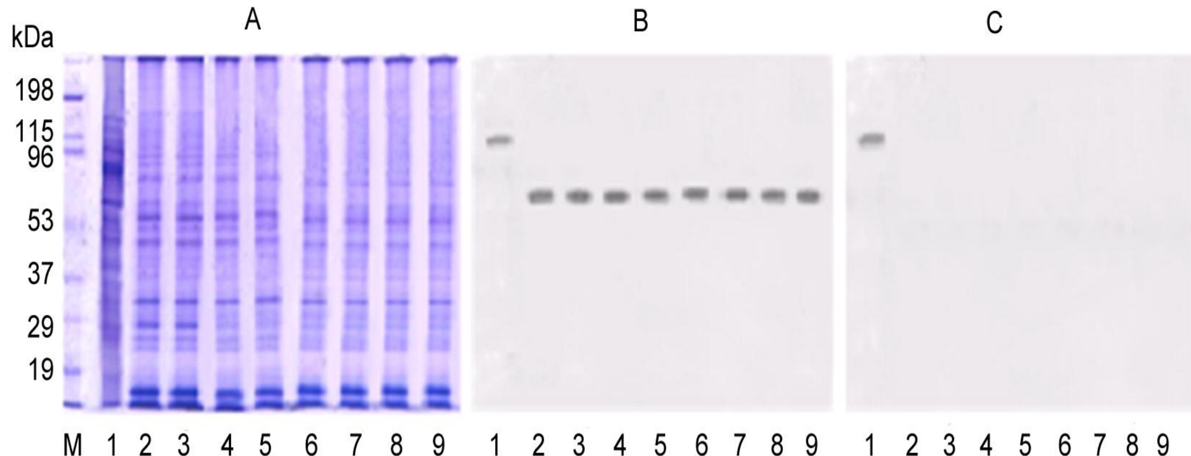

Figure 4.4.1A-C. SDS-PAGE of nuclear fractions from *Pelophylax bergeri* testis (A); Western blotting with anti PARP (H-250) on nuclear fractions from frog testis (B);anti-PARP (N-20) antibodies on nuclear fractions from frog testis (C). Buffalo testis nuclear fraction (1); frog nuclear fraction a. from Sarno River in reproductive phase: April (2), May (3) and in spermatogenetic regression: October (4) and November (5); b. from Matese Lake in reproductive phase: April (6), May (7) and in spermatogenetic regression: October (8) and November (9).

To confirm this hypothesis, the same samples of *Pelophylax bergeri*, were subjected to western blotting with anti-PARP1 (N20) antibodies able to recognize the N-Terminus zinc finger DNA-binding domain, characteristic of the only PARP-1. No immunopositivity is evident in correspondence of frog samples *Pelophylax bergeri* while in the nuclear fraction from bull testis, used as control, a clear immunopositive band corresponding to PARP-1 (113kDa) is evident (**Figure 4.4.1C**).

CHAPTER 5

DISCUSSION

5.1. POLLUTANTS IN SARNO RIVER AND ADOPTED CONTROL STRATEGY OF THE ENDANGERED SPECIES OF THE GENUS *Pelophylax*

To assess the biological site and targets, documentation related to the toxic pollutants of Sarno River (Arienzo et al., 2000; De Pippo, 2006, Albanese et al., 2013) was examined starting from considering the industries present on the territory, their discharges and all the human activities (**§1.1 and §1.3**). During the study specific issues have emerged, related to urban discharges, continue spills by industries, but at the same time the desire of the inhabitants of the areas around the Sarno river, and local and national authorities, to tackle the problem of the environmental impact of the Sarno river pollution (**see §1.3**). As evidence of this, it is worth noting the interest of the Association "Amici del Sarno", the Province of Naples and Salerno, the ARPAC and the Government Commissione for the Emergency of Sarno River, for the activities conducted by the laboratory of Comparative Endocrinology EC*lab* (certificate UNI EN ISO 9001:2008), directed by Prof. Giulia Guerriero, where I've conducted my research.

