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Are Antarctic fish adapted to face global changes? A study on antioxidant enzymes gene expression in two species from Ross Sea, in environmental natural conditions or exposed to PFOA pollution

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RIASSUNTO (Versione italiana)

Negli ultimi anni, l'impatto dell'inquinamento chimico di origine antropica sugli ecosistemi ha suscitato un crescente interesse da parte della comunità scientifica. A tal proposito, l'Antartide, grazie al suo isolamento, è considerato un " insostituibile laboratorio naturale", essendo l'unico continente non costantemente antropizzato, soggetto quindi a scarse fonti locali di inquinamento, ma a relativamente alti carichi di contaminanti provenienti da altre zone. In particolare, le sostanze *per-* e *poli-*fluoroalchiliche (PFAS), come inquinanti emergenti, suscitano particolare preoccupazione a causa delle loro sempre più diffuse applicazioni industriali, attitudine al trasporto, al bioaccumulo, persistenza ambientale, e potenziale tossicità. A tal fine, il progetto di ricerca "AntaGPS", finanziato dal PRNA, all'interno del quale si inserisce il presente lavoro di tesi, utilizza l'Antartide come sensore dell'inquinamento globale ed i suoi organismi endemici come bioindicatori. Nello specifico, l'analisi dell'accumulo di mRNA (tramite RT-PCR) ci ha permesso di valutare l'espressione genica a livello trascrizionale di varie isoforme di SOD (1 e 2) e GPx (1 e 4), in diversi organi di due specie di pesci antartici.

In *Trematomus borchgrevinki*, la più alta espressione di tutti gli enzimi antiossidanti è stata misurata nel fegato e nel cuore, dove il gene più espresso è quello codificante per l'enzima mitocondriale SOD2, mentre nei muscoli scheletrici (bianco e rosso), con espressione genica più bassa, l'isoforma più espressa è la citoplasmatica SOD1.

In *Trematomus newnesi*, il rene mostra sempre un livello di espressione più elevato rispetto al fegato, per ciascun enzima antiossidante, ma il più espresso è SOD1.

In questa specie, i livelli di mRNA sono stati valutati anche in pesci esposti a 1,5 µg/l di PFOA per 10 giorni. Nel fegato, il trattamento ha indotto un aumento dell'espressione genica per tutti gli enzimi considerati (soprattutto di GPx4), mentre nel rene una diminuzione generale (soprattutto di SOD1).

I risultati ottenuti costituiscono un punto di partenza per utilizzare l'espressione di enzimi antiossidanti come biomarker, sia di stress ossidativo che di esposizione a PFAS, utilizzando i nototenioidei come bioindicatori in future campagne di biomonitoraggio sull'ambiente marino antartico. Sarebbe in futuro opportuno ampliare il numero di organi e il tipo di enzimi antiossidanti testati, integrando anche l'analisi cellulare con saggi biochimici, come la quantificazione del contenuto di ROS o della proteina attiva nel tessuto.

SUMMARY (English version)

In recent years, the impact of anthropogenic chemical pollution on ecosystems has acquired increasing interest from the scientific community. In this regard, Antarctica, thanks to its isolation, is considered an "irreplaceable natural laboratory", being the only not constantly anthropized continent, therefore subject to scarce local sources of pollution, but to relatively high loads of contaminants from other areas. In particular, per- and poly-fluoroalkyl substances (PFAS), as emerging pollutants, are of particular concern due to their increasingly widespread industrial applications, aptitude for transport, bioaccumulation, environmental persistence, and potential toxicity. For this purpose, the "AntaGPS" research project, funded by the PRNA, within which this thesis is inserted, uses Antarctica as a global pollution sensor and its endemic organisms as bioindicators.

Specifically, the analysis of mRNA accumulation (by RT-PCR) allowed us to evaluate the gene expression at the transcriptional level of various isoforms of SOD (1 and 2) and GPx (1 and 4), in different organs of two species of Antarctic fish.

In *Pagothenia borchgrevinki*, the highest expression of all antioxidant enzymes was measured in liver and heart, where the most expressed gene is that coding for the mitochondrial enzyme SOD2, while in skeletal muscles (both white and red), with the lowest gene expression, the more expressed isoform is the cytoplasmic SOD1.

In *Trematomus newnesi*, the kidney always shows a higher level of expression than the liver, for each antioxidant enzyme, but the most expressed is SOD1.

In this species, mRNA levels were also assessed in fish exposed to 1.5 µg/L of PFOA for 10 days. In the liver, the treatment induced an increase in gene expression for all the enzymes considered (especially of GPx4), while in the kidney a general decrease (especially of SOD1).

The obtained results constitute a starting point for using the expression of antioxidant enzymes as biomarkers, both of oxidative stress and exposure to PFAS, using notothenioids as bioindicators in future biomonitoring campaigns on the Antarctic marine environment. It would be appropriate in the future to expand the number of organs and the type of antioxidant enzymes tested, also integrating cellular analysis with biochemical assays, such as the quantification of the content of ROS or of the active protein in the tissue.

1. INTRODUCTION

1.1. THE NATIONAL PROGRAM OF RESEARCH IN ANTARCTICA (*PNRA*)

The "*National Program of Research in Antarctica*" also known as PNRA (Figure 1.1) was established in 1985, after Italy joined the *Antarctic Treaty* (1981).

Figure 1.1: The PNRA logo.

PNRA's function is to promote and support national research in Antarctica, guaranteeing the maintenance of adequate research infrastructures (both in Antarctica and in Italy), defining and coordinating a plan of logistical and scientific activities carried out in Antarctica each year.

Activities are planned on a three-year programme, based on a proposal from the *National Scientific Commission for Antarctica* (*CSNA*), which indicates the strategic guidelines and evaluates projects. The three-year programme is approved and funded by a decree of the *Ministry of Universities and Research* (*MIUR*), which also supervises its implementation, in compliance with the rules of the *Antarctic Treaty*. The *National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA*) is responsible of carrying out the expeditions, of the operational planning and technical-logistical management of research activities (italiantartide.it). The planning and coordination of scientific activities is entrusted to the *National Research Council (CNR)*, which prepares the *Annual Executive Plan* (*AEP*). In agreement with the *MUR* and the *CSNA*, the *CNR* provides for the dissemination of results and the management of scientific data **(PNRA, 2019).**

By supporting the activities of Italian researchers in the Antarctic continent, the PNRA ensures that Italy contributes both to deepening the national knowledge base of the climate system and ecosystems and to monitoring the climate changes occurring in the polar regions of the Southern Hemisphere. One of *PNRA's* tasks is the internationalisation of national polar research.

The *Scientific Committee on Antarctic Research* (*SCAR*) indicates the priorities of Antarctic science for the future decades **(PNRA, 2019).**

The government programme has completed 37 national scientific/logistics campaigns between 1985 and 2022, setting up two permanent research station in Antarctica over the years - one entirely Italian (*Mario Zucchelli*) and the other in collaboration with France (*Concordia*) - and developing scientific and technological activities of national and international importance. Currently, the 38th Italian research expedition to Antarctica is underway **(PNRA, 2019).**

The *Mario Zucchelli Station* (MZS) (Figure 1.2) is located in the Baia Terra Nova area along the coast (74°42' South and 164°07' East) at 15 m a.s.l. It is open from mid-October to mid-February, with temperatures between -25°C and +5°C. It represents the privileged observatory for biological and ecological research on the marine and terrestrial trophic networks of the Antarctic ecosystem and the adaptation of living organisms to extreme temperatures **(PNRA, 2019).**

Figure 1.1: The Italian "Mario Zucchelli" station. Photo by Gianfranco Santovito.

1.2. ANTARCTICA

1.2.1. THE ANTARCTIC CONTINENT

Antarctica (Figure 1.3) is the southernmost continent on planet Earth. Its territory is equal to about 9% of the planet's land area, making it the fifth largest continent by surface area **(PNRA, 2019).**

It surrounds the geographic South Pole and lies almost entirely within the Antarctic Circle (latitude 66°30' south) **(Turner et al., 2009).**

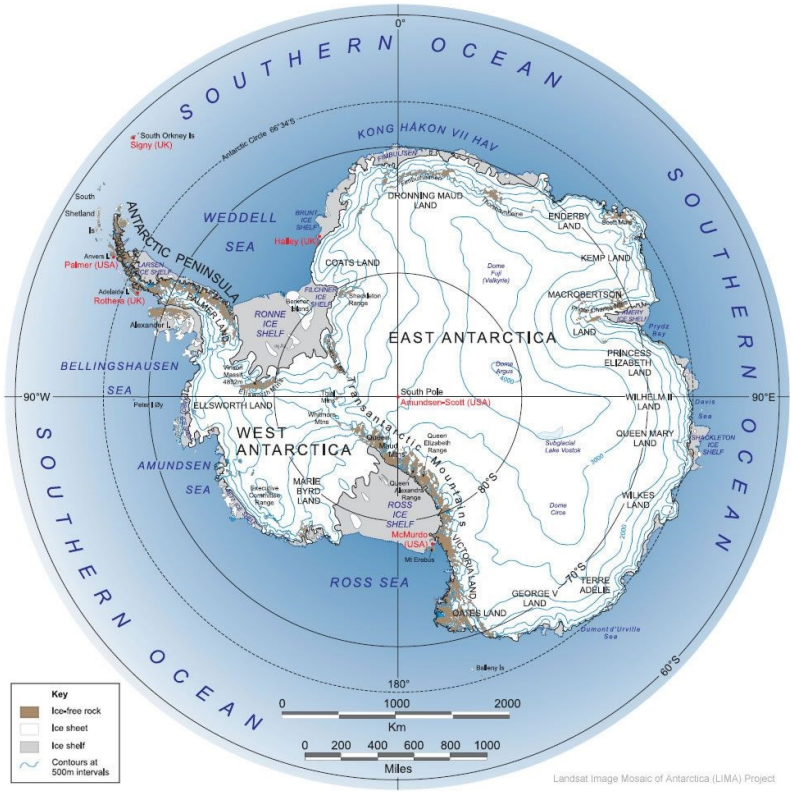


Figure 1.2: The Antarctic continent.

The almost centred position with respect to the pole means that the Sun rays arrive at a sharp angle to the vertical and therefore have a lower calorific power.

Antarctica it's also the planet area that receives the least amount of heat from the Sun, since it has mountainous reliefs that can exceed 4,000 metres and it is covered by a thick blanket of ice, that acts like a mirror (it has an albedo ≥ 80 %) **(Colacino, 1996).**

In fact, due to the inclination of the Earth's axis of rotation (23°27') with respect to the plane of the orbit, the Antarctic region, like the Arctic, receives sunlight mainly during two periods of the year: spring and austral summer (from 21 September to 23 March) when the Antarctic ice cap faces the Sun; during the rest of the year, however, it remains in shadow (polar night) **(PNRA, 2019).**

The alternation of these periods of light and darkness is reflected in the thermal regime and thus contributes to determining the climate of the polar regions, which are among the most hostile on Earth: the radiation balance is strongly negative and, consequently, temperatures are extremely low. During the austral summer, temperatures fluctuate between 0° C in the coastal zone and -32° C in the higher inland areas, whereas in the winter season, the range widens, as in the coastal zone

the temperature drops to around -20° C and on the continental areas values of around -70° C are recorded.

Normally, most of the central plateau has an average annual temperature of -55ºC. It is also worth mentioning that, right in the centre of the continent, at the Russian research station Vostok, in July 1983, the absolute lowest temperature of -89.6 ºC was recorded **(Colacino, 1996).**

Linked to the temperature regime is the trend in other climatic parameters such as air humidity and precipitation. Relative humidity values generally fluctuate between 50 and 80 % and, given the low temperatures in the region, they correspond to a low water content in the atmosphere. Consequently, precipitation is also low: the average value over the entire continent is around 130 mm/year, with a maximum of 500 mm/year along the Antarctic Peninsula and a minimum of 50 mm/year in the inner continental zone.

The wind regime is also conditioned by the temperature trend: in the central area of the continent, where thermal inversion conditions often occur and the atmosphere is extremely stable, the winds are generally weak, with speeds of around 4-5 m/s throughout the year **(Colacino, 1996).**

The intense cooling that the air experiences on the continent (constant high-pressure area) makes it denser than that of the coastal areas (low-pressure area): as a result, the air tends to drop, causing the so-called katabatic wind, one of the best-known characteristics of the Antarctic climate. These winds reach very high speeds, sometimes exceeding 300 km/h (~80 m/s) and raise snow flurries (blizzards). When they reach the sea, they encounter warmer and less dense air masses and cause high waves, resulting in violent storms along the Antarctic convergence line **(Pozzobon, 2019).**

The total extent of the continent, including land and sea, is around 13.8 million square kilometres and is delimited to the south by the Southern Ocean (merging of the Atlantic, Pacific and Indian Oceans) and to the north by the Antarctic Polar Front (or Antarctic Convergence). The true boundary of Antarctica is an oceanographic boundary, consisting of a 40-80 km wide strip of sea, located about 1600 km from the coast, between 45° and 60° south latitude, traversed by an intense permanent marine current, which rotates clockwise. The transition between water masses, distinguished by differences in temperature and other chemical and

physical properties, is sufficient to create a barrier between the Antarctic Ocean and the other oceans (Atlantic, Pacific and Indian), largely isolating the continent, as the Antarctic surface waters, which are cold and not very salty, sink beneath the sub-Antarctic waters, which are less cold and more salty.

The Antarctic Convergence, existed for about 25 million years, prevents the exchange of marine life from one side to the other, as the two bodies of water do not mix, but rather create a natural barrier to the migration of marine life in both directions, resulting in a separate marine ecosystem. This makes Antarctica a unique geographical area for studies on the evolution and adaptation of organisms.

Looking at a map, it is possible to notice that Antarctica has a sub-circular appearance shaped by the Antarctic Peninsula, which juts out to the north (it faces South America), and by the two large inlets, located on opposite sides of the continent, called the Weddell Sea and the Ross Sea. They are connected by imposing huge mountain system, the Trans-Antarctic Chain, 3500 km long, which crosses the continent and divides it into two unequal parts **(PNRA, 2019)**:

- East Antarctica is a single continental plate of about 10 million km^2, the oldest and largest, with altitudes reaching 4000 metres.
- West Antarctica is a collection of smaller plates joined by ice in an archipelago and is structurally more recent.

98% of the surface of Antarctica is covered by the largest glacial system on Earth: an ice cap, which constitutes 68% of the freshwater reserve of our planet and accounts for 91% of the Earth's ice **(Cattaneo-Vietti R., 1998).**

The average thickness of the ice cap is about 1990 m, but, in some parts, it exceeds 4000 m, reaching a maximum at 4700 m in the centre of the continent. This means that Antarctica has an average altitude three times higher than any other continent, i.e., 2300 m. The highest point on the continent is the Vinson Massif, 4892 m above sea level, while the lowest point is the Bentley subglacial trench in West Antarctica, 2538 m below sea level.

However, there are also about 250000 km^2 of territory uncovered by ice (about 2%): the largest rock outcrops are found on the Antarctic Peninsula and in the Trans Antarctic Mountains. Then there are the countless nunataks, the small rocky outcrops of the highest mountain peaks **(PNRA, 2019).**

Geophysical measurements have shown that the polar ice cap reflects the characteristics of the underlying landmass and is divided into two portions: the East Ice Cap, which is larger, lies mostly east of the Greenwich meridian, is more than 4,500 metres thick and rests on the East Antarctic substrate; the East Ice Cap, which is smaller, rests mostly directly on the seafloor and corresponds to West Antarctica. The ice caps are not static: the ice generally flows from the centre of the continent towards the surrounding ocean, at an average speed of 1 m/year. At the end of its course, it juts out to sea and forms extensive ice shelves which can break and drift away like icebergs. They have a flat surface and emerge for about 40-50 m, but about 8/10 of their height remain submerged below the water surface **(Cattaneo-Vietti, 1998).**

The loss of ice to the sea is continually supplied again by snowfall, which is modest and mostly concentrated along the coast and with direct ice fall on the plateau (hidden precipitation): about 2,400 km^3 of ice is estimated to form on the continent annually.

In addition to fresh ice, sea ice (ice floe) is also formed by the freezing of seawater. The ice floe has a maximum thickness of 3-4 metres and an extremely variable extent over the year: it reaches a winter maximum, in September, of 20 million km^2 with an estimated volume of more than 60,000 km^3 **(Cattaneo-Vietti, 1998).** It separates sea water from the atmosphere and contributes to regulating the global climate and the balance of the marine ecosystem. However, it is interrupted in some places by areas of ice-free sea, the polynya: in these areas inside the ice shelf, the sea never freezes due to the continuous vertical circulation of ocean water **(PNRA, 2019).**

The continuous supply of fresh ice to the sea not only greatly influences temperature, salinity, currents and mean sea level, but also shapes the coastline and littoral bottoms through continuous ablation. The ice cap functions as the main cold reservoir of the global thermodynamic system, influencing the climate of the Southern Hemisphere and the entire Earth. This impressive phenomenon is caused by the whiteness of the continent's surface and the surrounding ice floe which, as mentioned before, results in the reflection of the Sun rays back to the Space (albedo > 80 %), resulting in a very low heat input. The ice cap can therefore be considered

a global indicator of the Earth's climate trend, highlighting and documenting changes in the trend towards warm or cold **(Cattaneo-Vietti, 1998).**

1.2.2. THE EVOLUTION OF THE ANTARCTIC CONTINENT

The geographical situation of Antarctica, isolated, polar and ice-covered, is the consequence of a long and complex geological evolution that is still ongoing. It is, at least in part, still to be understood and reconstructed.

Until the Jurassic period, 140 million years ago, Antarctica occupied temperate latitudes and was part of Gondwana, the supercontinent that also included Africa, the Arabian Peninsula, India, Australia, New Zealand and South America.

At the end of the Jurassic period, the break-up of Gondwana began and, during the Tertiary period (around 20 million years ago), Antarctica became completely detached from South America. As a result of the isolation, a permanent circumpolar ocean current was established around the continent, fed by convective motions of water masses of different temperature and salinity and favoured by atmospheric circulation, all influenced by the Earth's rotation. Figure 1.4 illustrates each of these steps. The circumpolar current hinders the mixing of temperate zone waters with cold continental waters.

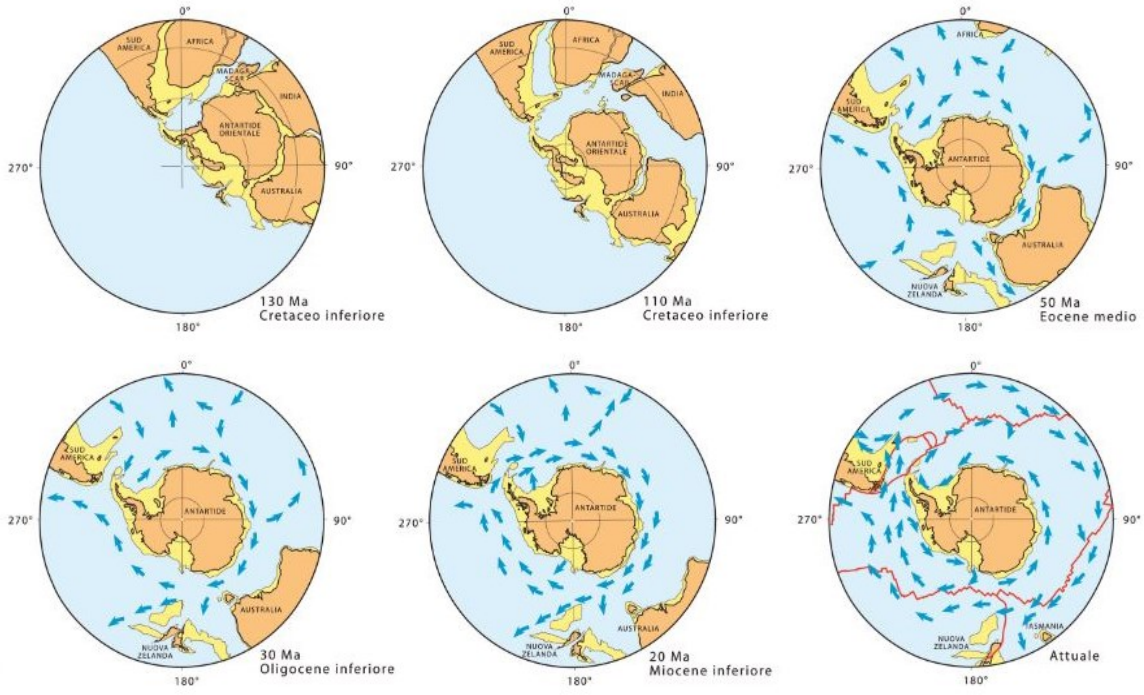


Figure 1.4: Subsequent stages of Gondwana fragmentation and following drift of the Antarctic continent, from 130 million years ago to the present and the establishment of the circumantarctic current.

The progressive cooling of the continent has therefore occurred due to the concomitance of the following factors: the polar geographic position, the separation from the other continental masses, the great extension, high average altitude and the albedo phenomenon. These conditions together led to the slow accumulation of an immense ice sheet over time **(Irianni et al., 2014).**

1.2.3. ANTARCTICA AND THE GLOBAL CLIMATE

As already mentioned, the Antarctic ice cap plays a key role in the Earth's climate system: its mass balance is the difference between snow accumulation and glacier runoff into the ocean. It has been estimated that the total melting of the cap would cause the sea level to rise by about 66 m.

At the current rate of mass loss, the Antarctic ice sheet could become the main component of sea-level rise within a few decades.