Starting by classic biomarker water monitoring, as described by the national law 152/06, and the resulting level of Macrodescriptors (LIM) and Extended Biotic Index (EBI), preliminary studies were conducted in Sarno at 40,8088N; 14,6079E and Scafati at 40.7460N; 14.5229E as polluted Sarno River area; and in Matese Lake unpolluted area, at a control site, 41.4079N,14.4042E.

On the basis of sightings and semi-quantitative indexes of abundance of potential biosentinel species per site (see **Table 1**), we chose to monitor the frogs belonging to the genus *Pelophylax*. The *Pelophylax* frogs, like all Amphibians, represent a good indicator of

the health of aquatic environments, as they are able to integrate stimuli from the abiotic and biotic component and translate them into forms of adaptation and benefit to the community (Ghetti, 1997); since they were the first tetrapods on earth, they occupy an important position from a phylogenetic point of view. One of the most interesting aspect is that their anatomical organization, as in other non-mammalian vertebrates, allows an access to tissues not readily approachable in mammals (Pierantoni et al., 2002).

They have long life cycles, showing temporal changes caused by perturbations, allowing a long-term analysis of both regular and intermittent discharges, of varying concentrations of pollutants, of single or multiple contaminants and synergistic or antagonistic effects (Venanzi, 2006); furthermore, they represent an important link in the food chain, as a predator of many insects. The weakening of this link would create an imbalance within the chain, with the loss of other elements (Blaustein, 1994), or the predominance of certain insects, with the establishment of some important diseases (Venanzi, 2004). Since they're bound to water in the larval stage, and to the sediment as adults, can provide answers related to a greater range of pollutants, with which they are constantly in contact (Kloas et al., 2009). These characteristics make *Pelophylax* excellent bioindicators, to efficiently represent the changes induced in a watercourse by environmental stress, than unpolluted area, using appropriate biomarkers.

From the morphological estimate of the sampling (**Tab 4.3**) during April/May, and October/November, the number of specimens per reproductive area gave the idea to monitor the most representative specie *Pelophylax*, ie *bergeri*, and to evaluate the effects of pollutants on appropriate non invasive biomarkers, which avoided the sacrifice. In addition, where possible, we carried out a parallel monitoring on other species belonging to the genus *Pelophylax*: *ridibundus, esculenta* and *lessonae*. The choice to use the biopsy and gonadectomy, related to the exiguous number of specimens on the territory, and according with the need to identify hibridous species, resulted to be suitable to evaluate biomarkers linked to the oxidative stress, induced by pollutants. This method, even if not

innovative was, for the first time, applied for environmental impact studies, and preserves the biodiversity.

The success of the skin biopsy, and one testis' gonadectomy, with a mortality of only 0,1% in April-May and 0% in October-November, allows not only to reintroduce specimens in nature but, above all, the continuity of the specie.

5.2. BARCODING OF THE REPRESENTATIVE AMPHIBIANS SPECIES ALONG THE SARNO RIVER AND CHOICE OF THE BIO-SENTINEL

The utilized frogs were identified basing on the DNA barcoding (Sanger sequencing of the standard barcode region, procedure reported in Guerriero et al., (2010) as a confirmation of the morphological identification. The analysis of the number of examined specimens permitted to define all the *Pelophylax* species presents along the Sarno river, and their representativeness.

The most present bio-indicator along the Sarno river resulted to be *Pelophylax bergeri*, as in the control area, the Matese lake. The study of the specie with molecular approach, by skin biopsy, also allowed the evaluation of further mitochondrial DNA traits, widening the database for this specie. Further sequences information, for 16S and cytb rRNA genes for *Pelophylax bergeri*, not previously published in the literature, were submitted on GenBank (see accession number JN689223 for cytb, JN689222 for 16S).

The approach through the use of barcoding, with the submission of the cytochrome oxidase I gene fragment (accession number KJ433966), will ease the monitoring even by analyzing eggs and tadpoles.

5.3. *Pelophylax bergeri* AND THE STUDY OF POLLUTION BY REACTIVE OXYGEN SPECIES (ROS) AND THE GENOTOXIC DAMAGE IN ENVIRONMENTAL MONITORING BY NON-INVASIVE BIOMARKERS

5.3a. Analysis of the effects of pollutants on Reactive Species Of Oxygen (ROS) in the selected bioindicator

Analysis of ROS, consequence of pollution oxidative damage in frog as in other biosentinels, is a challenging task because of their low concentration and short lifetime, which prevents direct ex-situ tests (Falfushinska et al., 2008; Zhang et al., 2013).

All the used spectroscopic approaches suffer the interference from the chemical matrix in which the analysis is embedded, thus requiring, in many cases, a preliminary purification step (Burns et al., 2012; Khono et al., 2010).

From this point of view, conversion of ROS into stable radicals is convenient, since these can be easily, sensitively and selectively detected by our selected approach. ESR methods used in our experiments, based on spin-trapping reagents, such as nitrone compounds which react with ROS to stoichiometrically, produced stable spin-adducts such as nitroxide radicals. In particular, the selected spin-trap, ά-phenyl-N-t-butylnitrone (PBN) resulted to be a very suitable spin-trap for ROS detection, in relation to its soluble and stable in aqueous solution, and because it’s able to form long-lived spin-adduct whose spectrum that permit easy analysis (Guerriero et al., 2013).

The procedure developed from the method Ashton et al (1998) provided the use of the ά-phenyl-N-t-butylnitrone (PBN) as spin-trapper. The advantage of this technique is that free radicals generated by chemical systems or biological systems can be detected and identified by observing the ESR spectrum of a spin adduct; the ESR spectrum of a spin adduct can be used for quantitative analysis of free radicals by comparing the peak area with those obtained from stable radicals. Studies are in progress to determine the kinetic and to determine the formation and elimination velocities of a free radical in both polluted and polluted sites.

The analysis of ROS, reported in the **table 4.3** (§4.3) includes only the data related to the non-reproductive phase of the species *Pelophylax bergeri* in the period 2011-2013, in relation to the metal measurements made by ARPAC, which show a greater increase of the contaminants in waters, effect referable to discharges of the tanning and agri-food industry (§1.1). The obtained samples, as reported in the experimental section, show an EPR signal characterized by three rows relatively narrow, as shown in a typical spectrum in **Figure 4.3.1**. From the average coupling constant, ie the average distance, measured in G, between the peaks (aN = 13.7 ± 0.2), it's deducible that the PBN adducts, present in these samples, are generated by ROS (Buettner, 1987). This is necessary to record these EPR signals, for instrumental reasons, in the derivative. So the first integration returns the absorption spectrum of the incident radiation, shown in **Figure 4.3.2**, while the second integration provides the underlying value in this, which is proportional to the concentration of radicals in the sample. The values of the double integrals obtained are reported in **Table 4.3**. The obtained values of double integrals show that the concentration in the testis is higher than that measured on the skin. Skin and corresponding testis from frogs sampled from the Sarno river average concentrations of ROS are greater than those seen in frogs in the Matese lake. This shows directly that these frogs are subjected to conditions of oxidative stress, certainly related to the conditions of pollution of their habitats (Albanese et al., 2013). Specifically, it clearly opens a way for an eco-monitoring by skin biopsy, avoiding the sacrifice of animals thanks to the development of this assay provided in frog.

5.3b. Genotoxic damage identification by DNA mobility shift and poly(ADP-ribose)polymerase (PARP) activity

The oxidative damage monitored and related to pollutants, and the small number of specimens in areas adjacent to the sampling sites (see **§4.1.a**) prompted us to test and track, in a spatial-temporal way (several sites; years 2011-2013), DNA integrity and

possible repair systems. Since the DNA is a polianionic molecule, it represent a useful substrate for infiltration through membranes and adherence of contaminants, thus facilitating the formation of HO° adjacent to these critical biological targets (Halliwell and Aruoma, 1991). Additionally, the heterogeneity of DNA molecules allows for HO° attacks, including the nucleobases and the sugar–phosphate backbone (Buxton et al., 1988). Hydroxyl radicals react with nucleobases approximately five times faster than with the nucleic acid backbone (Cadet et al., 1997). Furthermore, recent studies identified more than 100 different products of DNA damage by various ROS (Kotz, 2012). The most important lesion of DNA, after radical and ROS attacks, that is used extensively as a biomarker for cellular oxidative stress and genotoxicity in living organisms.