Another important component of the climate system is the ocean, and the Antarctic Ocean, being connected to the southern portions of the Atlantic, Pacific and Indian oceans, constitutes the only circumpolar sea on the globe. This characteristic has a profound influence on the global climate, mainly due to the presence of the Antarctic Circumpolar Current (Figure 1.5), which regulates the exchange of water masses, heat and other properties between the main ocean basins and the Antarctic seas, through the so-called *Great Conveyor Belt*, i.e., the complex circulation system that crosses all the oceans, ensuring the renewal and mixing of waters **(PNRA, 2019).**

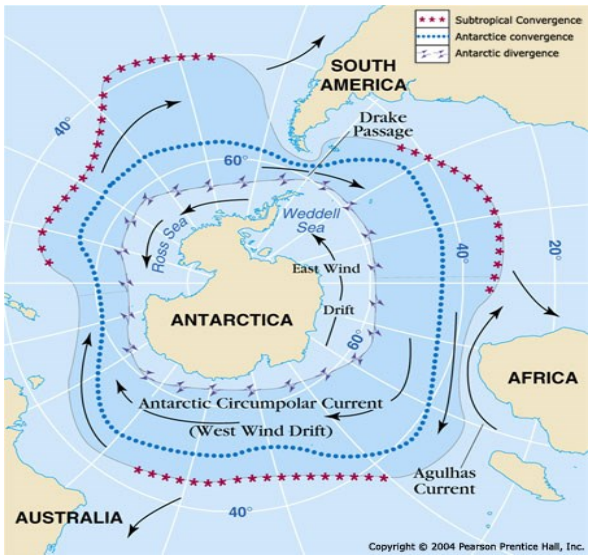


Figure 1.5: The Antarctic Circumpolar current.

In addition, the Antarctic atmosphere has characteristics that are particularly suitable for research into the effects of climate change on the continent and its connections with the rest of the planet. The conditions of strong atmospheric stability allow for very accurate studies of energy exchange at the interface with the surface, the interaction of local dynamic phenomena with atmospheric circulation at different space-time scales, the transport of energy and mass from mid- to low-latitudes, atmospheric composition, aerosols and contaminants.

In addition, for measurements in the upper atmosphere, Antarctica is the preferred observatory for monitoring the ozone depletion process and polar stratospheric clouds, but also for making clear astronomical observations **(PNRA, 2019).**

The Antarctic atmosphere is, in fact, particularly sensitive to the phenomenon known as the 'ozone hole'. 90% of the atmospheric ozone is found in the stratosphere, at a height of between 10 and 50 km: it acts as a shield for solar UV radiation that is harmful to life on Earth.

In Antarctica, the natural major depletion is related to the special atmospheric and chemical conditions, generated by the very low temperatures, that take place in an isolated region of the polar stratosphere, called the Polar Vortex. Here, the polar stratospheric clouds react with halogens in the atmosphere, mainly Chlorofluorocarbons (CFCs), promoting ozone depletion. At the beginning of the austral spring (our autumn) about 50% of the ozone column in the Antarctic atmosphere disappears, only to recover as spring progresses **(PNRA, 2019).**

Of particular interest are the drilling of the ice cap, with the extraction and analysis of ice 'cores', since micro-samples of the Earth's ancient atmosphere can be found in the layers at different depths. Through their examination, it is possible to learn about changes in atmospheric composition and past climatic conditions (palaeoclimatic studies), which are extremely useful for better understanding future climate changes.

Through drilling, climate changes over the last 900,000 years have so far been studied, highlighting the close interdependence between changes in temperature, changes in the amount of carbon dioxide and atmospheric circulation (in turn connected with ocean circulation) **(Irianni et al., 2014).**

In contrast to ice core drilling, which has allowed us to extend our knowledge of the Earth's climate up to about a million years ago, with the study of sediments and

rocks we can go back several tens of millions of years, i.e., when extensive ice sheets did not yet exist in Antarctica. With these analyses, data were obtained for a portion of this sediment core, which can be traced back to a period called the Lower Pliocene (3 to 5 million years ago), in which the planet's atmosphere had CO_2 concentration and temperature values similar to those predicted by the IPCC by the end of the 21st century, due to anthropogenic temperature increases. The Lower Pliocene can therefore be used to acquire useful data for predicting the future behaviour of the West Antarctic ice sheet. This region, in fact, possesses morphological characteristics that make it particularly sensitive to global temperature increases: large portions of the cap have their base below sea level and therefore small variations in the temperature of the surrounding ocean are sufficient to make the cap unstable. The scientific value of these data is huge because in these 600 metres of core lies the detailed record of 38 cycles of advance and retreat of the entire Ross Shelf in response to climate change, and each of these cycles has left a trace and evidence in the type of sediment deposited **(Florindo and Boschi, 2009).**

Antarctica is, therefore, the most suitable territory for the study of processes aimed at understanding the changes that regulate the evolution of the Earth's environment and to understand the relative weight of the natural and anthropogenic components, since the ice and soil contain more than a million years of history and information on past geological eras and climatic changes. This is because the polar regions are considered a natural laboratory, as they are relatively isolated and untouched areas; consequently, there are fewer variables in the study of natural processes. Moreover, processes related to change occur more rapidly and more markedly here than elsewhere **(Pozzobon, 2019).**

1.3. THE ANTARCTIC BIODIVERSITY

The geographic isolation of Antarctica (with the formation of the Antarctic Convergence about 25 million years ago), its progressive cooling and the formation of the ice cap, caused the extinction of many of the species that previously populated both the continent and the surrounding marine waters, thus creating empty ecological spaces. It was precisely this availability of numerous different ecological niches that led, in some cases, to rapid diversification from a single ancestral species to the formation of a large number of new species, each adapted

to occupy a particular ecological role (the phenomenon of adaptive radiation) and able to cope with the changed climatic conditions.

These newly formed species, both animal and plant, are therefore endemic, i.e., they have the characteristic of being present only in Antarctica, mainly due to the considerable degree of geographical isolation of this region, which acts as a natural barrier to the dispersal of organisms and has evolved physiological and biochemical mechanisms to survive and grow in this environment **(Battaglia et al., 1996).** In fact, the Antarctic environment shows various examples of evolutionary convergence (penguins and seals: very distant species have adopted a similar adaptive strategy to live in the same environment, such as a hydrodynamic silhouette and modified flipper-like forelimbs) and parallel evolution (Antarctic fish: production of antifreeze proteins to adapt to the very low temperatures, similar to Arctic fish) **(Di Prisco, 1998).**
The trophic networks have also changed and are considerably simpler than those found in temperate seas **(Battaglia et al., 1996).**

Furthermore, in the Antarctic region there is a huge contrast between the terrestrial and marine ecosystems. Life, in order to establish itself on the continent, must adapt to extremely hostile environmental conditions: a surface almost completely covered by ice (about 98%), very low temperatures, continuous darkness for six months of the year followed by intense and continuous solar radiation in the remaining six months, and consequent large annual temperature fluctuations. But the main obstacle to life is the extreme scarcity of water in the liquid or vapour state, also due to the action of the wind **(Azzolini et al., 2006).**

The Antarctic continent can be subdivided into 3 zones, which differ in variety and complexity of ecosystems in a decreasing manner as the latitude and severity of climatic conditions increase:

- the sub-Antarctic area, which includes the islands between 46° and 55° latitude, north of the Convergence,
- the Maritime Antarctic, which includes the Antarctic Peninsula and surrounding archipelagos,
- continental Antarctica, which consists of most of the continent and is entirely enclosed by the polar circle (66° 33' south latitude).

We can state that, due to the harsh environmental conditions, the Antarctic terrestrial habitat is one of the least populated and differentiated on Earth.

Contrary to this condition, however, the Antarctic marine environment is populated and productive, in both the pelagic (water column) and benthic (seabed) compartments. Life is, in fact, characterised by an abundant number of individuals (compared to the terrestrial fauna), although the number of species is small, thanks to certain characteristics of Antarctic seawater:

- high thermal stability: just south of the Antarctic Convergence, the surface water temperature does not vary significantly throughout the year and remains constant between -1°C and -2°C.
- average salinity around 34-35‰, although there are seasonal variations due to ice formation and melting.
- abundance of dissolved oxygen and carbon dioxide in water: the low water temperature favours the solubility of carbon dioxide and oxygen, allowing them to be immediately used by algae for photosynthesis and by marine animals for respiration. This results in an abundance of marine microorganisms that form the basis of the food chain.
- abundance of nutrients, especially in deep waters and with a seasonal presence, linked to production processes in the summer season.
- transparency of the water, which allows the sun's rays to penetrate deeply. **(Azzolini et al., 2006; Albertelli and Chiantore, 1996).**

Demonstrating the importance of the biodiversity of this area, in 2017 the Ross Sea became the largest marine protected area in the world. It was established by the *Commission for the Conservation of Antarctic Marine Living Resources* (*CCAMLR*), in which all member countries agreed to approve the proposal to safeguard this area, particularly from human activities. This area constitutes the largest ecosystem on the Antarctic continent, with unique features and a higher level of biodiversity than other polar areas. The main feature that makes the area so unique is its physical conformation: it remains isolated from the rest of the Southern Ocean because most of its surface water remains within its area and mixes little with external water. This isolation ensures that the fish and larvae present do not disperse or mix with other organisms **(Marrone et al., 2021).**

And it is precisely in this area, overlooking the Ross Sea, that the *Mario Zucchelli* station is located, in the Terra Nova Bay area, a permanent Italian scientific base that has been operational since 1985.

Furthermore, a key element of the Antarctic marine ecosystem is the annual sea ice cycle, which controls salinity, light availability, phytoplankton production and, consequently, the organisms that feed on it **(Azzolini et al., 2006).**

1.3.1. THE MARINE ENVIRONMENT

Trophic networks are numerous in both water and sediment and encompass a wide variety of organisms in terms of function and dimension, including prokaryotic and eukaryotic organisms as well as both autotrophic and heterotrophic ones.

Plankton is dominated by prokaryotes (Bacteria and Archaea), present in the waters both in summer and during the cold months. Phytoplankton, on the other hand, consisting of autotrophic, predominantly eukaryotic organisms ranging in size from 1 to 200 microns, develop consistently in the spring/summer period and support the development of krill (zoo-planktonic crustaceans, belonging to the order *Euphausiacea*, whose size varies from less than one to several centimetres), for which they are the preferred source of food. Krill is at the centre of the trophic chain, so the survival of organisms at the top of Antarctic food webs, such as cephalopods, fish, penguins, seals and whales, depends on it **(PNRA, 2019).**
Sponges, molluscs, coelenterates, echinoderms and other invertebrates populate the seabed where various species of fish also abound: the Southern Ocean is home to about 200 species of bottom fish. Among the deep-sea species, the endemic ones are only 25%, because in the lower layers of the ocean the Convergence does not hinder the movement of water masses and the chemical and physical conditions are more homogeneous. In coastal waters, on the other hand, endemic species make up 85% because of the environmental barrier posed by the Convergence. Equally abundant is the fauna of large mammals such as cetaceans **(Azzolini et al., 2006).**

1.3.2. THE ADAPTATION OF MARINE BIODIVERSITY TO THE ANTARCTIC COLD

Since Antarctica separated from the other continents around 40 million years ago, Antarctic organisms have evolved physiological and biochemical mechanisms to survive and thrive in the cold, resulting in a highly endemic and adapted fish fauna. They exhibit slower metabolic and growth rates, delayed reproductive maturity, a longer life span and are the most stenothermic species on the planet, unable to survive above 2-3 °C.

Until not so many years ago, the biodiversity of Antarctic marine species was considered very poor, given the extreme environmental conditions, but this was only true for a few groups, including fish and gastropod molluscs. Thanks to international research projects, hundreds of new species have been discovered: current data tell us that the Southern Ocean is home to more than 8000 of them, most of which belong to the benthos.

Studying the ecophysiology of individual organisms and monitoring the response of the trophic network to ongoing global changes are indispensable tools for developing models of Antarctic ecosystem transformation and designing mitigation and protection measures **(PNRA, 2019).**

1.3.3. ANTARCTIC FISHES

During the increasing geographical and climatic isolation of Antarctica, which began 65 million years ago, the physiology of Antarctic fish, which survived the extinction of a pre-existing fauna, adapted to the progressive cooling of the environment **(Di Prisco, 1998).** They evolved about 30 million years ago from perciform progenitors that inhabited the temperate coastal waters of the ancient supercontinent Gondwana. The original coastal fish fauna underwent strong selective pressures, which led to a drastic decrease in the variety of species present, and thus diversified, developing adaptations that were indispensable for surviving the increasingly harsh environmental conditions **(Vacchi, 1996).**

At the same time, other groups, unable to migrate to warmer waters due to the presence of the Antarctic Convergence, became extinct, thus freeing numerous ecological niches **(Clarke and Johnston, 1996).**

In the course of their evolution in Antarctic seas, fish have therefore developed the necessary adaptations to cope with stable, but extreme, chemical and physical conditions: temperatures lower than the freezing point of their body fluids, but also scarce and sporadic amounts of nourishment, strong hydrostatic pressures and large quantities of oxygen on the deep shelf bottoms, and the constant presence of a substrate, ice, in which and with which to live **(Vacchi, 1996).**

Precisely because of its stable conditions, Antarctica has a less variety of life forms than the Arctic Ocean which, on the contrary, has more variable conditions **(Eastman and De Vries, 1987).**
In fact, it is not so much the low temperature per se, but rather the stability of the low temperature, hence the continuous evolution with a smaller number of variables, that makes the Antarctic environment an ideal study area.

Antarctic fish are mostly small (75 % less than 35 cm in length): the only exceptions are the so-called Antarctic cod. Specimens of this group can reach conspicuous sizes such as the Dissostichus mawsoni, also caught in Terra Nova Bay near the Italian Base. Unlike those of tropical and temperate seas, Antarctic fish are more abundant at depth than at the surface, where the environment is more protected from the destructive action of glaciers. Only a few species are pelagic; most Antarctic fish in fact live, feed and reproduce on the bottom **(Vacchi, 1996).**

1.3.4. THE ANTARCTIC NOTOTHENIOIDS

The modern Antarctic fish fauna is dominated by a single, highly endemic group of teleosts: the suborder *Notothenioidei*, which comprises 91% of the biomass of the benthic fish fauna on the continental shelf and 45% of the species (120 species out of 272), known to date, in the Southern Ocean **(O'Brien et al., 2022).** Of these, 174 are found in the Antarctic region (95 are notothenioids*)*. Given the vastness of the habitat, the number of species is very low.

As discovered by **Eastman and Eakin in 2000**, the suborder *Notothenioidei* includes 8 families, 43 genera, with 122 species. As summarized in Table 1.1, 5 families and 96 species are Antarctic, whereas 3 families and 26 species are non-Antarctic. The non-Antarctic notothenioid fauna is composed of both phylogenetically basal lineages and species from clades that are thought to have

originated in Antarctica but have dispersed to non-Antarctic areas of the Southern Ocean, after Antarctica's continental separation from Australia, New Zealand, and South America. **(Near et al., 2004).**

Family	Genera	Antarctic	Non-	Total
Bovichtidae		1	9	**10**
Pseudaphritidae			1	**1**
Eleginopsidae			1	**1**
Nototheniidae		33	15	**48**
	Patagonotothen	1	13	
	Notothenia	3	2	
	Paranotothenia	2		
	Lepidonotothen	4		
	Gobionotothen	4		
	Trematomus	11		
	Pagothenia	2		
	Cryothenia	1		
	Aethotaxis	1		
	Dissostichus	2		
	Gvozdarus	1		
	Pleuragramma	1		
Harpagiferidae		6		**6**
Artedidraconidae		25		**25**
Bathydraconidae		16		**16**
Channichthyidae		15		**15**
Totals		**96**	**26**	**122**

Table 1.1: Species diversity and geographic distribution within notothenioids families and among nototheniid genera ***(Eastman, 2000).***

The *Cannictiidae*, often known as "icefish," are the only vertebrates in the world with colourless blood, because it lacks hemoglobin and red blood cells, while all other families have red blood.

In the evolution of the notothenioids, the formation of the Antarctic Convergence had a great importance, and they are also the most striking example of the phenomenon of adaptive radiation, as they were so evolutionarily successful **(Battaglia et al., 1996).** Cartilaginous fish (sharks and rays), on the other hand, are very rare and their presence is limited to sub-Antarctic areas **(Vacchi, 1996).**

Notothenioids are potentially vulnerable to warming because of their long evolution (12-22 MY) at temperatures less than 5°C, which has diminished their thermal plasticity compared with temperate fish species. Moreover, 88% of notothenioids are endemic to the Southern Ocean and unlike fishes elsewhere, there is little to no opportunity for migration to more suitable habitats. Therefore, plasticity in key traits that influence thermal tolerance and fitness, will likely be paramount to the survival of Antarctic fishes in a changing climate **(O'Brien et al., 2022).**

The ecological importance of notothenioids and their adaptation mechanisms have therefore prompted scientists to study their physiology and phylogeny, with a focus on the oxygen transport system.

1.3.5. MORPHOLOGYCAL AND PHYSIOLOGICAL ADAPTATION OF NOTOTHENIODS

In the course of evolution, the adaptation of fish to the Antarctic environment has led to a variety of physiological specialisation that characterise them as unique organisms, evolutionarily interesting because they have differentiated over a well-defined time span. The main physiological adaptations of Antarctic fish are:

- Absence of the swim bladder (the organ that fills and empties with oxygen to allow ascent to the surface or descent to the depths) and, consequently, compensation through modification of the bones, which have become partially cartilaginous and accumulation of fat deposits, so that their density in the water is reduced: this greatly reduces energy consumption when moving along the water column.
- Ability to biosynthesise antifreezing compounds: these molecules circulate in the blood and other body fluids, lowering the freezing point to below - 1.87°C. They are generally glycoproteins, the most common of which has a particular amino acid sequence, formed by the succession of a repetitive unit of three residues (-Alanine-Alanine-Threonine-) in a linear structure. The disaccharide residues combined with the threonine residues form hydrogen bridges with the water molecules of the ice microcrystals as they form, preventing their growth and the subsequent freezing of the tissue.

- Molecular specialisation of tubulins (the proteins that associate with each other to form microtubules, subcellular structures used in the cell for primary processes). Whereas in warmer-water fish and homeothermic organisms the microtubules are unstable at low temperatures and dissociate into tubulins, the molecular structure of Antarctic fish tubulins has changed so that they can build biologically functional microtubules at -1.87°C (the temperature of equilibrium between ice and seawater, characteristic of Antarctic coastal waters).
- Reduction in the concentration of haemoglobin (the protein that carries oxygen to tissues) and the number of erythrocytes (the cells that contain it), in order to reduce the viscosity of blood. At temperatures close to zero the viscosity would increase to extremely high levels necessitating levels of heart energy that the body could not support. The low temperature allows the reduction of these two components as it also slows down all metabolic processes (thus decreasing the demand for oxygen) and increases the solubility of gases by ensuring oxygen-rich waters. This physiological modification therefore produced no negative results on respiratory capacity.

  The extreme stage of this evolutionary pathway is found in the family of *Cannictiidae*, which are the only vertebrates to have completely eliminated haemoglobin from the blood (while retaining vestiges of its gene) without replacing it with an alternative specific transporter. The oxygen brought into circulation is physically found in solution in the blood (whose relative volume is much greater than that of the red-blooded notothenioids, just as the heart is much larger) and is taken from the water either through the gills or through the skin, via a highly developed network of capillaries **(Di Prisco, 1998).**
- Finally, a permanently cold environment, such as that of Antarctic waters, increases the solubility of gases and thus promotes an increase in the concentration of oxygen within the body fluids of fish (and other aquatic organisms) with a consequent increase in the production of reactive oxygen species (ROS) and the risk of oxidative stress.

  These aspects are specifically addressed in the following paragraphs.

1.4. THE IMPACT OF GLOBAL WARMING

In recent years, the problem of global warming and the contribution of modern civilization to the increase in greenhouse gas levels on Earth, has become increasingly important within the scientific and political community. During the 20th century, the average global warming was 0.7 ° C and one of the latest projections implemented by the Intergovernmental Committee on Climate Change (IPCC, 2007) indicates that, by 2100, the temperature will be similar to or higher than that present on Earth 40 million years ago, when the ice sheet covering Antarctica was not yet formed.

In these decades of profound climate change, Antarctica has functioned as a heat sink for our planet. Nonetheless, the effects of global warming can be seen even in this remote continent, affecting, above all, the region of the western Antarctic Peninsula **(PNRA, 2019).** Here, it was measured a temperature rise of about 1°C in the surface waters during the past 50 years and also subsurface waters are projected to increase by 0.4–0.6°C during the next century with an increase of as much as 1°C by the year 2200.