Although we can not speak about a strictly linear response between ROS and DNA damage, the analysis of oxidative damage show that the tissue mostly affected by stress is the gonad, in frogs from the Sarno site where, remaining stable the DNA, we can suppose the action of the several repair systems, including the PARP system, is more effective.

Previous studies showed that PARP enzymes are widely distributed among different classes of eukaryotes (Aoufouchi and Shall, 1997).The broad distribution of PARP genes indicates that the ancestor of all extant eukaryotes, including invertebrates, encoded proteins of this type and that the ancestral PARP proteins had different functions and activities (De Lisa, 2012).

In liver and oocytes of female frogs (*Rana esculenta*) PARP activity was detected and active peptides of different molecular weight (100 kDa, 80 kDa, 52 kDa and 45 kDa) were evidencied (Scovassi et al., 1986).

It has been known for many years that both oocytes and eggs of *Xenopus laevis* is expressed a PARP enzyme. No detectable enzyme activity was observed in the oocytes, whereas the enzyme is active in the eggs. Enzyme activity appears during oocyte maturation, where the PARP activity is regulated by phosphorylation (Aoufouchi and Shall, 1997).

In the examined pools of skin biopsy and corresponding pools of testis two ADPribosylating enzymes with different molecular weight (66 kDa and 150 kDa) were recognized by anti-PARP(H-250) antibody. **Figure 4.3.6 B** shows the results in *Pelophylax bergeri* skin homogenates.

We hypothesized that the protein of 66 kDa, defined PPARP2 (for *Pelophylax bergeri*, as in the other species) might correspond to human PARP2 (HsPARP2), since it is localized into nucleus (**Figure 4.3.6 B**, **Table 4.3.1**), activated by DNA damage (**Table 4.3.1**) and devoid of the “zinc finger” domain at the N-terminal (**Figure 4.3.6 C**).

The protein of 150 kDa (PPARP10) (**Figure 4.3.6 B**), instead, might correspond to HsPARP10, a new member of PARP family, which does not function as polymerase but as mARTs. It was also reported that HsPARP10 can be localized in both nucleus and in cytoplasm and is not activated by DNA damage (Yu et al., 2005).

We evidenced the cytoplasmic PPARP10 is not activated by DNA damaged (**Table 4.3.1**).

Both PPARP2 and PPARP10 are covalent acceptors of ADPR (**Figure 4.3.11**). By Western Blotting with anti-PAR antibodies it was possible to evidence that PPARP2 is also endogenously modified (**Figure 4.3.12**C), whereas, using the same antibody, which is able to recognize olygomers of ADPR at least 5 units, not evident endogenous modification of PPARP10 was observed (**Figure 4.3.12**C).

Evidence of an endogenous modification of PPARP2 allows us to suppose that in the skin of examined frogs, this protein is active. This activity seems not to be correlated to DNA fragmentation (**Figure 4.3.5**), thus we hypothesized that it is responsible for the normal DNA metabolism. The same results were evidenced in frog’s testis (**Figure 4.3.4**).

In both skin and testis of frogs reproductive phase, collected from polluted sites, PARP activity levels are higher than those measured in the same tissues from frogs collected in Matese Lake (**Figure 4.3.14**).

PARP activation in skin might be explained on the basis of the evident DNA fragmentation (**Figure 4.3.5**) due to the constant exposure of this tissue to contaminants (ARPAC result,

www.arpacampania.it). Thus, in skin, PARP enzyme might be continuously engaged to repair oxidative DNA damages.

In testis, instead, where no DNA fragmentation occurs (Figure DNA) PARP activation might be correlated to other events, as chromatin remodeling during meiosis and spermatid nuclear condensation, (see for review Celik-Ozenci & Tasatargil, 2013) and be necessary for maintaining normal spermatogenesis.

In this perspective, the measurement of PARP activity in frog's skin might be use as sensitive "early warning" tools in monitoring the health state of these organisms.