The signs of climate warming are evident not only in the collapse of ice shelves, but also in the sharp declines of Adélie penguin populations, in the shifts in phytoplankton distribution and in the reduction in krill habitat and abundance **(O'Brien et al., 2022).**

In the last decade, in fact, two enormous ice shelves have disintegrated in just a few days: in 2002 the Larsen B with 570 km^2 of extension and 220 m of thickness and in 2008 the Wilkins platform with 3250 km^2 of extension and a thickness of about 200 m **(Florindo and Boschi, 2009).** At the moment, the data show a significant increase in sea level rise (3.2 ± 0.4 mm per year) due for the 40% to the thermal expansion of the seas, but for the 60% to variations in the cryosphere **(PNRA, 2019).**

However, if we analyse the map of the temperature variations of the atmosphere on the Antarctic continent, it appears quite uneven. In the Antarctic Peninsula, the temperature increase in the period 1957-2006 was about 1.75 °C and in the last 50 years it was recorded an average temperature increase of 2-3 °C. This is

increasingly threatening the balance of the floating ice shelves that surround the continent: since 1950, in fact, their extent has been reduced by 8,000-10,000 km^2. Temperatures in the rest of the continent and in the Ross Sea region, on the other hand, show a substantially stable situation and the variations are very modest and statistically insignificant, while East Antarctica even shows signs of cooling **(PNRA, 2019).**

The Antarctic marine ecosystem, in particular, is exposed to many impacts deriving from rapid climatic changes, mainly due to the increase in temperature and acidity of marine waters (due to the excess of dissolved carbon dioxide), to the modification of the general circulation, to the decrease in sea ice and in the concentration of water oxygen.
These changes induce reactions on the part of ecosystems, ranging from the molecular and cellular level to the level of organism and population, in times that can vary from hours or days up to millions of years. Since its foundation, the PNRA has successfully addressed the issue of the mechanisms of evolution and adaptation of marine organisms in an international specialist context **(PNRA, 2019).**

1.5. THE IMPACT OF POLLUTION

Although it is located at the southernmost point of the world, the Antarctic continent is not immune to human activity damages. Long-range atmospheric transport is the primary way by which contaminants reach Antarctica, as the Antarctic Polar Front's presence significantly decreases the pollutants sea transfer. Due to the low internal pollutant emissions but high external contamination levels, Antarctica is an excellent sensor for global pollution trends, thus useful for ecotoxicological studies **(Marrone et al., 2021).** In fact, polar regions are ideal research sites for studying the environmental behaviour of pollutants, because of their relative isolation from other ecosystems, thus the concentration of a pollutant in different trophic levels can reflect the trophodynamics along the food chain. Compared to the Arctic region, Antarctica's food chains are simpler and shorter, so some toxic pollutants can cause significant effects even at low levels on local ecosystems. In addition, the bioaccumulation and biomagnification behaviours of certain pollutants can potentially threaten the health of high trophic level organisms **(Gao et al., 2020).**

Moreover, cool temperatures (high latitudes) favour pollutants deposition from the atmosphere onto land/water. Climate change and global warming may increase the transport/deposition of contaminants in polar areas and may be particularly problematic for species living at the limit of their physiological tolerance range thus limiting the ability to acclimatise. Monitoring contamination levels is necessary to evaluate the efficacy of global measures adopted to mitigate pollutant release into the environment.

Fish are widely recognised as the most relevant model for monitoring pollution in aquatic ecosystems. In particular, Antarctic organisms, due to their endemic and high capacity to adapt to extreme conditions, are excellent bioindicators for analysing trends in pollutant bioaccumulation over time **(Marrone et al., 2021).**

In addition, the impact of global pollution in Antarctica is evidenced by the marked decrease in the ozone layer in the atmosphere. First revealed in 1985 by British scientists, as mentioned above, the phenomenon normally occurs in the stratosphere above Antarctica during the austral spring (September-October). However, it was calculated that from 1979 to 1987, the ozone layer was depleted by about 50 per cent, and in 1994, the World Meteorological Organisation declared that the ozone layer had reached its minimum value. For this reason, measures were taken to limit the use of chlorofluorocarbons (CFCs) used in certain industries, and subsequently, production came to a virtual standstill. Despite this, ozone above the polar region continues to thin and this may lead to negative effects due to increased ultraviolet radiation on the ground. More cataracts and skin cancers are likely to occur, so the classes most at risk are mammals and polar birds, including penguins **(Willmer et al., 2003).**

Another dramatic effect of pollution on this area of the Earth is global warming, a complex phenomenon caused by the interaction of natural causes and human activity. The Great Ice Continent, as temperatures rise, increasingly experiences massive ice masses breaking off and of surface glaciers melting, which in turn is a large-scale cause of global sea and ocean level rise **(Pozzobon E., 2018-19).**

The vulnerability of polar ecosystems is determined both by the prevailing environmental conditions, such as very low temperatures and low incidence of sunlight, and by the typical characteristics of these ecosystems, such as the low level of complexity and generally slow growth rates of the organisms that populate them. Ecosystem alterations caused by toxic substances can be substantial, with even very long recovery times.

1.5.1. THE PFAS

A prominent role is played by the analysis of organic compounds with a clear anthropogenic footprint, and thus considered markers of anthropogenic activity: the Persistent Organic Pollutants (POPs). These contaminants can reach Antarctica through Long-Range Atmospheric Transport (LRAT) mechanisms from heavily anthropogenic areas of the planet, although a minimal contribution is made by local emission sources, such as scientific research facilities, fishing and tourism, present along the coasts and on the Antarctic Peninsula. The control of local sources of pollution is regulated by the Environmental Protection Protocol in the Antarctic Treaty, while the control of non-local and long-range sources is much more complex **(Fedon, 2019).**

Among POPs, one of the most hazardous classes is PFAS (per- and polyfluoroalkyl substances), a heterogeneous group of industrially produced chemicals that are highly persistent in the environment. In particular, the aquatic ecosystem is suggested to be one of the major sinks of environmental PFAS, because of their high water solubility compared to traditional POPs **(Liu, 2014).** PFAS are considered “emerging contaminants”, i.e., chemicals that are not commonly monitored in the environment but have the potential to enter it and cause known or suspected adverse ecological and human health effects **(Piva et al., 2022).** In particular, two PFAS are currently restricted under the Stockholm International Convention on POPs and the EU POPs Regulation, as they were among the first to be detected in human blood and the environment and are considered some of the most prevalent and widespread PFAS **(Bonato et al., 2020)**:

- PFOS (perfluoroctanesulphonic acid) and its derivatives, since 2009/2010. PFOS was the first PFAA to have a global restriction, but not a ban on its production and use, being added to Annex B of the Stockholm Convention.
- PFOA (perfluorooctanoic acid), its salts and related compounds, since 2019, in Annex A with specific exemptions.

  (EPA, 2022; Secretariat of the Stockholm Convention, 2022)

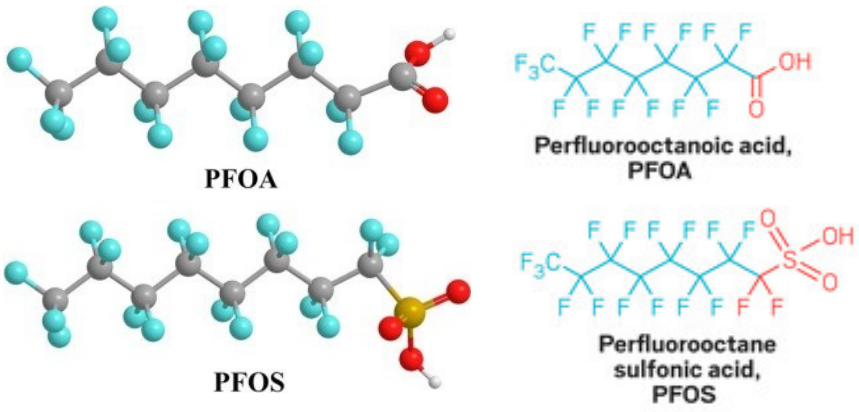


Figure 1.6: Chemical structure of the two best known PFAS congeners: PFOA (perfluorooctanoic acid) and PFOS (perfluorooctane sulfonic acid). The carbon atoms are represented in grey, in blue the fluorine, in red the oxygen, in white the hydrogen and in yellow the sulfur.

Perfluorooctanesulphonate (PFOS) and perfluorooctanoate (PFOA) acids, represented in Figure 1.6, are two common long-chain perfluoroalkyl acids (PFAA), used in many industrial applications and in large quantities, e.g. as surfactants, flame retardants, surface treatments, water and oil repellents, lubricants and coating materials **(Cerro-Gálvez et al., 2020).**

Many of these synthetic chemical compounds are known to be both hydro- and lipophobic, as well as chemically and thermally stable, due to the high chemical stability of their C-F bonds. They have also dielectric properties, low surface energy, low friction properties and a surfactant nature. PFAS are extremely resistant to hydrolysis, photolysis, biodegradation and metabolism. Furthermore, their characteristics of high persistence and mobility allow them to travel great distances, dispersing in surface and groundwater bodies, as well as in the air in the form of dust particles **(Piva et al., 2022).**

PFOS and PFOA are, thus, globally transported mostly by oceanic currents (OCTs) and by sea-salt aerosols, but also long-range atmospheric transport (LRAT) of volatile neutral precursors and subsequent oxidation and deposition, is a potential

source of PFAS to remote regions, such as Antarctica **(Cerro-Gálvez et al., 2020).** Since the early 2000s, in fact, PFAS began to be detected in the environment, even far away from where they were originally used or manufactured and, above all, in different matrices: from aquatic ecosystems (surface and groundwater) to sediments, flora and fauna **(Piva et al., 2022).**

The effect of exposure to PFOS, PFOA and other PFAA on the immunologic system, fertility, foetus development, carcinogenesis and the thyroid system, among others, is a matter of increasing concern, as it has been observed in both fish and mammals. Exposure to PFAA by microorganisms can originate an increased permeability of cell membranes, alteration of membrane fluidity, and membrane disruption by PFAA penetration into the membrane bilayer. These effects are compound and concentration dependent **(Cerro-Gálvez et al., 2020).**

1.6. THE REACTIVE OXYGEN SPECIES (ROS) and THE OXIDATIVE STRESS

The constantly low temperature of the Southern Ocean (around the average value of $-1.18 \pm 0.68°C$) determines a high concentration of dissolved gasses, in particular O_2, in the Antarctic seawaters, producing a consequent increase of pO_2 also in organism's tissues and cells **(Zuo et al., 2015).**

All aerobic organisms rely on the oxygen presence in the environment, using it mainly for energy generation via oxidative phosphorylation. O_2 is the final acceptor in the formation of ATP at the mitochondrial level. This process consists in the reduction of oxygen molecules to water by cytochrome oxidase, which uses over 90% of oxygen consumed by an organism **(Lushchak and Bagnyukova 2006):**

$$\mathbf{O_2 + e^- \rightarrow {\bullet}O_2^- + e^- \rightarrow H_2O_2 + e^- \rightarrow {\bullet}OH + e^- \rightarrow H_2O}$$

This chain can be interrupted at various levels, therefore, during these (and other) electron transfer reactions, may be formed partially reduced by-products of oxygen metabolism: the so-called reactive oxygen species (ROS) **(Thannickal e Fanburg, 2000).** Under normal circumstances, it has been calculated that the 3% of the O_2 used by the cell is improperly converted into undesirable intermediates, but the particular condition of Antarctica can increase the formation rate of these ROS. Some studies in experimental mammals, verified that more than 90% of the ROS

are generated in mitochondria and approximately 2% of the whole O_2 uptake is accounted by ROS generated as obligatory byproducts of oxidative metabolism **(Wilhelm Filho, 2007).**

ROS are chemically highly reactive molecules, which consist of both radical species (which contain an unpaired electron) and non-radical species: the first ones include superoxide anion radical ($\bullet O_2^-$), peroxyl radical ($\bullet ROO^-$), hydroxyl radical ($\bullet OH$) and the singlet oxygen ($^*O_2$), instead, among the others, we only have hydrogen peroxide (H_2O_2) **(Santovito et al., 2005).**

There may be three sources of ROS **(Noori et al, 2012)**:

a. Endogenous sources.
b. Exogenous sources.
c. Pathological sources.

a. Endogenous sources.

This group includes essential metabolic processes that are regularly going on in the organism.

Non-enzymatic endogenous sources of ROS are *Fenton's* and *Haber's reactions.*

Enzymatic sources apply as NADPH oxidase, xanthine oxidoreductase, arachidonic acid and cytochrome P_{450} oxidase.

Some others endogenous sources of ROS are a series of biochemical reactions within cellular compartments: mostly mitochondria and endoplasmic reticulum, but also peroxisomes, lysosomes and phagocytes.

Endogenous ROS are physiologically produced, therefore, at normal levels, they are necessary for the organism, because they are involved in mediating many biological responses, including cell growth, migration, necrosis, apoptosis, phagocytosis. They also play an essential role in maintaining the redox balance and in activating various cellular signalling pathways, such as gene expression, protease activities, immunity and wound healing. Under normal conditions, the steady-state ROS concentration is kept within a homeostatic range, and it is free to fluctuate between the extremes of this interval **(Piva, 2020).** Despite a certain amount of oxidative damage takes place

even under normal conditions, such as aging, cells normally act to counteract the oxidant effects and to restore redox balance **(Bonato et al., 2020).**

On the other hand, their increased levels may lead to a condition which is called "oxidative stress". Oxidative stress is a particular form of chemical stress, defined as "the imbalances in the equilibrium between pro-oxidants and anti-oxidants status in cellular systems, which results in damaging the cells" **(Noori, 2012).** Cells have an intact oxidation process to detoxify the cellular environment from oxidants, and thus create the equilibrium in oxidants and antioxidants from aerobic metabolism: the formation of pro-oxidants is readily balanced by antioxidants by a similar rate. Indeed, cells react by resetting critical homeostatic parameters, activating or silencing genes encoding defensive enzymes, transcription factors, and structural proteins. A consequence of this action can be the accumulation of oxidation products of lipids, nucleic acids, proteins, sugars, and sterols causing cellular dysfunction **(Bonato et al., 2020).**

However, the failure in the neutralization events of oxidative status (which occurs when oxidative action of ROS is stronger than antioxidant responses of the cellular defense system) result in oxidative stress. It leads to irreversible cell damage and eventually to cell death, because, due to their reactivity, ROS can produce deleterious effects in tissues, like lipid peroxidation, carbohydrates oxidation, protein oxidation and nucleic acid oxidation**.** In particular, the damage caused by ROS can lead to membrane alteration, inactivation of enzymes and receptors, protein modification, genomic damages or mutations and activation of apoptotic and/or necrotic cell processes **(Zuo et al., 2015; Noori, 2012; Bonato et al., 2020).**

In relation to this, in fact, we speak of the oxygen paradox, because complex organisms require oxygen for their existence, but, at the same time, oxygen, being a highly reactive molecule, can damage the organisms themselves by producing ROS **(Acworth et al., 1997).**

b. Exogenous sources.

This group involve all the external sources that can lead to ROS formation, such as mechanical and thermal stress, ionizing radiation, viral and bacterial infections, hypoxia and hyperoxia (as in the case of Antarctic notothenioids),

but also the biotransformation of xenobiotic compounds such as toxins, drugs and chemical contaminants **(Poli et al., 2014)**, for example the exposure to industrial waste products, cigarettes smoking, asbestos fibers and ozone. The main environmental pollutants which can induce oxidative stress are dioxins and heavy metals, but also the exposure to PFOS and PFOA is known to cause an increase in ROS formation and, consequently, cellular oxidative stress **(Bonato et al., 2020).**

c. *Pathological sources.*

This group refers to ROS production due to diseases. Some examples are immune cell activation, inflammation, cardiovascular diseases, ischemia, infections cancer, metabolism of environmental pollutants and certain drugs.

Therefore, when produced in excess, ROS can cause tissue injury. However, tissue injury can itself cause ROS generation (e.g., by causing activation of phagocytes or releasing transition metal ions from damaged cells), which may (or may not, depending on the situation) contribute to a worsening of the injury **(Aruoma, 1998).**

The toxicity of ROS is related to the fact that they are molecules with one or more unpaired electrons, and this makes them highly unstable and ready to react with atoms or molecules from which they can steal electrons **(Acworth et al., 1997),** leading to the formation of other radicals. Therefore, ROS can interact with virtually all cellular components such as lipids, carbohydrates, proteins and nucleic acids, modifying them into degradable elements or toxic products.

1.6.1. LIST OF THE MAIN ROS

- **Superoxide anion radical $\bullet O_2^-$**

It is formed by the addition of one electron to dioxygen, caused either by metabolic processes or following oxygen “activation” by physical irradiation. Regarding the first method, it is produced within the mitochondrial membrane in the electron transport chain, but also in the sarcolemma and in the sarcoplasmic reticulum by NADH oxidase, during oxidative phosphorylation:

$$\mathbf{O_2 + e^- \rightarrow \bullet O_2^-}.$$

Superoxide anion is the first intermediate of the electron-transport chain, considered the "primary" ROS. it is a radical with rather low reactivity, which is unable to permeate biological membranes.

Consequently, it can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalysed processes **(Valko et al., 2007)**, for example hydrogen peroxide H_2O_2**:**

$2{\bullet}O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$.

Superoxide anion radical, together with hydrogen peroxide H_2O_2, are not considered to be particularly reactive in aqueous solutions. However, being combined with each other, they considerably increase the risk of oxidative stress, because they are involved in the *Haber-Weiss reaction* and produce the most reactive hydroxyl radical •OH.

$$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + O_2 + OH^-$$

(Haber-Weiss reaction)

The Haber–Weiss reaction provide a means to generate more toxic radicals from the less reactive superoxide and hydrogen peroxide, that could be generated enzymatically **(Kehrer, 2000).**

This radical •OH reacts instantly with all organic molecules, it is considered the most destructive reactive oxygen species **(Santovito et al., 2005)** and one of the main causes of damage to the nervous system, proteins and DNA.

- **Hydrogen peroxide H_2O_2**

It is the second non-radical intermediate. Hydrogen peroxide is a common product of many biological processes that underlie our metabolism. This molecule is formed mainly in peroxisomes, whose membranes are rich in enzymes that transfer hydrogen from other molecules to oxygen.

H_2O_2 can be beneficial to organisms, as it can be used as a potent antimicrobial against infectious pathogens. Furthermore, among the physiological functions there is also that of second messenger in many signal transduction pathways (especially in plants and animals), mainly for the activation of the immune response, inflammation, cell proliferation and apoptosis.

On the other hand, although it is not a radical and is characterized by limited reactivity and therefore considered a weak oxidizing agent, it can still be dangerous for the body. This is due to its ability to easily permeate cell membranes and spread between different cell compartments, where it has a relatively long half-life inside the cells. Another harmful thing is its ability to interact with some complexes of transition metal ions in their reduced form (such as Fe^{2+} and Cu^{2+}), to generate the hydroxyl radical •OH in the *Fenton reaction* **(Bayr, 2005).**

$$\mathbf{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\bullet}OH + OH^-}$$

(Fenton reaction)

When H_2O_2 is produced, it needs to be quickly converted into less harmful molecules.

- **Hydroxyl radical •OH**

It is the third radical intermediate, produced by tri-electronic reduction of molecular oxygen.

The redox state of the cell is largely linked to an iron (and copper) redox couple and is maintained within strict physiological limits. Iron regulation ensures that there is no free intracellular iron. However, in vivo, under stress conditions, an excess of superoxide releases free iron from iron-containing molecules. The released Fe^{2+} can participate in the *Fenton reaction* **(2)**, generating highly reactive hydroxyl radical. Thus, under stress conditions, $\mathbf{{\bullet}O_2^-}$ acts as an oxidant of [4Fe–4S] cluster-containing enzymes and facilitates radical **•OH** production from H_2O_2 by making Fe^{2+} available for the *Fenton reaction*. The superoxide radical participates then in the *Haber–Weiss reaction* **(1)**, which combines a *Fenton reaction* and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen ($Fe^{3+} + {\bullet}O_2^- \rightarrow Fe^{2+} + O_2$) **(Valko et al., 2007).** This reaction is thermodynamically unfavourable in biologic systems, having a second order rate constant of zero in aqueous solution, for this reason it requires a metal ion catalyst to proceed: the iron. The iron-catalysed *Haber–Weiss reaction*, which makes use of *Fenton* chemistry, is considered to be the major mechanism by which the highly reactive hydroxyl radical is generated in biological systems **(Kehrer, 2000).**

The hydroxyl radical is the most reactive ROS, because it has a very short in vivo half-life (approximately 10^{-9} s): this means that it reacts even before the antioxidant enzymes can stop it and so it has a very limited diffusion. For this reason, it is a very harmful radical, which can cause a lot of damage to the site where it is produced **(Valko et al., 2007).**

- **Peroxyl radical •ROO-**

It is an additional reactive radical, derived from oxygen, that can be formed in living systems.

It is not a specific radical, but it represents a group of radicals that are very often found within biological membranes. Here, the fatty acid chains can form radicals of this type on the lipids that are oxidized, creating damage that can propagate along the entire membrane. This happens because, once a lipid oxidizes, it is less stable and can easily tear electrons from other molecules, transforming the adjacent lipids into ROS and thus forming an additional radical, which can in turn oxidize the adjacent molecule and so on. This therefore causes damage that spreads to the entire membrane structure, both in the plasma membrane and in the membranes of cellular organelles **(Bayr, 2005).**

The simplest peroxyl radical is the hydroperoxyl radical **HOO•**, which is the protonated form of superoxide (**$•O_2^-$**) **(Valko et al., 2007).**

1.7. THE ANTIOXIDANT DEFENCES

Aerobic organisms evolved adaptive metabolic strategies in order to reduce the toxicity of ROS and protect the cells against their potentially damaging effects. For this reason, they possess several antioxidant defences in their cells, both at the cytoplasmic and mitochondrial levels. They consist in small molecules and enzymes with the function of detoxifying and scavenging the ROS, that are continuously produced as a by-product of aerobic metabolism **(Santovito et al., 2005)**, and so maintaining the redox equilibrium and inhibiting the propagation of oxidative stress.

ROS, having a very low half-life, may not even require the presence of molecular components responsible for their elimination, but it is also true that the low half-life is caused by the high reactivity, which is capable of damaging the molecules.

Therefore, although the spontaneous reduction of ROS is of the order of 10^{-6} sec, the presence of antioxidant enzymes, lowers this time by, at least, two orders of magnitude.

The problem with antioxidant enzymes is that they evolved to counteract only some ROS, such as H_2O_2 and $\bullet O^{2-}$, while the more reactive and dangerous ones, such as •OH, do not have an enzymatic counterpart. Fortunately, there are other non-enzymatic molecules in the cells, which play a very important role in the elimination of these particular chemical species **(Demple, 1999).**

The main enzymatic and non-enzymatic antioxidants are listed in the Table 1.2.

Enzymatic Antioxidants	**Non-enzymatic**
Superoxide dismutases (SOD)	Vitamin E
Catalase (CAT)	Vitamin C
Glutathione peroxidases (GPx)	β-carotene
Glutathione reductase	Vitamin A
Glutathione-S-transferases	Glutathione (GSH)
Peroxiredoxins (Prdx)	Metallothioneins
Tioredoxine (TxR)	Flavonoids
Methionine sulfoxide reductase	Thiols
Aldokheto- reductase	Coenzyme Q
Aldehyde dehydrogenase	Uric acid

Table 1.2: Enzymatic and Non-enzymatic antioxidant defenses ***(****Adapted from* ***Chowdhury and Saikia, 2020).***

The group of enzymatic antioxidants represent a first line of defence from the risk of oxidative stress, but the combined action with other antioxidant responses is needed **(Santovito et al., 2012).**

The class of non-enzymatic antioxidants is represented by low molecular weight molecules, among which there are those biosynthesized by the cell (e.g. GSH and metallothioneins) and those assumed only by diet (e.g. vitamins, β-carotene, ubiquinol, polyphenol). These molecules are considered chain-breaking antioxidants, because they can stop the propagation of the catalytic reactions **(Storey, 1996).**

1.7.1. THE ANALYSED ANTIOXIDANTS

Different vertebrate groups exhibit antioxidant enzymes which are very similar to each other: most interspecies differences rely on the quantitative distribution of antioxidant enzymes in different tissues and at different sub-cellular levels **(Wilhelm Filho, 2007).**

The most important components of the antioxidant system are depicted in Figure 1.7, which is followed by a description of the enzymes considered in this work.

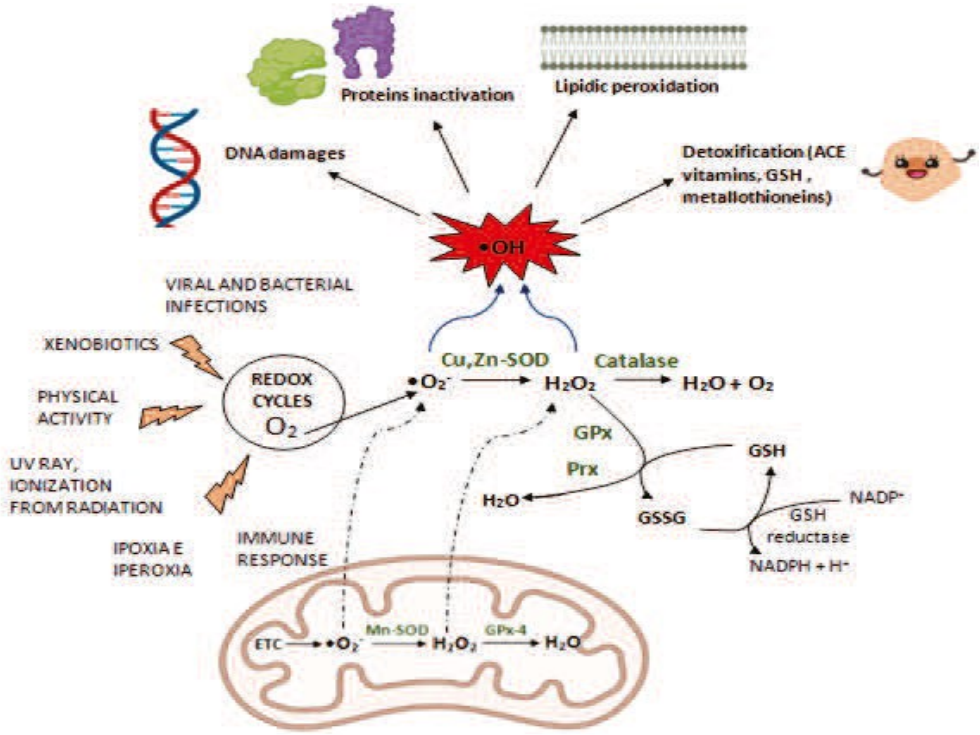


Figure 1.7: Production and inactivation of various reactive oxygen species by the enzymatic and non-enzymatic components of the antioxidant defence system. Adapted from ***Poli et al., 2014.***

- **Superoxide dismutase (SOD)**

Primary antioxidant responses include superoxide dismutases, enzymes present in various isoforms, virtually in all oxygen-dependent organisms. It is a family of metalloenzymes that catalyse the dismutation of the superoxide anion ($\bullet O^{2-}$) into dioxygen (O_2) and hydrogen peroxide (H_2O_2). The physiological role of this protein appears to protect cells by scavenging superoxide anions produced under oxidative conditions by energy-generating processes **(McCord and Fridovich, 1969).**

The reaction occurs in two steps:

E-me (ox) + $\bullet O^{2-}$ → E-me (red) + O_2

E-me (red) + $\bullet O^{2-}$ + $2H^+$ → E-me (ox) + H_2O_2

In the first step, the protein molecule with the metal in the oxidized form interacts with a superoxide radical, leading to the formation of molecular oxygen and the simultaneous reduction of the metal in the active site.
In the second step, the E-me (red) enzyme interact with a second superoxide radical molecule and this results in the oxidized form of the enzyme, together with the formation of one hydrogen peroxide.
The sum reaction is $\bullet O^{2-} + \bullet O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2$. Therefore, overall, this enzyme is able to eliminate two molecules of superoxide anion, but the production of hydrogen peroxide is an example of why the enzymatic antioxidant defences do not provide a complete coverage for the oxidative stress, since H_2O_2, although less harmful than other ROS, need to be eliminated as well **(Buettner et al., 2006).** Consequently, the SODs work in synchrony with other enzymes that catalyse the elimination of H_2O_2: GPxs, CATs and Prdx.

Four classes of SOD have evolved with distinct protein folds and different catalytic metal ions present in the active sites *Cu,Zn*-SOD, *Mn*-SOD, *Fe*-SOD and *Ni*-SOD. Cu and Zn are present in the catalytic centre of two isoforms of SOD which are localized to either intracellular cytoplasmic compartments (*Cu,Zn*-SOD or SOD1) or to extracellular elements (EC-SOD or SOD3). SOD1 is a homodimeric enzyme, which has a molecular mass of about 32 kDa and is predominantly present in the cytoplasm, nuclear compartments, and lysosomes of virtually all eukaryotic cells, but also in some prokaryotes. It is very sensitive to cyanide and H_2O_2 **(Santovito et al., 2006).** SOD3, on the other hand, is the most recently discovered and least characterized member of the SOD family. The enzyme exists as a homotetramer of molecular weight 135 kDa, first detected in human plasma, lymph, ascites, and cerebrospinal fluids. It is a glycoprotein present in the circulating fluids or in the plasma membrane **(Poli et al., 2018).**
A third isoform of SOD has manganese (Mn) as a cofactor and has been localized to mitochondria of eukaryotic aerobic cells (*Mn*-SOD or SOD2), but also in the cytoplasm of some prokaryotes. It exists as a homotetramer with an individual subunit molecular weight of about 23 kDa. (**Zelko et al., 2002).**

Fe-SOD and *Mn*-SOD appear to have evolved from a common ancestral gene, with the *Fe*-SOD gene observed in primitive unicellular eukaryotes, in the cytosol of

bacteria and in the plastids of some plants. *Fe*-SOD is a homodimeric or homotetrameric protein, which is not sensitive to cyanide, but is, however, inhibited by H_2O_2. The distribution of the two proteins is not similar, because phylogenetic analysis of *Mn*-SOD indicates that it occurs in all the major domains of life, but it is mainly typical of eukaryotes and, in particular, it is located within the mitochondrial matrix, where it is particularly required, since in this organelle 90% of cellular ROS can be generated **(Perry et al., 2010).** *Fe*-SOD and *Mn*-SOD have diverged significantly from each other, so that the two metals cannot functionally substitute for each other in *Mn/Fe*-SODs from most species.

Finally, *Ni*-SOD is the more recently discovered isoform, a SOD with only nickel in the active site. It was purified from several aerobic soil bacteria of the *Streptomyces* species. It is a small homotetrameric protein encoded by the gene *sodn* and located in the cytoplasm. However, it also occurs in other bacteria and is predicted to be present in some cyanobacteria, but never in eukaryotic cells **(Wuerges et al., 2004).**

- **Glutathione peroxidase (GPx)**

Glutathione peroxidases (GPxs) are a family of phylogenetically related metalloproteins, mainly characterized by the presence of selenium**.** GPxs are widely distributed in animal tissues: mainly present in the cytosol, while the mitochondrial matrix contains about 10% of GPx of the total.

These enzymes catalyse the reduction of H_2O_2 or organic hydroperoxides (R-OOH) to water or corresponding alcohols (R-OH), using reduced glutathione (**GSH**) as an electron donor (**Sattin et al., 2015).** The general equations are the following:

$H_2O_2 + 2$ **GSH** $\rightarrow$ GSSG $+ 2\ H_2O$ GSSG + NADPH + H^+ $\xrightarrow{GR}$ 2 **GSH** + $NADP^+$

R-OOH + 2 **GSH** $\rightarrow$ GSSG + R-OH

The efficiency of the systems that use these enzymes to remove hydrogen peroxide and other organic peroxides depends on the specific activity of GPX, the concentration of GSH and the catalytic intervention of the antioxidant enzyme glutathione reductase (GR). The latter catalyses the reduction from GSSG to GSH, using, as a reducing agent, NADPH, produced in the pentose-phosphate cycle. The GSH/GSSG ratio may be used as a marker of oxidative stress, because reduced glutathione (GSH) is considered to be one of the most important scavengers of ROS.

Under physiological conditions, the GR is able to maintain the GSH/GSSG ratio at a relatively high level (> 10: 1) while in various models of oxidative stress, this ratio has been demonstrated to decrease to values of even 1:1) **(Zitka et al., 2012).**

The GPxs family comprises more than hundreds of members, spread over all domains of life. In vertebrates, up to 8 distinct GPxs were identified. Most of them are selenoproteins (mammalian GPx1, GPx2, GPx3, GPx4, and human GPx6), while, in the remaining isoforms (GPx5, GPx7, GPx8), the selenocysteine residue (Sec) is replaced by cysteine (Cys). Selenium-containing GPxs (*Se*-GPxs) were also discovered in other organisms such as parasitic trematodes, protists, and bacteria. However, the overwhelming majority of non-vertebrate GPxs contain cysteine in their active site **(Sattin et al., 2015).** The difference between these two types of enzymes is that Se-GPx enzymes can catalyze the reduction of both organic and inorganic peroxides (like H_2O_2) while non-Se-GPx enzymes act only on organic peroxides **(Ren et al., 2009).**

Phylogenetic analyses revealed that, in mammals, the GPx gene family evolved from a common ancestral gene by duplication events, followed by random mobilization in the genome. It has also been postulated that GPx1, 2, 3, 5, and 6 may have a common ancestral gene, probably distinct from GPx4 **(Sattin et al., 2015).** GPx1, 2 and 3 act as homotetramers, whereas GPx4 is functional as a monomer **(Margis et al., 2008).**

The 4 *Se*-GPxs isozymes are distributed in different mammalian tissues: GPx1, which is the most abundant isoform, is found in red cells, liver, lung and kidney; GPx2 in the intestinal epithelium; GPx3 in different organs such as kidney, lung, epididymus, vas deferens, placenta, seminal vesicle, heart and muscle, and GPx4 is a phospholipid protein, therefore broadly distributed in different tissues **(Margis et al., 2008).** GPx5 is *Cys*-GPx, a protein secreted in the epididymus. GPx6 is present in humans, but not in rats or mice and is expressed in the olfactory epithelium. Also GPx7 and GPx8 are *Cys*-GPxs with low GPx activity **(Brigelius-Flohé and Maiorino, 2013).**

GPxs have an antioxidant function also at different cellular compartments: GPx1 was identified mainly in the cytosol of almost all mammalian tissues, but also in nucleus and mitochondria; GPx2 accumulates in the cytosol and nucleus, GPx3 is a secreted protein, also found in the cytosol. All three thus work in the water phase,

whereas GPx4 appeared to protect membranes from oxidative stress, because is present in phospholipids, but it can be also found in the nucleus, cytosol, mitochondria and bound to membranes **(Margis et al., 2008).**

1.8. BIOMARKERS

Modern eco-toxicological studies are focusing on evaluating how an organism, a community or a population, physiologically responds to an environmental chemical stress. The measurement of chemicals, metabolites, enzymes and other biochemical substances is used to determine the interaction between chemicals and biological system. These substances are referred to as "biomarkers" **(Piva et al., 2022).** The term "biomarkers" includes any measurement that shows an interaction between a biological system and an environmental agent **(WHO, 1993).** Biomarkers can thus be defined as measurable alterations, induced by a contaminant (or in any case by a state of stress) and incident on different levels of biological structural complexity **(Stebbing, 1985 and Garrigues et al., 2001).**

Biological and ecological responses to stressors may range from changes at the molecular level, where genetic integrity and subcellular processes are evaluated, up to damage to the level of organisms (of structure or function), and then also at population and community levels (influence on dynamics and structure of entire food chain). Indicators of stress at several levels of biological organization have been used to evaluate effects of contaminants at the organism level. **(Teh et al., 1997).** Biomarkers measured at molecular and cellular levels represent sensitive early warning tools to assess the biological effect in environmental biomonitoring programs **(Ferreira et al., 2021).**

Toxicants, such as PFAS, act at the molecular level by causing an alteration in the production of reactive oxygen species (ROS), followed by a cellular redox imbalance. This can subsequently cause DNA damage, enzyme activity modifications and a subsequent cascade of oxidative damage to the entire organism. To protect cells from these damages, organisms have evolved various antioxidant multi-enzymatic systems. Some of these enzymes can be used as good molecular biomarkers for contaminant-mediated oxidative stress and may also represent good markers of physiological response in organisms chronically exposed to xenobiotics **(Piva et al., 2022).**

2. AIM OF THE THESIS

In recent years, the scientific community is increasingly focusing the attention on how climate change and chemical pollution, due to anthropogenic origin, affects the ecosystems.

Antarctica is known to be an "irreplaceable natural laboratory" for this topic. In fact, this continent is considered to be the least affected by human activity, hence, samples from this environment are often considered as reference in studies on environmental global changes. On the other hand, the expansion of human activity, mainly related to research activity (with the all-year-round activity at Antarctic bases), the exploration of resources and the increasing tourism, have already introduced considerable levels of local pollution.

In addition to this, however, the majority of contaminants (including PFAS) can reach Antarctica through Long-Range Atmospheric Transport (LRAT) mechanisms from the other polluted areas of the planet. Therefore, many investigations indicate the need for continuous monitoring of the levels of environmental pollutants in Antarctica, for future impact assessment of anthropic pollutants in this remote area **(Olech et al., 1998).**

Special attention should be paid to those organisms that can be sensitive to environmental changes and that are well represented in the Antarctic marine environment **(Regoli et al., 1997).**

The particular interest in the antioxidant system of Antarctic marine organisms arises from the unique characteristics of the waters in which they live and have evolved, such as the presence of ice, extreme seasonal changes in light intensity and availability of food, low water temperature and salinity. In particular, these last two chemical-physical conditions can determine an increase in the solubility of oxygen in water, with a consequent increase in the bioavailability of the gas and greater probability of the formation of reactive oxygen species (ROS) and oxidative stress risk.

Many studies focus on the enhancement of ROS as a general pathway influenced by toxicity induced by xenobiotics and associated with oxidative stress **(Di Giulio, 1991).** For detoxification and removal of ROS, the cell has evolved a complex defence system consisting of both low molecular weight scavengers and antioxidant

enzymes. Variations in the content and the activity of antioxidant enzymes, can be used as biomarkers for contaminant-mediated oxidative stress in several marine organisms **(Regoli et al., 1997).**

Fish belonging to the *Notothenioidei* suborder are unique organisms, because they have evolved peculiar physiological adaptations over a well-defined period of time. In particular, their antioxidant system has been rated very efficient by some studies on antioxidant enzymes from Antarctic fish.

Some previous biomonitoring work has shown that some species of the genus *Trematomus* can be used as a bioindicator of the impact of pollutants on the Antarctic environment **(Campos, 2007).**

My thesis work is part of AntaGPS, a research project fund by the PRNA, aimed at studying the components of the antioxidant system of Antarctic fish and how this system can be influenced, at a biomolecular level, by the exposition to some chemical stressors.

In particular, the main object of my thesis was the study of the expression levels of genes coding for 4 antioxidant enzymes (SOD1, SOD2 and GPx1, GPx4), in 5 different organs of two Antarctic fish species.

In *T. borchgrevinki*, the aim was to characterize for the first time the expression levels of genes encoding antioxidant proteins. The analysis was carried out using 5 untreated organisms and 4 different target organs (liver, heart, white muscle and red muscle) in order to determine tissue-specific responses (quantity and type of antioxidant enzymes expressed in each tissue), that may be related to the physiology of these animals. Among notothenioids, the high-latitude bald notothen *T. borchgrevinki* is particularly notable as the sole cryopelagic species, exploiting the coldest and iciest waters of the Southern Ocean. **(Bilyk and Cheng, 2013).**

In fact, many studies **(Eastman and DeVries, 1985)** demonstrate that, compared to closely related benthic species, *T. borchgrevinki* exhibits morphological differences also in some organs, allowing a better adaptation to life in the water column, as well as the development of a trophic relationship with the ice shelf.

In *T. newnesi*, instead, the expression levels of the same antioxidant enzymes were analysed in liver and kidney of 5 individuals sampled from a control group and 5 individuals exposed for 10 days (acute stress) to 1.5 µg/l of PFOA.

In this way it was possible to assess the physiological differences between organs again, but also the differences between untreated and treated organisms.
Precisely PFOA was analysed because it is part of the emerging PFAS, in fact, in 2019 it was included in the list of POPs regulated under the Stockholm Convention. Another reason is that ionizable PFAS also manage to reach the most remote areas of the planet, thanks to their solubility, which is higher than that of chlorinated POPs, therefore, oceanic currents (OCTs) are considered the mayor transport vector of PFAS to remote marine environments like Antarctica **(Casal et al., 2017).**
Furthermore, the increasingly widespread industrial and commercial applications of these pollutants, together with environmental persistence, potential toxicity, bioaccumulation and long-range transport behaviours, leads to detect them in several matrices of Antarctica, like ocean seawater, sediment and also biota **(Gao et al., 2020).** PFAS can bioaccumulate and magnify along food webs, acting at the molecular level by causing an alteration in the production of ROS, followed by a cellular redox imbalance, which can cause a subsequent cascade of oxidative damage to the entire organism **(Piva et al., 2022).**
However, to our knowledge, there are very limited data on the occurrence of PFAS in Antarctica biota and no information on their trophic transfer. For these reasons, more studies are needed to investigate the occurrence and trophodynamic behaviours of PFAS, especially emerging PFAS, in Antarctic ecosystems **(Gao et al., 2020).**

The present work represents a further contribution to the characterization of the antioxidant system of Antarctic fish, leading to an increase in knowledge on the molecular and functional evolution of these organisms.
Furthermore, the detection of variations in biomolecular responses will provide a useful tool for early assessment of environmental disturbance, improving the usefulness of these organisms in biomonitoring the Antarctic marine environment.
Therefore, the obtained results constitute an important starting point in order to use the expression of antioxidant enzymes as biomarkers, both of oxidative stress and of exposure to PFAS, using notothenioids as bioindicator organisms in biomonitoring campaigns of the Antarctic marine environment.

3. MATHERIALS AND METHODS

3.1. TARGET SPECIES AND TREATMENT

The organisms used in the experiments were sampled near the Italian *Mario Zucchelli* research station, in Terra Nova Bay facing the Ross Sea (74°42' S, 164°7' E), during the XXXVII Italian Expedition to Antarctica (2021-2022).

The specimens of *Trematomus borchgrevinki* were fished on 11/15/2021 at Silverfish Bay (74°38,031'S, 164°41,351'E), close to the ice sheet, at a depth of about 3 m below the surface and their physical parameters were: weight 81-95,9 g and length 20-22 cm.

Instead, the specimens of *Trematomus newnesi* were fished on 09/11/2021 at Tethis bay (74°42,001'S, 164°02,640'E) at a depth of about 62 m and their physical parameters were: weight 83,8-149,7 g and length 20-23,5 cm. All fish were female. Then, both species were put in thermostated aquariums (Figure 3.1) at -2 ° C, where they were kept for 4 days, in order to eliminate the sampling stress.

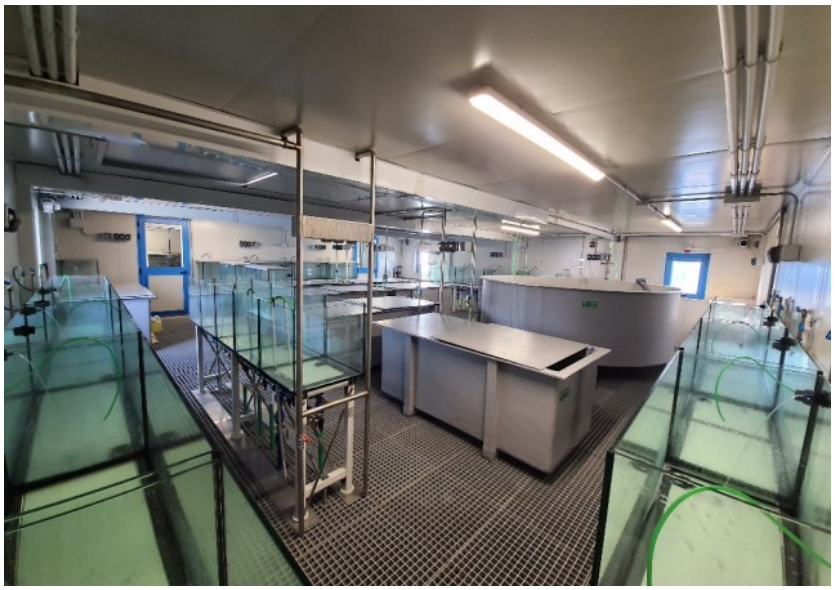


Figure 3.3: The aquarium laboratory at the Mario Zucchelli station, inaugurated during the 2020-2021 campaign. The infrastructure consists of a series of tanks of different volumes, which can also be used individually, in which it is possible to adjust the temperature and dissolved gases, and continuously monitor the pH, dissolved oxygen, conductivity and temperature values ***(PNRA).***

Once this period was over, 5 individuals of the species *T. borchgrevinki* were sacrificed, using an overdose of anesthetic, and then dissected for the removal of some organs: in particular liver, heart, white muscle and red muscle. The collected

organs were immediately frozen in liquid nitrogen and stored at -80°C, before being shipped back to Italy for laboratory analysis.