5.4. PARP TESTIS ACTIVITY AND ENDOCRINE DAMAGE IN *Pelophylax*: AN INNOVATIVE METHOD FOR BIO-PRESERVATION

As the DNA strand breaks directly and immediately activate PARP1 and PARP2 (Amé et al., 2004), in this contest, first we proposed to identify which PARP was expressed seasonally in testis of all three species frogs hybridogenic complex *Pelophylax* collected from Sarno river (polluted site) and Matese Lake (control site), during the main spermatogenesis phases. Subsequently, in the same frogs, we verified whether variations of PARP activity occur, to correlate DNA damage to PARP activation and its repair capacity. It is known that both PARP1 and its cognate PARP2 are essential in DNA repair mechanisms, being involved in the base excision repair pathway, and exert their functions in both mild and extensive DNA damage (Schreiber et al., 2002). PARP2 is preferentially expressed and activated in response to exposure to heavy metals, for this reason, on the basis of our analysis by Western Blotting, we suppose that frogs collected from Sarno river are subjected to this type of pollution. The metals are reported as endocrine disruptors (Iavicoli et al., 2009) and their effects on reproduction could vary by individual differences in metabolism, body composition and susceptibility due to genetic polymorphisms (Diamanti-Kandarakis et al., 2009, 2010).

The evidences, reported only for *Pelophylax bergeri*, since statistically significative, show in all examined tissues collected from Sarno river, the PARP activity in April and May is always higher than measured in October and November, lead us to suppose that, during the active spermatogenesis the genomic material is damaged by the overexpressed free radicals inducted by the stressor insult of pollution. It appears in agreement with what is known from the literature (see for detail Guerriero & Ciarcia 2006; Baarends et al., 2001 review).

Despite the activation of PARP, no DNA fragmentation was observed in testis, also if the concentration in the testis of ROS is higher than that measured on the skin (see **table 4.3**;

Guerriero et al., 2013). The major activity of PARP in Sarno river frogs might be due to exposure to pollutants.

On the basis of the present results, we suggest PARP2 as useful genotoxic marker to monitor the DNA damage entity in *Pelophylax bergeri, ridibundus* and *lessonae* that live in polluted environments. Further studies carried out on the other frog populations could contribute to extend knowledge.

In testis, in which a DNA fragmentation is not observable, the PARP activation might represent a strategy to guarantee DNA repair, indispensible for the health of species and their reproductive capacity.

Furthermore, studies in mammalian reported by Catriona et al., (2007) seem confirm the seasonal relevance of our data in all species of frogs "*Pelophylax*" and highlight the importance on the differences in the timing of the vulnerability of genotoxic event.

In fact, levels of proteins involved in detection of DNA damage and in DNA repair are very high in the germ cell precursors for sperm and eggs too, and remain elevated in spermatocytes up until a transition period that precedes spermiogenesis. After meiosis is completed, haploid spermatids enter a period during which they are repair-deficient, and the condensed DNA is transcriptionally inactive, and inaccessible to DNA repair enzymes. Thus, it is critical for any DNA damage, incurred during crossing over and other phases of meiosis, to be detected and repaired prior to spermiogenesis (Marchetti & Wyrobek, 2005).

Although direct causal links between exposures to EDCs, PARP activity and disease states in our biosensor *Pelophylax* are difficult to draw (we have only evidence of animal three gonads or damaged arts, data not shown), results from basic research and previous geomorphology studies (De Pippo et al., 2006) make it clear that more screening for exposures.

CONCLUSIONS

In a growing deterioration of the ecosystem both in terms of biodiversity and ecological features, even more problematic is the bio-preservation of the species. For this purpose the detection and monitoring the state of the health of the Plain of the Sarno river was performed basing on the characterization of physical, chemical, biological and eco-toxicological components, which permitted to assess the ecological vulnerability of the Sarno River ecosystem, by using non-destructive biomarkers: skin biopsy, and use of only one testis by gonadectomy of the bio-sentinel organism, *Pelophylax bergeri*. The identification of the several species belonging to the genus *Pelophylax* permitted, moreover, to widen information in the database; thus this will, in the future, allow to monitoring the biodiversity.

The analysis carried out in sites with a different degree and type of pollution has allowed the development of an eco-procedure for the evaluation of a sensitive biomarker for oxidative stress, and the effectiveness of the DNA repair systems, in testis and skin. ROS and PARP-2 seem useful oxidative and genotoxic and/or endocrine marker to monitor respectively, the induction of DNA damage, and the effectiveness of the repair systems, both necessary conditions for the bio-preservation of species.

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