At the same time, two experimental groups of another species, *T. newnesi,* were set up: a control group (untreated) and a group of treated organisms, which were exposed to 1,5 µg/l of PFOA for 10 days (Figure 3.2).

Figure 3.2: A PNRA researcher who injects into the tank the dose of PFOA to which some specimens of T. newnesi will be exposed. Photo taken by Gianfranco Santovito, during the XXXVII Italian Expedition to Antarctica (2021-2022).

After the treatment period, also these organisms were sacrificed and then dissected for the removal of liver and kidney. Also these organs were frozen and stored (Figure 3.3) before being sent to Italy.

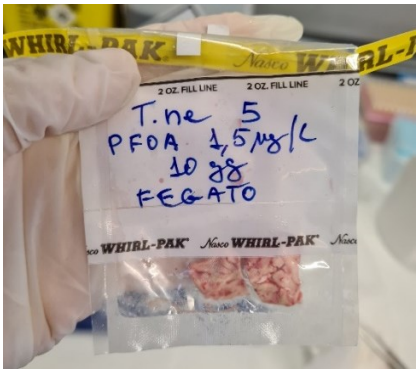


Figure 3.4: A liver sample (in this case from individual 5 of T. newnesi, exposed to 1.5 µg/L of PFOA for 10 days) after being frozen at -80 ° C in Italy.

3.1.1. Trematomus borchgrevinki

Figure 3.5: A specimen of Trematomus borchgrevinki. Photo by Gianfranco Santovito.

Trematomus borchgrevinki (Figure 3.4) is a species of marine ray-finned fish (Actinopterygii) belonging to the family *Nototheniidae*. Its classification, from the World Register of Marine Species, is illustrated in Table 3.1.

Kingdom	**Animalia**
Phylum	Chordata
Subphylum	Vertebrata
Gigaclass	Actinopterygii
Class	Actinopteri
Subclass	Teleostei
Order	Perciformes
Family	Nototheniidae
Genus	Trematomus
Species	Trematomus borchgrevinki

*Table 3.2: Trematomus borchgrevinki classification **(WoRMS Editorial Board, 2022**).*

T. borchgrevinki is native to the Southern Ocean, is a cryopelagic species and feeds on amphipods and copepods **(Phleger et al., 1999).** It has a circumantarctic distribution in surface water, beneath fast and floating ice and it differs in many essential characters from other members of the family *Nototheniidae* **(Eastman and DeVries, 1985).**

Trematomus borchgrevinki was originally described as a trematomid by **Boulenger in 1902**. Then, **Andriashev and Jakubowski (1971)** placed the cryopelagic species *Trematomus borchgrevinki*, together with *T. brachysoma*, in *Pagothenia*, a monotypic genus originally erected for *P. antarctica* by **Nichols and LaMonte**

(1936). This realignment was based on the morphology of the cephalic lateral line system. It was found that the canal and pore arrangements were similar in all trematomids, except the cryopelagic species, where most canals were interrupted and reduced. **Jakubowski (1971)** noted that these traits were also evident in pelagic species, maybe because, without canals, the neuromasts of these species are better able to detect slight turbulence in the water caused by floating ice. In recent years, the genus *Pagothenia* (with the two species *P. borchgrevinki* and *P. brachysoma*) was integrated into the genus *Trematomus* **(Dettai et al., 2012).** Mitochondrial and nuclear sequence analyses suggested that *Pagothenia* and *Cryothenia* are more closely related to the *Trematomus* genus **(Fields and DeVries, 2015).** Therefore, the genus *Trematomus* contains 14 species: 11 species classified in the genus *Trematomus*, 2 in the genus *Pagothenia* and at least one of the other two species in genus *Cryothenia* **(Lautrédou et al., 2012).** *Trematomus* is monophyletic only when it includes both *Pagothenia* and *Cryothenia* **(Kuhn and Near, 2009).** Then, **Near et al. (2018)** stated that *T. newnesi* and *T. borchgrevinki* are sister taxa and all other species of *Trematomus* form a monophyletic group.

There are also substantial morphological differences in several organs between the cryopelagic *T. borchgrevinki* and related benthic species. Assuming that the ancestral notothenioid stock was benthic, *T. borchgrevinki* evolved from this condition, as evidenced by some derived characters, that are probably specializations for life in the water column. In spite of being well adapted for this pelagic life, *T. borchgrevinki* is still dependent on the substrate for reproduction, as are most other notothenioids **(Eastman and DeVries, 1985).**

As all notothenioids lack swim bladders, they are supposed to be confined to predominantly benthic habitats. However, there has been evolutionary diversification in their presence in the water column and now it is possible to recognize also pelagic, cryopelagic and benthopelagic species. *T. borchgrevinki* organisms live in the water column, swim beneath the surface of the platelet ice and use it as a shelter. Their tissues contain less lipid than the neutrally buoyant *Dissostichus mawsoni,* but more than the benthic *Trematomus bernacchii*. Liver, spleen and muscle are rich in lipid, especially triacylglycerol which provides static lift and serves as a metabolic reserve.

A relatively high oxygen consumption rate in red pectoral muscle is indicative of the active labriform swimming mode in T. *borchgrevinki*.

Certain hematological parameters may also be correlated with its activity pattern and position in the water column. **Wells et al. (1980)** found that *T. borchgrevinki* had higher hemoglobin concentrations and hematocrits than other trematomid. The risk of freezing is very high for cryopelagic fishes, because they frequently come into touch with ice crystals of the platelet ice in their habitat. For that reason, they have antifreeze glycoprotein in their body fluids, which lower the freezing point of these fluids. Therefore, among all species of the Ross Sea, *T. borchgrevinki*, has more antifreeze than benthic species **(Eastman and DeVries, 1985).**

The sea ice microalgae bloom contributes a significant amount of new carbon to both the pelagic and benthic communities of Ross Sea. High productivity at the ice-water interface may have served as a stimulus for the evolution of cryopelagic invertebrates and fishes. During the austral spring, *T. borchgrevinki* fed exclusively at or near this interface and there was no dietary overlap with a closely related benthic species **(Eastman and DeVries, 1985).**

3.1.2. Trematomus newnesi

Figure 3.6: A specimen of Trematomus newnesi. Photo by Gianfranco Santovito.

Regarding species diversity, numerical abundance, and ecological diversity, *Trematomus* species are the dominant component of the high Antarctic shelf fish fauna and are especially characteristic of the subzero waters of East Antarctica. *Trematomus* consists of 15 species and is a relatively young clade when compared to the entire Antarctic notothenioid clade **(Kuhn and Near, 2009).**

The nototheniid fish *Trematomus newnesi* (Figure 3.5) has a circumantarctic distribution in cold, shallow shelf waters of Antarctica and adjacent islands. Its classification is depicted in Table 3.2.

Kingdom	**Animalia**
Phylum	Chordata
Subphylum	Vertebrata
Gigaclass	Actinopterygii
Class	Actinopteri
Subclass	Teleostei
Order	Perciformes
Family	Nototheniidae
Genus	Trematomus
Species	Trematomus newnesi

Table 3.3: Trematomus newnesi classification ***(WoRMS Editorial Board, 2022****).*

It is the only known example of phenotypic plasticity in Antarctic notothenioid fish, existing as populations of a typical common morph (benthic morph versus semipelagic morph), because it presents intermediate forms between the two morphs. The depth range of this species varies from shallow waters to 400 m, but it is more abundant in inshore waters within the 20–50 m depth range, mainly over rocky bottoms or macroalgae beds **(Mahé et al., 2016).**

Therefore, initially recognized as semipelagic (both cryopelagic and benthic), *T. newnesi* exhibits considerable trophic plasticity, feeding in the water column and occasionally also on the substrate: mainly on amphipods, polychaetes, gastropods, isopods, copepods, and euphausiids (*E. superba*). **(Phleger et al., 1999; Dewitt et al., 1990).**

Recent research indicates, however, that *T. newnesi* differs from the other predominantly benthic and epibenthic species of *Trematomus* also in features of the skeleton and cephalic lateral line, and that there are reasons for considering generic isolation.

Additionally, also the blood vascular system revealed differences. For example, *T. newnesi* is the only species of *Trematomus* without an ocular rete mirabile **(Eastman and Devries, 1997).**

Another characteristic of fishes of the family *Nototheniidae* normally have a single major Hb (*Hb 1*), often a second, minor component (*Hb 2*, about 5 % of the total) and traces of a third component (*Hb C*, less than 1 %).

However, the haematological features of *T. newnesi* are remarkably different. It has two functionally distinct major Hbs: *Hb 1* (70 %) and *Hb C* (20-25 %) **(Mazzarella et al., 1999),** which is present in only trace amounts in other notothenioids**.** This haemoglobin multiplicity is consistent with an attitude to active swimming **(Eastman and Devries, 1997).**

Furthermore, the blood of Antarctic fishes, normally contains less hemoglobins, rather than fishes from temperate latitudes probably, because of the relatively stable conditions of their habitat. In contrast, other fishes have in their blood a variety of hemoglobins with different ligand binding properties, to meet the physiological requirements imposed by the variability of the environment **(Giangiacomo et al., 2001).**

3.2. RNA EXTRACTION

Total RNAs were extracted from the sampled species with the purpose of further analysis on gene expressions (*sod1*, *sod2* and *gpx1*, *gpx4*).

In five specimens of *T. borchgrevinki,* the RNAs were extracted and purified starting from the entire organs: liver, heart (Figure 3.6), red muscle and white muscle. At the same time, also in five specimens of *T. newnesi,* the RNAs were extracted and purified, but from liver and kidney (both treated and untreated organs).

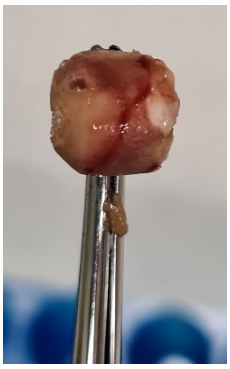


Figure 3.7: The just defrosted heart of a specimen of Trematomus borchgrevinki,

Ribonucleic acid, unlike DNA, is an easily degradable structure, mostly due to RNAse (nucleases that catalyze the hydrolysis of RNA), for this reason its isolation requires a special accuracy and precision. It involves 5 steps:

1. HOMOGENIZATION. Every tissue sample was cut in pieces, transferred into a 1.5mL Eppendorf tube and manually homogenized, using pestles, in 1mL of PRImeZOL™ per 50-100mg of tissue. This reagent keeps the RNA intact while it destroys the cells and dissolves its cellular components. Figure 3.7 illustrates the various steps listed above.

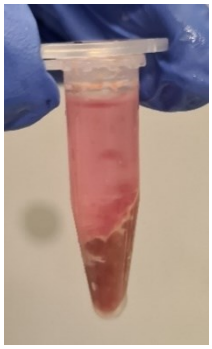
(a)  (b)  (c)  (d) 

Figure 3.8: Illustration of consequent steps of the homogenization phase: (a) pieces of tissue transferred into the Eppendorf tube; (b) addition of PRImeZOL™ reagent; (c) Manual homogenization using pestles; (d) homogenized sample.

PRImeZOL™ (AN1100) is composed by phenol 25-50%, which acts like a strong denaturing of proteins, and guanidinium thiocyanate 10-25% to ensure optimal results. The latter has a lysing action, that causes the release of cellular components and also prevents the activity of RNase enzymes and DNase enzymes by denaturing them. These enzymes would otherwise damage the extract.

2. PHASE SEPARATION. Homogenized samples were incubated for five minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. Then, 200 µL of chloroform were added per 1 mL of PRImeZOL™ used. Chloroform enhances the denaturation of the proteins, improves the solubilisation of lipids and the recovery of the total RNA and facilitates the phase separation.

   The samples were then incubated for 3 minutes at room temperature, afterwards they were centrifuged at a speed of 13000 rcf for 15 minutes at a temperature of 4°C.

The centrifugation leads to the effective separation of the samples in three different phases (illustrated in Figure 3.8): a red lower organic layer containing proteins and lipids, a white and thin interphase containing denaturated proteins and DNA and a colourless upper aqueous layer that contains the RNA.

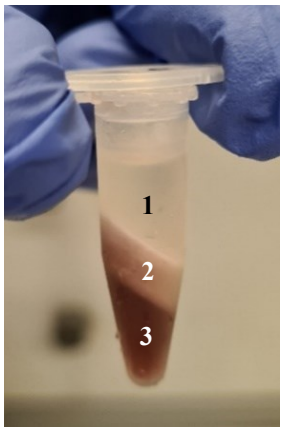


Figure 3.9: The separation into 3 different phases after the centrifugation.

3. RNA PRECIPITATION. For every sample, the upper phase containing RNA was transferred to another tube with proper label. This step was performed with cut tips. being very careful not to touch the interphase. The precipitation of RNA then occurs by mixing it with 500 µL of cold isopropyl alcohol per 1 mL of PRImeZOL™ used. After an incubation at room temperature for 10 minutes, the samples were centrifuged at 13000 rcf for 15 minutes at 4°C. After this step, it is possible to observe the formation of a pellet in the bottom of the Eppendorf tube (Figure 3.9). The size of the pellet depends on the initial amount of tissue used, as well as on the type of the tissue itself.

Figure 3.9: pellet formation at the bottom of the tube.

4. RNA WASHING. In the next step the supernatant was removed from each sample being very careful not to touch the pellet. Then the pellet was washed twice with 75% ethanol, adding at least 1 mL of ethanol per 1 mL of

PRImeZOL™ used. After every wash the samples were centrifuged at 7600 rcf for 5 minutes at 4°C.

5. RNA RE-SUSPENSION. The final steps involve the air-drying of the pellets under the laminar flow hood for 10 minutes (or more) and then the re-suspension of the pellet, in an appropriate volume of RNAse free water. In order to improve the dissolution, the samples were kept 2 hours at room temperature.

3.2.1. RNA purification

This step, particularly suggested when working with Antarctic fish, provides the purification of RNA using lithium chloride (LiCl), in order to eliminate peptides or carbohydrates that can be possibly present in the samples.

The samples were incubated for 5 minutes at 40°C and then centrifuged at 13000 rcf for 15 minutes, in order to allow the precipitation of the remaining carbohydrates. Subsequently the RNA precipitation occurs by mixing the supernatant with 1/3 (of the water volume) of LiCl 8M and then keeping the samples incubated at 4°C *overnight*.

The day after, a further centrifugation at 13000 rcf for 20 minutes results in the formation of the RNA pellet again, this has to be washed twice with 200 µL of cold ethanol 75%. At the end, after the air-drying of the pellet under laminar flow hood, RNA was re-suspended in RNAse free water. The purified samples were then analyzed at the NanoDrop spectrophotometer.

Finally, the integrity of the RNA was verified, using an electrophoresis in 1% agarose gel and formaldehyde, by running an aliquot of RNA (1000 ng/µL) on a denaturing gel stained. This technique allows, in the presence of intact RNA, to visualize two clear bands, corresponding to ribosomal RNA (28S and 18S) following the staining of the samples. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band. Only RNA that showed fair or good quality was then used for the next steps.

3.2.2. RNA quantification

The quantification of RNAs was performed using the NanoDrop spectrophotometer (Figure 3.10). Its technology is based on an innovative sample retention system, that uses the surface tension to hold and measure microvolume samples between

two optical pedestals, without the use of cuvettes or capillaries **(Desjardins et al., 2009).** The spectrophotometer is, in fact, capable of analyzing microvolumes of samples (1 µL of total RNA), working in a range of concentration between 2 and 3000 ng/µL referring to RNA.

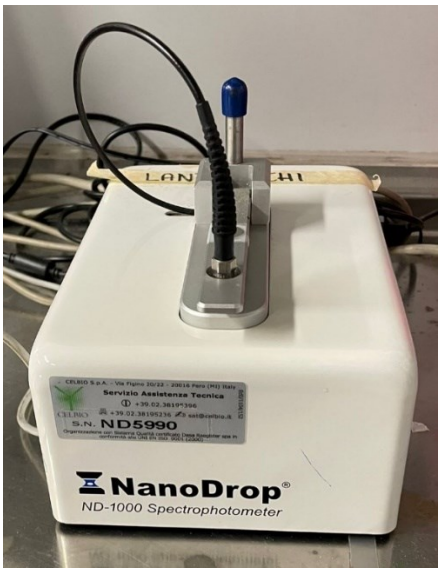


Figure 3.10: The NanoDrop spectrophotometer.

Every reading is carried out at 230, 260, 280 nm, in order to evaluate the A260/A230 and A260/A280 ratios. If the result of the first ratio is less than 2 it means that there is a phenol contamination or a contamination due to aromatic compounds, peptides or carbohydrates. If the second ratio is between 1.8 and 2.2, it indicates purity of the nucleic acids; if higher it is possible that there is a contamination by phenol and genomic DNA.

3.3. RNA REVERSE TRANSCRIPTION AND cDNA SYNTHESIS

The purified total RNAs samples, extracted from *T. borchgrevinki* and *T. newnesi* were reverse transcribed into cDNA with the purpose of quantifying the gene expression of *sod1, sod2* and *gpx1*, *gpx4* using the quantitative real-time PCR technique (Polymerase Chain Reaction).

Gene expression analysis is among the most commonly used methods in modern biology, which provides quantitative information about the population of RNA species in biological systems (cells and tissues). It is an exceptionally powerful tool of molecular biology for investigating the transcriptional behaviour of biological systems, for classifying cell states in disease and for many other purposes. **(Lovén et al., 2012).**

However, before the expression of any gene can be measured, as RNA cannot serve as a template for PCR, the mRNA in the sample must be copied to cDNA by reverse transcription. This step is then followed by the exponential amplification of the cDNA template in a PCR reaction. **(Bustin, 2000).**

The cDNA synthesis was performed using a Biotechrabbit^{TM} cDNA Synthesis Kit which is highly efficient in synthesizing long cDNAs (≥19 kb). The kit contains RevertUP^{TM} II Reverse Transcriptase, that enables highly efficient reverse transcription with increased thermostability, an RNAse Inhibitor which is a potent non-component inhibitor of RNAses. Oligo (dT) primers were used for the synthesis of cDNA from only poly(A) tailed mRNA. Finally, Ribonuclease inhibitor is needed, in order to prevent RNA degradation.

The cDNA synthesis reaction was performed as described below (Table 3.3):

COMPONENT	**VOLUME (µL)**
dNTP Mix (10 mM each)	2
RNase Inhibitor, 40 U/ µL	0.5
Oligo (dT)$_{12-18}$ (10 µL)	0.5
5x Reverse Transcriptase Buffer	4
RNA Template	1
RevertUP^{TM} II Reverse Transcriptase	1
PCR Grade Water	Up to 20
Total Volume	20

Table 3.4: Reagents for cDNA synthesis reaction. Those underlined are enzymes.

Every test tube was then incubated into a PCR thermocycler (Figure 3.11) at 50° for 60 minutes and at 99°C for 5 minutes for the enzyme inactivation.

Figure 3.1: Thermocycler display showing the time and temperature for each stage of the retrotrascription.

The product can last for several weeks if stored at -20°C. The obtained cDNA is 50 ng/µL concentrated.

3.4. PRIMER DESIGN TO AMPLIFY *sod* AND *gpx* GENES

With the purpose of analyzing the gene expression trends of *sod1, sod2, gpx1, gpx4* and *gapdh* in *T. borchgrevinki* and *T. newnesi,* we needed to perform a bioinformatic job. Primer designing means to build a primers pair with which is possible to amplify a segment of the coding sequence for the specific antioxidant enzyme (SOD1, SOD2, GPx1, GPx4) in the mRNA of our species. The same procedure of primer design was performed to obtain primer pairs for *gapdh*, that was chosen as housekeeping gene.

First of all, we obtained the gene sequence (if present the complete one, otherwise the partial one) from the GenBank database on the NCBI website, but we were able to integrate the missing information also thanks to a colleague's collection of gene sequences, from the Department of Life Sciences in the University of Trieste.

The sequences were uploaded into the Primer3 web program (primer3.ut.ee/), where some specific parameters were set (Figure 3.12).

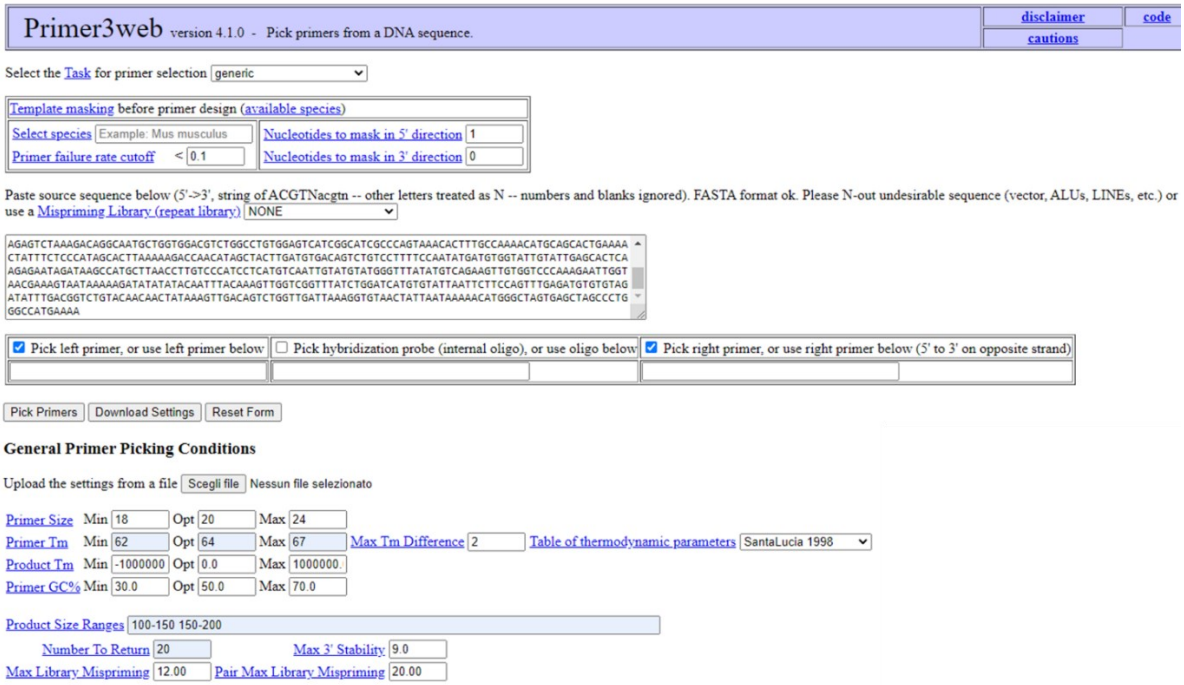


Figure 3.12: Screenshot example of the Primer3 webpage, with the specific set of the main parameters.

The main set parameters are listed below:

- Primer size: 18-23°C (optimal 20°C)
- Melting temperature: 57-63°C (optimal 60°C)
- Difference between Tm of the two primers: max 2°C
- GC content: 55%
- Product length: 100-200 bp
- C/G at the 3'- end: max one or two
- No dimers neither *loop* structures

Given these conditions, the program returns some primer pairs as output, which were further analyzed using the IDT OligoAnalyzer™ Tool (eu.idtdna.com/calc/analyzer). The program, as shown in the Figure 3.13, performs hairpin analysis (required condition: $\Delta G < 3$), self-dimer analysis (required condition: $\Delta G > -9$), and hetero-dimer analysis (required condition: $\Delta G > -9$). In addition, no bind at 3'-end is preferable.

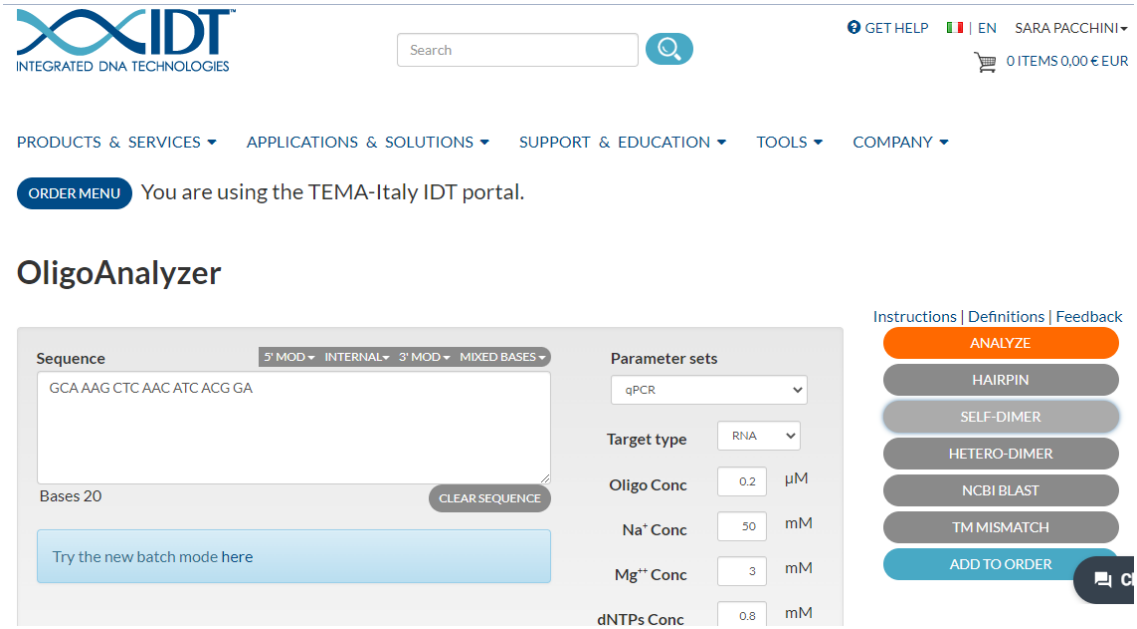


Figure 3.13: Screenshot example of the OligoAnalyzer™ Tool program, with the appropriate set of parameters. Sequence analysis of the primer sod1_T.eulepidotus_FW.

Only the primer pairs that respect the set parameters were ordered (listed in Table 3.4), to be consequently tested with *T. borchgrevinki* and *T. newnesi* cDNA.

isoform	PRIMER	Sequences 5' → 3'	Amplicon (bp)	T_a (°C)
sod1	SOD1_T.eulepidotus_FW	GCAAAGCTCAACATCACGGA	125	60
	SOD1_T.eulepidotus_RV	CCAGCATTGCCCGTCTTTAG		60
sod2	SOD2_T.bernacchii_FW	GTGGAAGCCTTCGTATCGCT	108	60
	SOD2_T.bernacchii_RV	GAAGGTAGTAGGCGTGCTCC		60
	7_SOD2_T.newnesi_FW	GCCTCAGCCAAACTTTAAACCTGG	135	60
	7_SOD2_T.newnesi_RV	CATGGTGCTTGCTGTGGTGC		60
gpx1	GPx1b_T.eulepidotus_FW	CCCTCCTCCCTCATGACTGA	145	60
	GPx1b_T.eulepidotus_RV	TGTCGCTGGTGAGGAACATC		60
gpx4	GPx4b_T.bernacchii_FW	TTTACGCATCCTCGCCTTCC	143	60
	GPx4b_T.bernacchii_RV	GATGAGCAGTGTCCCCGTTC		60
gapdh	GAPDH_T.bernacchii_FW	AAGTATGACTCCACCCACGG	113	60
	GAPDH_T.bernacchii_RV	ATGTTAGCGGGGTCCTTCTC		60

Table 3.5: Primer pairs used for qRT-PCR. The amplicon size and melting temperature (T_a) is also indicated.

3.5. cDNA AMPLIFICATION

To verify the reverse transcription's efficiency, the cDNA obtained by reverse transcriptase was amplified through PCR, using specific *primers* for the *gapdh* gene. As it is, in fact, an endogenous gene expressed constitutively, it represents a control for the correct success of the reaction.

Once the quality of the obtained cDNA has been verified, it is necessary to verify also the *primer* pairs functionality, proceeding with the amplification of the coding region of the two isoforms of *sod* and *gpx*, using specific *primers*. The thermal program is still the same, except for the annealing temperature (T_a), which vary depending on the used *primer*.

The table below (Table 3.5) lists the different reagent types and their corresponding dosages.

Component	**Volume**	**Final concentration**
2X YourTaq PCR Master Mix	12.5 µl	1×
5× PCR Enhancer (**optional-** BR1900201)	10 µl	1×
Forward primer	1.5 µl	0.2–1 µM
Reverse primer	1.5 µl	0.2–1 µM
Template DNA	3 µl	10 pg–1 µg

Use 0.01–1 ng for plasmid or phage DNA and 0.1–1 µg for genomic DN

Nuclease free water	6.5 µl	
Total volume	**25 µl**	

Table 3.6: Reagents for the PCR reaction.

The tubes were then placed in the thermocycler, imposing the following general cycling conditions:

a) **Initial denaturation**: 5 minutes at 95°C for the complete denaturation of template DNA, including removal of all secondary DNA structures, such as hairpin.

b) **Annealing**: 35 cycles for 20 seconds at 95°C, 30 seconds at T_a and 30 seconds at 72°C for the extension. For each primer pairs analysis three different annealing temperatures (T_a) were tried at once (usually 57°-60°-63°). The best results were usually obtained at 60°. Optimizing the annealing temperature is crucial, as a too low temperature might result in non-specific amplification, whereas a too high temperature results in no amplification. The melting temperature (T_m) is defined as the temperature in which 50% of the primer and its complementary sequence of the target DNA are present as duplex DNA.

c) **Extension:** 5 minutes at 72°C to ensure that all amplicons are fully extended and include 3'-A-overhang.

The PCR results were then analyzed after a run in a 2% agarose gel electrophoresis (the percentage of the gel was the best suggested for the DNA ladder 50bp used). Only the amplicons for which a positive result was observed underwent the next step, namely qRT-PCR.

3.6. GENE EXPRESSION ANALYSYS ON qRT-PCR

Reverse transcription quantitative PCR (RT-qPCR) is considered today as the gold standard for accurate, sensitive and fast measurement of gene expression **(Derveaux et al., 2010).** It is the most sensitive and reliable technique for the quantification of nucleic acids, which is based on the identification and quantification of the fluorescence emitted by a reporter molecule (in this case the SYBRGreen intercalating dye). The measurement takes place in real time during the accumulation phase of the PCR product and at each amplification cycle, thus allowing us to monitor the amplification during the initial phase and especially during the exponential phase of the PCR reaction **(Dale et al., 2013).**

To evaluate the target gene expression rates, cDNAs of 5 specimens per species, per organs and per treatment (for *T. newnesi*), were tested in qRT-PCR analysis.

For every analysis it was necessary to compare the gene expression rates of target genes to that of an housekeeping gene. The latter are ubiquitous genes, constitutively expressed, which are not affected by experimental treatments and which maintain their expression constant in different cell types. Therefore, in this

way we were able to normalize the expression levels in different samples and so, to obtain a relative quantification of the expression of our target genes.
In the present study, *sod1, sod2* and *gpx1, gpx4* expression rates were normalized onto *gapdh* expression rates.

To test the primer pairs efficiency, it is preferable to create a pool containing the cDNAs og the 5 specimens of the species of interest, in order to be sure that the primer pairs work for all the samples, avoiding an individual-specific response. The volumes of cDNAs pools were obtained according to the number of primer pairs that had to be tested. The efficiency of each primer pair was evaluated by setting up a qRT-PCR reaction where every cDNAs pool was diluted, following the concentrations shown in the Table 3.6, with the aim of building a standard curve. An example of the qRT-PCR set up is provided below.

PRIMER	**Concentrations of cDNAs samples per well (ng/ µL)**											
sod1	100	100	100	50	50	50	25	25	25	12.5	12.5	12.5
sod2	100	100	100	50	50	50	25	25	25	12.5	12.5	12.5
gpx1	100	100	100	50	50	50	25	25	25	12.5	12.5	12.5
gpx4	100	100	100	50	50	50	25	25	25	12.5	12.5	12.5
gapdh	100	100	100	50	50	50	25	25	25	12.5	12.5	12.5

Table 3.7: Example of a diagram of the dilutions on the RT-plate to build the standard curve.

Obtained the amplification curves and fixed the threshold cycle (Ct), the specific acquired Ct values, relating to certain samples, are inserted in a graph as a function of the logarithm of the cDNA concentration. By interpolating the points, we obtain a standard line, from which important information about the performance of the reaction, as well as various reaction parameters, can be derived:

- **The slope** is a measure of reaction efficiency. To obtain accurate and reproducible results, reactions should have an efficiency as close to 100% as possible, equivalent to a slope of about -3.32. The slope is, in fact, directly related to the amplification efficiency, through the relationship $E = 10^{-1/m}$ (m is the angular coefficient of the straight line).
- **The correlation coefficient R^2** reflects the linearity of the standard curve. The closer R^2 is to 1, the better the data fit the standard curve.

The qRT-PCR cycle was set as follows:

- Initial denaturation: 2 minutes at 95°C

- Amplification stage: 38 cycles of 95°C for 20 seconds and 60°C for 60 seconds. This stage provides an amplification curve that should be coincident for each technical replicate and shifted by one Ct at each subsequent dilution.

- Dissociation stage: 15 seconds at 95°C, 1 min at 60°C, 15 seconds at 95°C and 15 seconds at 60°C. This stage provides a dissociation curve as output, which can be analyzed in order to verify the specificity of the primer pair. If only one peak is present, it means that the primers amplified a single template and are specific. On the contrary, if more peaks are observed, it means that the primers have not only amplified the gene of interest, but also other non-specific portions of DNA. In this last case, the primer cannot be used for the subsequent steps.

The primer pairs that showed specificity were subsequently used for qRT-PCR analysis of each sample, according to the following dosages (Table 3.7):

Reagent	**Volume for sample (µL)**
2x qPCRBIO SyGreen Mix (PCRBIOSYSTEM®)	5.0
Forward Primer 10 µM	1.0
Reverse Primer 10 µM	1.0
cDNA	1-2
RNAse free water	Up to 10
Total Volume	**10**

Table 3.8: Reagents for the real time qPCR reaction.

3.7. CALCULATION OF EXPRESSION LEVELS WITH THE $2^{-\Delta\Delta Ct}$ METHOD (RELATIVE QUANTIFICATION)

QRT-PCR provides, as a result, a relative quantification value (RQ), given by the ratio describing the expression level of the target gene in the sample compared to the reference (control) sample. RQ is determined initially by normalizing the expression levels of the target with respect to the reference gene (HKG) and then by comparing this value relative to the sample with the normalized value relative to the control.

From a technical point of view, having initially verified that the *primer* amplification efficiency (E) was 100% or nearly, the relative quantification values

(RQ) were obtained through the mathematical Pfaffl's model **(Pfaffl, 2001),** which is reported below:

$$2^{-\Delta\Delta Ct} = \frac{2^{-[Ct(TARGET)-Ct(HKG)](sample)}}{2^{-[Ct(TARGET)-Ct(HKG)](control)}}$$

3.8. STATISTIC ANALYSIS

The results have been shown as the mean value ± SD (Standard Deviation). For the statistical analysis of the data, the Primer.exe program (Version 1.0, Stanton A. Glantz, Italy) was used (Figure 3.14).

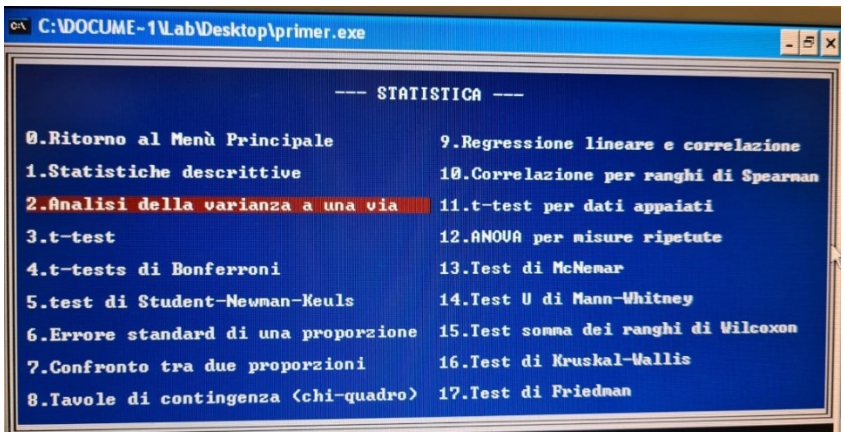


Figure 3.14: Primer.exe program screenshot: various statistical analysis options.

First of all, we applied the analysis of one-way variance (ANOVA), where we entered the results of each data group analyzed, in the form of means and standard deviations (Figure 3.15).

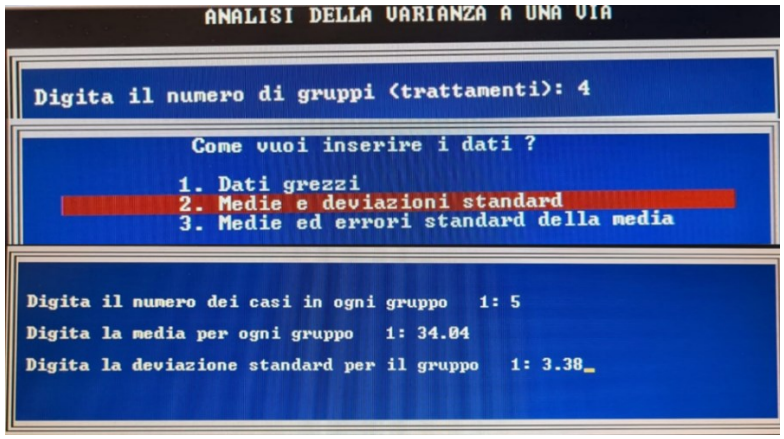


Figure 3.15: Primer.exe program screenshot. Required data for ANOVA analysis: number of groups (treatments), number of cases in each group and their respective means and standard deviations per group.

Afterwards, the Student-Newman-Keuls test was performed, with the aim of determining the significance of the difference between the various groups. The program returned a window as shown in the Figure 3.16, where a *p*-value < 0.05 is considered statistically significant.

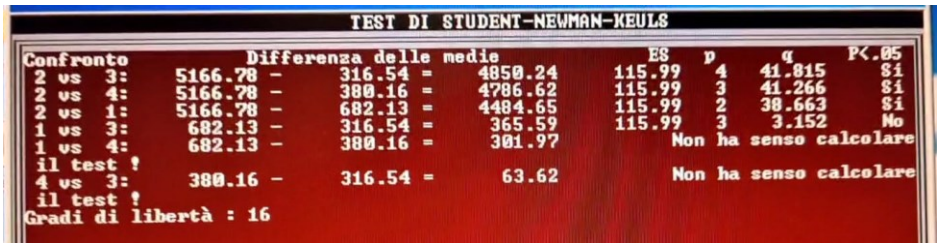


Figure 3.16: Primer.exe program screenshot. Example of the Student-Newman-Keuls test. The final column indicates whether the difference between the two compared groups is significant ("SI") or not ("NO" or "Non ha senso calcolare").

4. RESULTS

The analysis of mRNA accumulation allowed us to evaluate the gene expression at transcriptional level of various isoforms of SOD (1 and 2) and GPx (1 and 4), in different organs and tissues of two Antarctic fish species: *Trematomus borchgrevinki* and *Trematomus newnesi*. The mRNA levels of those antioxidant enzymes have been evaluated for both species by qRT-PCR analysis. The data were normalized against the expression of *gapdh*, used as a housekeeping gene.

4.1. mRNA EXPRESSION in *T. borchgrevinki*

The evaluation of basal expression levels was determined in liver, heart and muscle (both white and red) of specimens of *T. borchgrevinki*, with the aim of verifying tissue-specific differences.

We considered two comparisons:

- between organs, referring to the same enzyme
- between antioxidant enzymes, within the same organ

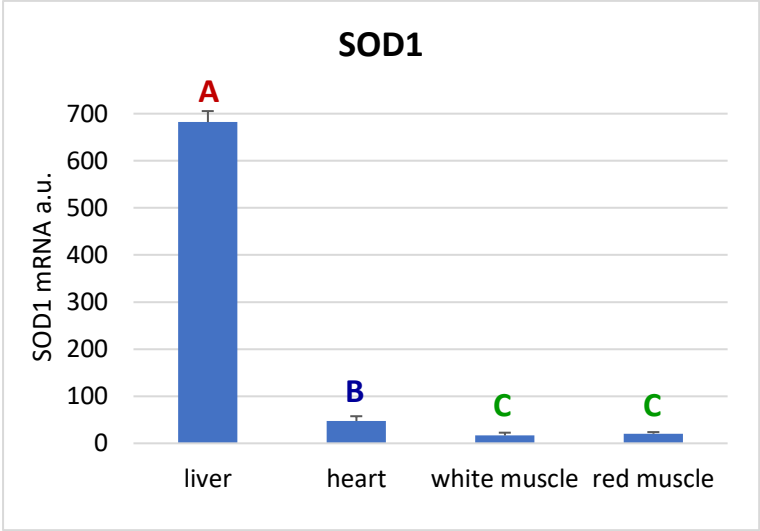
4.1.1. Difference between organs

The highest expression of all antioxidant enzymes was measured in the liver, with a statistically significant difference ($p < 0.05$) compared to all other organs (Figure 4.1). The hepatic mRNA levels are at least 14-time higher than other organs for SOD1 enzyme (Figure 4.1A), at least 2-time higher for SOD2 (Figure 4.1B), at least 6-times for GPx1 (Figure 4.1C), and at least 18-times for GPx4 (Figure 4.1D).

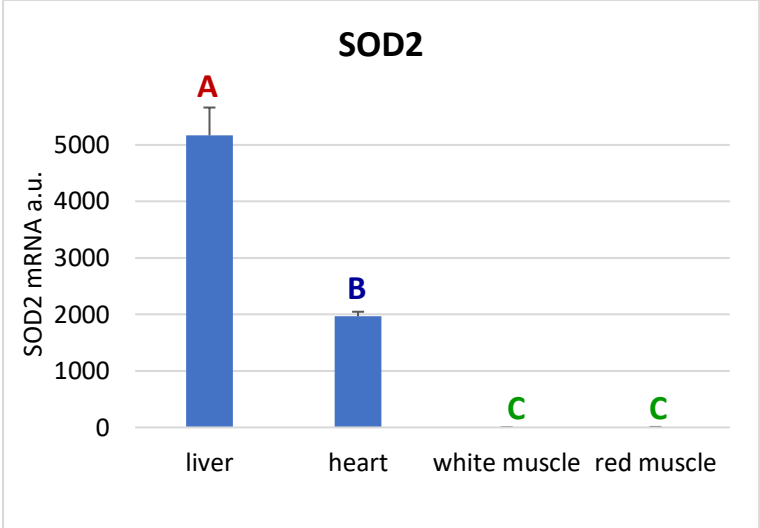
After the liver, the heart expresses the highest levels of all considered antioxidant enzymes (Figure 4.1). Also in this case the differences with the other organs are statistically significant ($p < 0.05$). The cardiac mRNA levels are about 2-time higher than muscles for SOD1 enzyme (Figure 4.1A), about 260-time higher for SOD2 (Figure 4.1B), about 10-times for GPx1 (Figure 4.1C), and about 7-times for GPx4 (Figure 4.1D).

White and red muscles are regularly the organs where gene expression at transcriptional level is the lowest and the differences between the two kinds of muscle were never statistically significant.

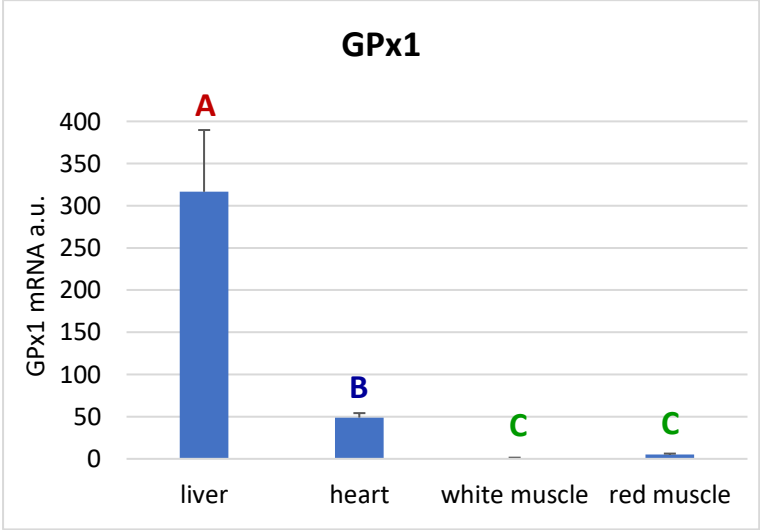
A)


SOD1
SOD1 mRNA a.u.
700
600
500
400
300
200
100
0
A
B
C
C
liver
heart
white muscle
red muscle

B)


SOD2
SOD2 mRNA a.u.
5000
4000
3000
2000
1000
0
A
B
C
C
liver
heart
white muscle
red muscle

C)


GPx1
GPx1 mRNA a.u.
400
350
300
250
200
150
100
50
0
A
B
C
C
liver
heart
white muscle
red muscle

D)

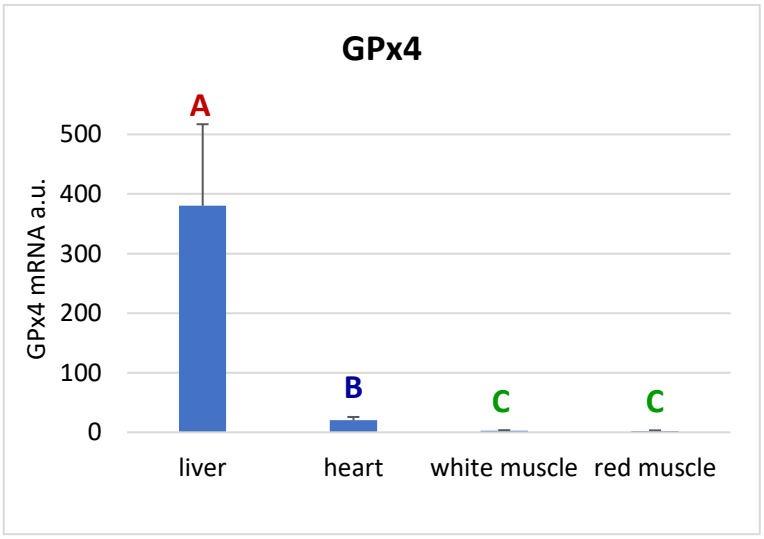


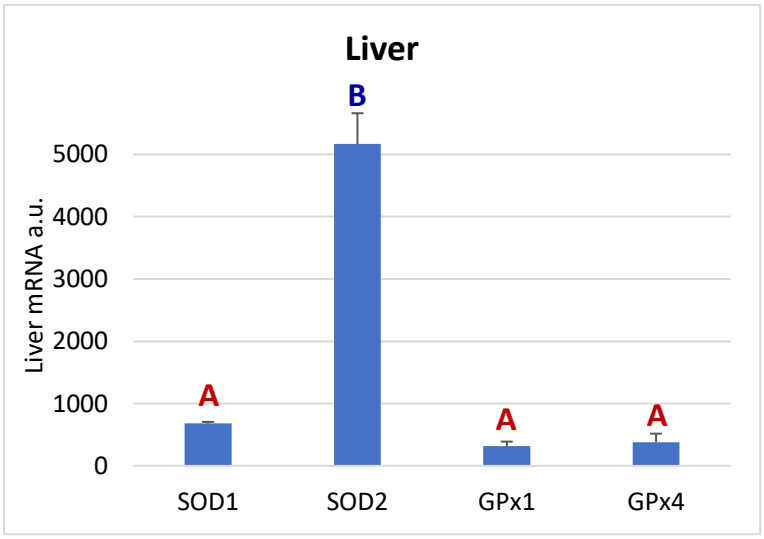
Figure 4.1: mRNA expression level (in arbitrary units: a.u.) of A) sod1, B) sod2, C) gpx1, D) gpx4, in 4 organs of T. borchgrevinki (means of five specimens with respective standard deviations). Different letters over the histograms indicate significant statistical differences (p <0.05) among means.

4.1.2. Difference between antioxidant enzymes

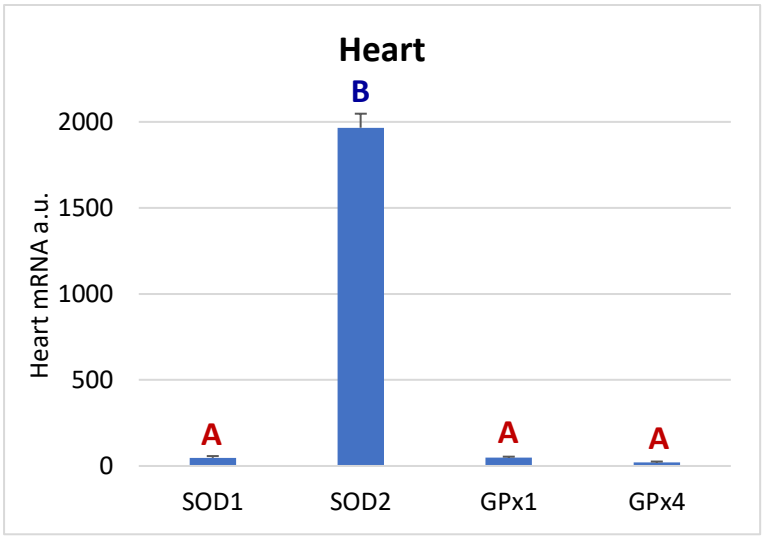
The Figure 4.2 shows the differences among the various enzymes related to a single examined organ. The most expressed gene in liver (Figure 4.2A) and heart (Figure 4.2B) is that coding the mitochondrial SOD2 enzyme, with a statistically significant difference ($p < 0.05$) compared to all other enzymes. The *sod2* mRNA levels is at least 7-time higher than other enzymes in the liver (Figure 4.2A) and at least 41-time higher in the heart (Figure 4.2B).

Different is the situation in white and red muscles (Figure 4.2), in which the most expressed isoform is the cytoplasmic SOD1 enzyme, always with a statistically significant difference ($p < 0.05$) compared to all other enzymes. The *sod1* mRNA levels is at least 5-time higher than other enzymes in the liver (Figure 4.2A) and at least 2-time higher in the heart (Figure 4.2B).

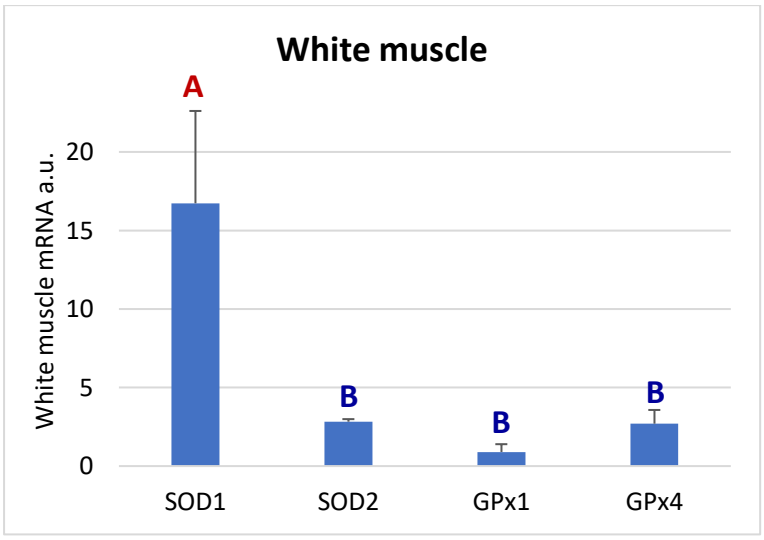
A)


Liver
Liver mRNA a.u.
5000
4000
3000
2000
1000
0
A
B
A
A
SOD1
SOD2
GPx1
GPx4

B)


Heart
Heart mRNA a.u.
2000
1500
1000
500
0
A
B
A
A
SOD1
SOD2
GPx1
GPx4

C)


White muscle
White muscle mRNA a.u.
20
15
10
5
0
A
B
B
B
SOD1
SOD2
GPx1
GPx4

D)

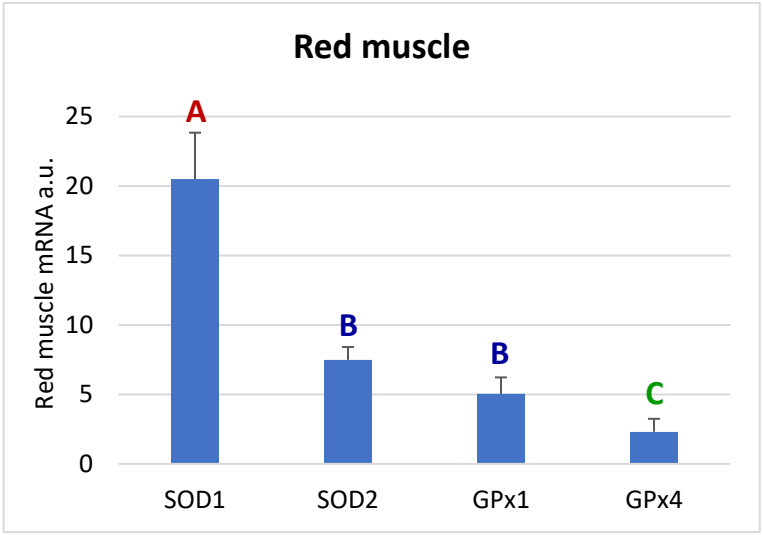


Figure 4.2: mRNA expression level (in arbitrary units: a.u.) in A) liver, B) heart, C) white muscle, D) red muscle, for 4 antioxidant enzymes of T. borchgrevinki (means of five specimens with respective standard deviations). Different letters over the histograms indicate significant statistical differences (p <0.05) among means.

4.2. mRNA EXPRESSION in *Trematomus newnesi*

In *T. newnesi*, the aim was to analyse some antioxidant responses against the exposure to PFOA. The mRNA levels were evaluated in fish exposed to 1.5 µg/l of PFOA for 10 days and compared to unexposed specimens. The considered organs were liver and kidney, because of their physiological role in xenobiotics' accumulation and excretion.

We considered two comparisons:

– between organs, referring to the same enzyme and the same experimental group
– between treatments, considering the same antioxidant enzyme

4.2.1. Difference between organs

Regarding the control group, the first thing to note is that the kidney always exhibits a higher level of expression with respect to the liver, for each antioxidant enzyme (Figure 4.3). These differences are always statistically significant ($p < 0.05$) and the renal mRNA levels are about 3-time higher than liver for SOD1 enzyme (Figure 4.3A), about 17-time higher for SOD2 (Figure 4.3B), about 7-times for GPx1 (Figure 4.3C), and about 2-times for GPx4 (Figure 4.3D).

The same situation also occurs in the specimens exposed to PFOA, but only for SOD2 (Figure 4.3B) and GPx1 (Figure 4.3C), with renal mRNA levels that are

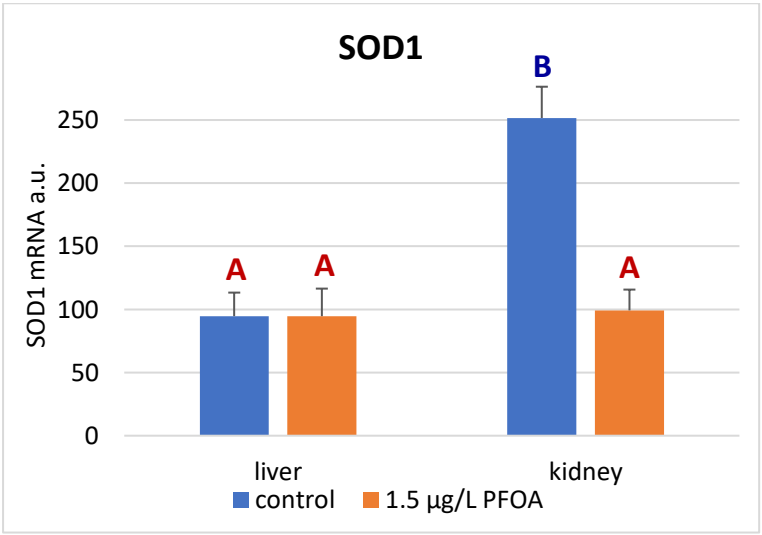
about 2-time higher than liver, but not for the other enzymes. In fact, for SOD1 enzyme the difference between the two organs is not statistically significant (Figure 4.3A), while for GPx4 there is a statistically greater accumulation of mRNA in the liver rather than in the kidney (about 6-time higher; $p < 0.05$).

4.2.2. Difference between treatments

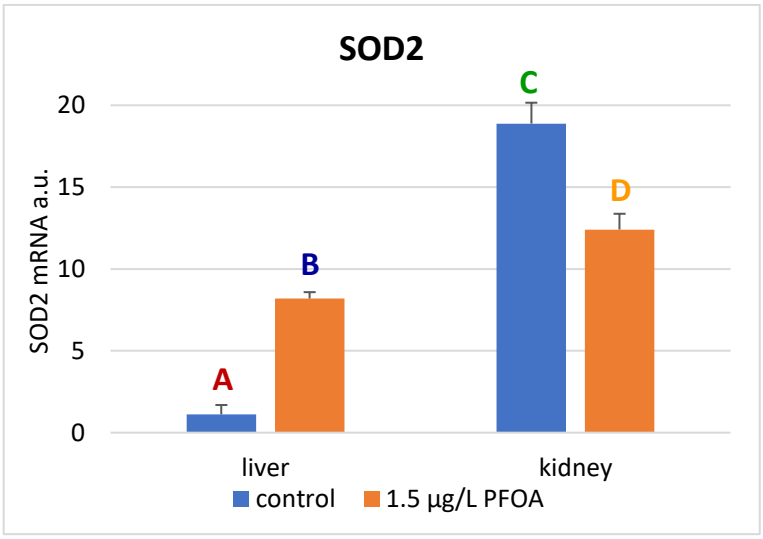
In the liver, the treatment with PFOA induces a statistically significant ($p < 0.05$) increase of gene expression for all considered enzymes (Figure 4.3), with the only exception of SOD1 (Figure 4.3A), whom mRNA levels remain unchanged after the treatment. In particular, the mRNA levels for SOD2 are about 12-time higher in specimens exposed to PFOA (Figure 4.3B), about 5-times for GPx1 (Figure 4.3C), and about 4-times for GPx4 (Figure 4.3D).

In the kidney, the exposure to PFOA leads a general statistically significant ($p < 0.05$) decrease of mRNA accumulation (Figure 4.3), with the only exception of GPx1, whom mRNA levels remain unchanged after the treatment (Figure 4.3C). In particular, the mRNA levels for SOD1 enzyme are about 60% lower in specimens exposed to PFOA (Figure 4.3A), about 34% lower for SOD2 (Figure 4.3B), and about 63% lower for GPx4 (Figure 4.3D).

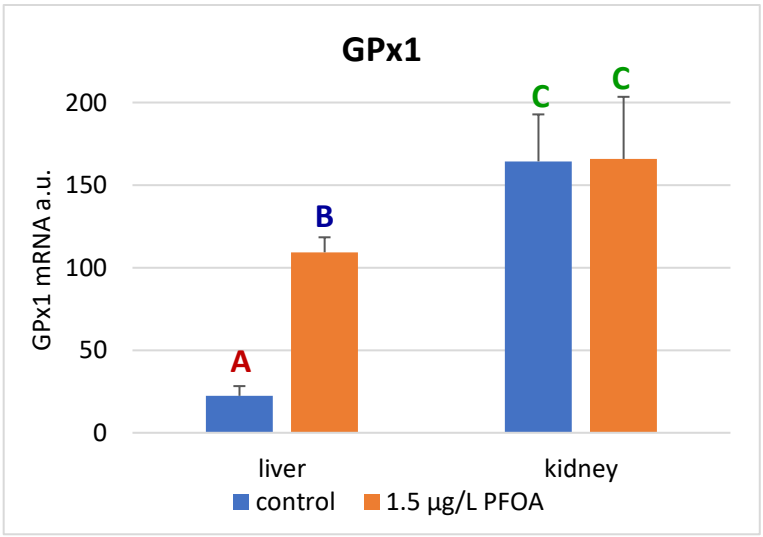
A)



B)



C)



D)

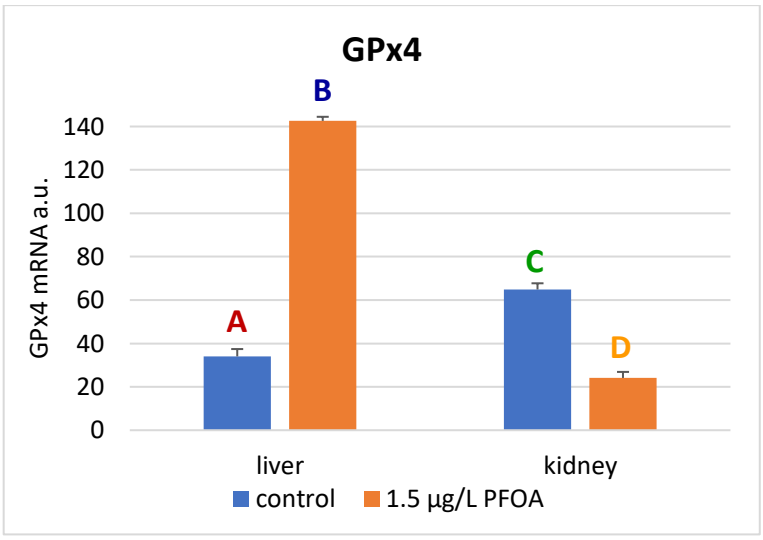


Figure 4.3: mRNA expression level (in arbitrary units: a.u.) of A) sod1, B) sod2, C) gpx1, D) gpx4, in liver and kidney of T. newnesi (means of five specimens with respective standard deviations), exposed to PFOA and unexposed (the control group).
Different letters over the histograms indicate significant statistical differences ($p < 0.05$) among means.

5. DISCUSSION

The antioxidant enzymes I considered (SOD and GPx), had already been previously characterized in our laboratory, both from a structural and functional point of view, in some species of Antarctic fish, such as *T. bernacchii* **(Santovito et al., 2006; Maschietto, 2008; Sattin et al., 2015)** and *T. eulepidotus* **(Sattin et al., 2008; Sattin et al., 2015).**

Although several studies have focused on the analysis of antioxidant enzymes activity in the Antarctic fish, the gene expression results presented here are among the first that consider the mRNA accumulation on these two species.

In fact, a publication presenting data on the relative expression levels of mRNA can be found only for *T. borchgrevinki* **(Carney et al., 2015)** that measures the relative expression levels of mRNA in the liver, and for *T. newnesi,* to our knowledge, there are no previous studies.

With regards to our evaluation of antioxidant expression levels in various organs of *T. borchgrevinki,* what emerges from the obtained results is that the liver is the organ that most accumulate the transcribed mRNA from the genes coding the considered antioxidant enzymes (SOD1, SOD2, GPx1, GPx4).

This was expected, as it is known from previous studies **(Ku et al., 2021)** that it is an organ which presents high metabolic activity, since it plays an important role in the maintenance of cellular homeostasis. The high metabolism of liver leads to the production of ROS, which presence is maintained under control by the remarkable ability to express antioxidant **(Ploch et al., 1999).** However, it should be noted that the conspicuous ROS production that characterizes the liver of Antarctic teleosts is not so much in relation to its digestive function, which is very limited and discontinuous due to the sporadic feeding of these fish, but more to its detoxifying function. In fact, the liver has a tendency to bioaccumulate xenobiotics, especially heavy metals such as Cd and Cu, which are present in relatively high concentrations in the Antarctic seabed as a natural condition **(Westerlund and Öhman, 1991; Bargagli et al., 1996)**.

In second place in terms of gene expression, for all the considered antioxidant enzymes, there is the heart, which statistically significantly differs from the skeletal muscles. The heart is also a muscle, so, in organisms from temperate latitudes, we

should find a high metabolism in both cardiac and skeletal muscles, but for Antarctic fish the situation is different. In fact, these fish have very low locomotor activity, due to very low polar temperatures and the need to save energy **(DeVries, 1971).** On the contrary, the energy consumption for the contraction of the cardiac tissue of the Antarctic teleosts is consistent, also in relation to the larger size of this organ compared to other fish. This also implies a high metabolism, high oxygen consumption and a greater probability of ROS formation, with consequent risk of oxidative stress, that leads to the need to implement antioxidant defences, with respect to less metabolically active organs **(Knox, 1994).** The high heart activity of Antarctic fish is also a way to avoid the risk of blood freezing. The maintaining of a sufficiently high blood flow (together with the action of anti-freeze proteins), reduces the probability of ice crystal formation **(DeVries and Wohlschlag, 1969).**

Finally, regarding the skeletal muscles, they are regularly the organs where gene expression is lower. This result was also expected, given the limited locomotor activity of these animals and, therefore, the low metabolism of skeletal muscle **(Knox, 1994).** In fact, at the mitochondrial level the formation of ROS derives from oxidative metabolism in order to produce ATP, but this only serves the muscles that are metabolically active, therefore not those of the Antarctic species. In fact, living in the cold, they have a very slow locomotion, they do not use the muscles so much and therefore do not have to produce large amounts of energy in this tissue.
There is also a slight tendency (although not statistically supported) to greater expression of antioxidant enzymes in the red muscle rather than in the white one. Even this derives from the different type of metabolism and so the different role which the two types of tissue play.

The red one is an oxidative muscle, which is located at the level of the pectoral fins and is active for most of the ordinary swimming of the animal, with a slow contraction **(Meyer-Rochow and Devine, 1986).**
In fact, the pectoral fins perform a specific function in the Antarctic teleost *T. borchgrevinki*, i.e. they allow the fish to detach itself from the colder substrate: for the benthics the bottom (and sometimes the anchor ice), for the pelagics the ice shelf. *T. borchgrevinki* is a pelagic fish, but it relates to the pack ice as if it were the bottom of the sea. If the animal's body came into contact with the ice, the probability

of formation of ice crystals in their body fluids, with consequent problems, would increase **(Eastman and DeVries, 1985).** Therefore, the pectoral adductor muscles, which are so important in aerobic movement, are well vascularized, contain high densities of mitochondria and have abundant lipid reserves, used as major source of energy for endurance efforts **(Davison et al., 1988).** Consequently, this type of muscle, being widely used because of the particular swim of these fish (labriform mode), has a greater consumption of O_2 and, so, a greater rate of ROS production.

On the other hand, the white one is an anaerobic muscle, which is collocated in the fish trunk, where it is held in reserve to contracts rapidly for fast starts and sprints, for example during escape or prey capture **(Meyer-Rochow and Devine, 1986).** In these moments, it converts glycogen to lactic acid to provide the necessary energy for a sharp and sudden effort.
In *T. borchgrevinki,* this muscle is poorly vascularised, with few mitochondria and extremely large fibres, making diffusion distances extremely long, so it has a poor blood supply. For this reason, white muscles only contribute to swimming thrust at the highest swimming speeds, and once recruited, are rapidly fatigued, so they are only occasionally used by Antarctic fish and this results in a minor ROS expression **(Davison et al., 1988).**

Within the same species (*T. borchgrevinki*), the obtained results allow to analyse also the comparison between the different levels of gene expression of the various types of antioxidant enzymes within each organ.

In heart and liver, the antioxidant enzyme which demonstrates the highest gene expression is SOD2. Since these organs have a higher metabolism than skeletal muscles, they contain a higher mitochondrial density, consequently a greater probability of ROS formation and the need of a proportional higher expression of mitochondrial antioxidant enzymes, such as SOD2. However, GPx4 is also a mitochondrial enzyme and then a high expression level is expected. Our results said that it is not the case. Mitochondria are the most important subcellular sites for the primary superoxide anion ($\bullet O_2^-$) production, with subsequent generation of hydrogen peroxide (H_2O_2) by the catalytic action of the mitochondrial SOD2 and the eventual production of significant amounts of the highly toxic hydroxyl radical (HO•) **(Wilhelm Filho, 2007).** Therefore, in presence of high-level expression of

SOD2 and GPx4, one possibility is that other enzymes can act as scavengers of H_2O_2, such as Prdx3 and/or Prdx5. A previous study on *T. bernacchii* demonstrated that these enzymes are expressed also in Antarctic fish and are involved in stress responses **(Tolomeo et al., 2019).** This also support the idea of an integrated system for the cell protection against the risk of oxidative stress **(Cassini et al., 1993).**

As already mentioned, the liver is one of the most metabolically engaged organs, because it works a lot to produce all the blood proteins, in addition to the fact that its tendency to accumulate xenobiotics can lead to an increase in the rate of ROS formation. However, these results suggest that the main reason for the high accumulation of mRNA in the liver is not its tendency to accumulate xenobiotics, since, if this were the case, we would have an equal increase in both the mitochondrial and cytoplasmic levels of antioxidant enzymes. Instead, the more consistent increase in the expression of the specific mitochondrial enzyme SOD2 in the liver suggests that the main reason is more plausibly the high metabolism of this organ compared to the others.

Regarding the skeletal muscles, they are the organs where a high expression of SOD1, which is a cytoplasmic protein, occurs. For the reasons said before, an expected result would be an equally high expression of other enzymes that eliminate H_2O_2 at the level of the cytoplasm. Not GPx1 that is low-level expressed, but probably some isoforms of peroxiredoxins, such as Prdx1, 2, 4, 6 **(Rhee et al., 2012)**.

As far as *T. borchgrevinki* is concerned, these are the first ever results relating to the expression of antioxidant genes in heart and skeletal muscles on this species.
Therefore, although they do not give us information on how fish relate to pollutants, they constitute a very important starting point for future studies, for example by exposing the animal to various pollutants (as we have already done on *T. newnesi*). To carry out this in-depth work, our results allow, for example, to deduce information about which organ is best as bioindicator. Indeed, the fact that the liver has been shown to have a very high antioxidant expression, much more than other organs, suggests that there may be some kind of regulation at the level of this organ.

As regards *T. newnesi,* the results are very interesting since they are the first obtained in an Antarctic fish experimentally exposed to PFAS.
First, in the untreated specimens SOD1 is the most expressed antioxidant enzyme both in the liver and in the kidney. This result may correlate with a high superoxide radical formation at the mitochondrial level, since SOD is the specific scavenger of this ROS **(Lushchak and Bagnyukova, 2006).**
On the other hand, in those exposed to PFOA we have a greater accumulation of mRNA coding for the two isoforms of glutathione peroxidase: GPx4 in the liver and GPx1 in the kidney. The obtained results are compatible with a possible increase in the formation of hydrogen peroxide at the mitochondrial level, as GPx have a specific scavenging action against this ROS **(Du et al., 2017).**

PFAS are suspected carcinogens and the fact that they generate oxidative stress can be a potential action mode **(Hu and Hu, 2009).** Previous studies, in fact, stated that the observed changes in activities of antioxidant enzymes indicated that PFAS exposure may disrupt the balance of the antioxidant system, boosting the generation of ROS, which impact the mitochondria leading to cascade reactions that end in the amplification of cell apoptosis **(Choi et al., 2017; Suh et al., 2017; Bonato et al., 2020).**
In accordance with these studies, the findings of this research show that, after the acute exposure to 1,5 µg/l of PFOA for 10 days, all antioxidant enzymes increase their expression levels in the liver (except SOD1, which shows no statistically significant difference). This consistent increase in the liver occurs due to the detoxifying function of this organ, already described in the previous paragraph in response to the presence in natural conditions of heavy metals in Antarctic waters. However, the liver further increases its detoxifying effect if organisms are exposed to a pollutant such as PFOA, because it is the first site where PFAS accumulates. Literature data show that the liver increases the concentration of pollutant in its own tissues, more than in other organs, as the environmental concentration of PFOA increases **(Piva et al., 2022).**

Furthermore, in the liver, the enzyme that increases the most its gene expression following exposure to PFOA is GPx4, followed by GPx1 and then SOD2, whereas SOD1 remains unaltered. Such a response confirms that this condition of PFOA

exposure may involve mitochondria **(Piva et al., 2022)** and that GPx4, as a mitochondrial-acting protein, plays an important role in the elimination of excess ROS, which are probably increasing in treated organisms.

On the contrary, the kidney is not responsive to PFOA exposure and the level of mRNA accumulation coding for all antioxidant enzymes (except for GPx1, where it remains the same) even tends to decrease in treated samples.
This organ also plays a detoxifying role and, so, it could also be involved in bioaccumulation phenomena (such as the liver), but our data show the opposite. This is probably because, in the short period of exposure (10 days), it is possible that the kidney would not be involved in the bioaccumulation of PFAS. This hypothesis could be verified in the future by evaluating the actual presence and measuring the concentration of these pollutants in this tissue.

However, not being involved in an accumulation, one would expect a non-variation, in reality we have a decrease. In fact, in the kidney, the enzyme that decreases the most its gene expression following exposure to PFOA is SOD1, followed by GPx4 and then SOD2, whereas GPx1 remains unaltered. The cell decreases the messenger biosynthesis for a given protein mainly on two occasions. The first occurs when the protein is not needed by the organism. The second occurs when the cell must save energy, which can instead be dedicated to other sites in the body, in particular in the liver, where it must instead counteract a high oxidative risk, especially through the action of GPx.

It is very probable that both these factors contribute to the result. In fact, the kidney is a target organ of xenobiotics, as it is involved in its excretion and it has been hypothesized that PFAS may damage kidneys because of its reabsorption across the renal tubules. This is hypothesized to occur due to renal tubule efflux transporters that actively transport PFAS back into systemic circulation, contributing to their long half lives in the organism **(Bonato et al., 2020).** However, in our experiment the exposure to PFOA is acute and not chronic, so we should expect a renal response in more than 10 days.

6. CONCLUSIONS

In this work, we analyzed the gene expression of various antioxidant enzymes (SOD1, SOD2, GPx1, GPx4) in the Antarctic fishes *Trematomus borchgrevinki* and *Trematomus newnesi*. Analyses were performed in liver, heart, white and red muscle and kidney, using RT-PCR, and the expression levels were related to the physiological role of these organs and tissues.

The collected data on gene expression show how the two analysed species are able to express molecular responses against the excessive formation of ROS, produced in a more pronounced way in natural conditions in the Antarctic marine environment. The results also provide information on the physiological mechanisms in response to an accumulation of PFAS (in particular PFOA) and on the tissues most involved in the detoxification of these chemical elements in Antarctic fish, leaving however the doors open to future studies on other tissues and proteins. In fact, within the antioxidant defence system, superoxide dismutase and glutathione peroxidase play a fundamental role in the detoxification of $\bullet O_2^-$ and H_2O_2 respectively, both in normal conditions and under stress, but it is only part of the complex system of defence against ROS. Therefore, to have a clearer picture, in the future, it is necessary to evaluate the played role also by other antioxidant components, both enzymatic and non-enzymatic.

The data presented in this study can improve the general knowledge about the molecular and functional evolution of Antarctic fishes, as they could be an important contribution to predict the physiological responses of these organisms to environmental changes.

Moreover, our expression analysis may be used as a starting point for using antioxidant enzymes as biomarkers both for oxidative stress and exposure to PFOA. Hence the possibility of using notothenioids as bioindicator organisms in future biomonitoring campaign of marine Antarctic environment, which will be increasingly necessary to assess the anthropic impact on the Antarctic ecosystem, unique but also extremely fragile.

As for the future prospects, first of all, it emerged the need to integrate the cellular analysis performed within this work with biochemical assays, in order to correlate the physiological response of the cell with quantifications of cellular processes: for example, the measurement of the quantity of ROS present in the tissue or also the enzymatic activity.

In particular, the quantification of enzymatic activity would be interesting since, from studies on other species, it is known that organisms living in naturally unfavourable conditions, but in the absence of acute stress, have developed post-transcriptional control of the gene expression of antioxidant enzymes **(Lavut and Raveh, 2012).** In fact, in the case in which high levels of mRNA are present without a corresponding presence of active protein, it can be deduced that a part of the transcript is not immediately translated.
The hypothesis is that it is stored in intracellular compartments such as P-bodies or stress granules **(Olszewska et al., 2012),** in which the messengers may undergo degradation or future translation, respectively **(Lavut and Raveh, 2012).** Stress granules are dense membrane-free cytoplasmic aggregations, composed of messenger ribonucleoproteins, translation initiation factors and mRNA binding proteins, which act by blocking the translation of messengers and increasing their stability **(Anderson and Kedersha, 2009).** This condition allows an extremely rapid response by the tissues to the sudden occurrence of acute stress, in this specific case with a greater biosynthesis of the specific antioxidant enzyme in response to a sudden increase in the rate of formation of the corresponding ROS.
This hypothesis has been deepened in recent studies on the nucleation proteins of stress granules also in Antarctic fish **(Nicorelli et al., 2018)**, in which this situation occurs in particular in organs such as liver and muscle, which can undergo stress also in very short time and therefore require a fast defensive action made explicit by antioxidant agents.
Therefore, both the quantification of the accumulation of the messenger and the expression of the active protein is interesting for our purposes and the latter could be performed using the Western blot technique.

Furthermore, the detection of variations in biochemical responses will provide a useful tool for the early assessment of environmental disturbance, which could

subsequently be correlated with the quantities of pollutants present in Antarctica, in order to evaluate the goodness of the physiological parameter of the specific organism as a biomarker of environmental stress.

Therefore, the obtained results constitute an important starting point in order to use the expression of antioxidant enzymes as biomarkers, both of oxidative stress and of exposure to PFAS, using notothenioids as bioindicator organisms in biomonitoring campaigns of the marine Antarctic environment. For these purposes, in the future it turns out to be essential to increase the types of biomarkers that should be investigated, such as the expression levels of several antioxidant enzymes, besides SOD and GPx.

Finally, as regards practical laboratory activities that could be performed to continue the study, an interspecific comparison between the levels of gene expression of the same antioxidant enzyme within the same organ of *T. borchgrevinki* and *T. newnesi* would be useful. In fact, as regards the analysis I carried out, the liver would be the only organ analysed in both species. However, the gene expression values of *T. borchgrevinki* and *T. newnesi* cannot be compared, since the measured values are not absolute, but relative, that is, we refer to analysis performed at different times, therefore, in different RT plates, containing different housekeeping on which to normalize the data. This condition opens up new opportunities to continue the study in this area, as further analysis could be performed by inserting samples of the same organ (not only liver, but also other common organs) of *T. borchgrevinki* and *T. newnesi* into the same RT plate.

In the future it will also be appropriate to expand research on the production of ROS by cells and on the detoxifying role performed by antioxidant enzymes both in physiological and non-physiological conditions, such as exposure to environmental contaminants of anthropogenic origin capable of carry out a pro-oxidant action, such as for example PFAS. Studies of this type are the object of the attention of the scientific community and continue to be carried out in our laboratories as well.

7. REFERENCES

A. Langberg H., E. Hale S., D. Breedveld G., M. Jenssen B., Jartun M. (2022). A review of PFAS fingerprints in fish from Norwegian freshwater bodies subject to different source inputs. Environmental Science: Processes & Impacts, 24(2), 330–342.

Acworth I. N., McCabe D. R., Maher T. J. (1997). The Analysis of Free Radicals, Their Reaction Products, and Antioxidants. In Oxidants, Antioxidants, and Free Radicals. CRC Press.

Albertelli G., Chiantore M. (1996). I popolamenti bentonici litorali. Collana *In Antartide., PNRA,* Museo Nazionale dell'Antartide Felice Ippolito. Istituto di Scienze Ambientali Marine Università degli Studi di Genova.

Anderson P., Kedersha N. (2009). Stress granules. Current Biology: CB, 19(10), R397-398.

Andriashev A.P., Jakubowski M. (1971). Morphological grounds for generic separation of the Antarctic broadhead-fishes (Trematomus borchgrevinki Boulenger and T. brachysoma Pappenheim) and a new status of the genus Pagothenia Nichols et LaMonte (Nototheniidae). *Zool Zh 50*:1041–1055.

Aruoma O. I. (1998). Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of the American Oil Chemists' Society, 75(2), 199–212.

Azzolini R. et al. (2006). 1985 – 2005, Venti anni di ricerche italiane in Antartide. PNRA.

Bargagli R., Nelli L., Ancora S., Focardi S. (1996). Elevated cadmium accumulation in marine organisms from Terra Nova Bay (Antarctica). Polar Biology, 16(7), 513–520.

Battaglia B. et al. (1996). L'evoluzione biologica. Collana *In Antartide., PNRA,* Museo Nazionale dell'Antartide Felice Ippolito. Dipartimento di Biologia - Università degli Studi di Padova.

Bayr H. (2005). Reactive oxygen species. Critical Care Medicine, 33(12), S498.

Bilyk K. T., Cheng C.-H. (2013). Model of gene expression in extreme cold—Reference transcriptome for the high-Antarctic cryopelagic notothenioid fish Pagothenia borchgrevinki. BMC Genomics, 14(1), 634.

Bonato M., Corrà F., Bellio M., Guidolin L., Tallandini L., Irato P., Santovito G. (2020). PFAS Environmental Pollution and Antioxidant Responses: An Overview of the Impact on Human Field. International Journal of Environmental Research and Public Health, 17(21), Art. 21.

Bonato M., Corrà F., Bellio M., Guidolin L., Tallandini L., Irato P., Santovito G. (2020). PFAS Environmental Pollution and Antioxidant Responses: An Overview of the Impact on Human Field. International Journal of Environmental Research and Public Health, 17(21), Art. 21.

Boulenger G. A. (1902). Pisces. Report on the collections of natural history made in the Antarctic regions during the voyage of the "Southern Cross". British Museum (Natural History), London, pp 174–189.

Brigelius-Flohé R., Maiorino M. (2013). Glutathione peroxidases. Biochimica et Biophysica Acta (BBA) - General Subjects, 1830(5), 3289–3303.

Buettner G. R., Ng C. F., Wang M., Rodgers V. G. J., Schafer F. Q. (2006). A new paradigm: Manganese superoxide dismutase influences the production of H2O2 in cells and thereby their biological state. Free Radical Biology & Medicine, 41(8), 1338–1350.

Bustin S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology, 25(2), 169–193.

Campos D. Y. F. (2007). Análise das respostas citogenotóxicas e histopatológicas do peixe Trematomus newnesi exposto à água do mar diante da Estação Antártica Brasileira Comandante Ferraz, Ilha Rei George, Antártica. Text, Universidade de São Paulo.

Carney Almroth B., Asker N., Wassmur B., Rosengren M., Jutfelt F., Gräns A., Sundell K., Axelsson M., Sturve J. (2015). Warmer water temperature results

in oxidative damage in an Antarctic fish, the bald notothen. Journal of Experimental Marine Biology and Ecology, 468, 130–137.

Casal P., González-Gaya B., Zhang Y., Reardon A. J. F., Martin J. W., Jiménez B., Dachs J. (2017). Accumulation of Perfluoroalkylated Substances in Oceanic Plankton. Environmental Science & Technology, 51(5), 2766–2775.

Cassini A., Favero M., Albergoni V. (1993). Comparative studies of antioxidant enzymes in red-blooded and white-blooded antarctic teleost fish. Pagothenia bernacchii and Chionodraco hamatus. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 106(2), 333–336.

Cattaneo-Vietti R. (1998). I ghiacci. Collana *In Antartide., PNRA,* Museo Nazionale dell'Antartide Felice Ippolito.

Cerro-Gálvez E., Roscales J. L., Jiménez B., Sala M. M., Dachs J., Vila-Costa M. (2020). Microbial responses to perfluoroalkyl substances and perfluorooctanesulfonate (PFOS) desulfurization in the Antarctic marine environment. Elsevier Enhanced Reader. Water Research, Volume 171.

Choi E. M., Suh K. S., Rhee S. Y., Oh S., Woo J.-T., Kim S. W., Kim Y. S., Pak Y. K., Chon S. (2017). Perfluorooctanoic acid induces mitochondrial dysfunction in MC3T3-E1 osteoblast cells. Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering, 52(3), 281–289.

Chowdhury S., Saikia S. K. (2020). Oxidative Stress in Fish: A Review. Journal of Scientific Research, 12(1), 145–160. https://doi.org/10.3329/jsr.v12i1.41716.

Clarke A., Johnston I.A. (1996). Evolution and adaptive radiation of Antarctic fishes. Trends in Ecology & Evolution, Volume 11(5), 212-218.

Colacino M. (1996). Il Clima. Collana *In Antartide., PNRA,* Museo Nazionale dell'Antartide Felice Ippolito.

Dale J. W., Schantz M. von, Plant N., Ginelli E. (2013). Dai geni ai genomi (3° edizione). Edises.

Davison W., Forster M. E., Franklin C. E., Taylor H. H. (1988). Recovery from exhausting exercise in an Antarctic fish, Pagothenia borchgrevinki. Polar Biology, 8(3), 167–171.

Demple B. (1999). Radical Ideas: Genetic Responses to Oxidative Stress. Clinical and Experimental Pharmacology and Physiology, 26(1), 64–68.

Derveaux S., Vandesompele J., Hellemans J. (2010). How to do successful gene expression analysis using real-time PCR. Methods, 50(4), 227–230.

Desjardins P., Hansen J. B., Allen M. (2009). Microvolume Protein Concentration Determination Using the NanoDrop 2000c Spectrophotometer. Journal of Visualized Experiments, 33, 1610.

Dettai A., Berkani M., Lautredou A.-C., Couloux A., Lecointre G., Ozouf-Costaz C., Gallut C. (2012). Tracking the elusive monophyly of nototheniid fishes (Teleostei) with multiple mitochondrial and nuclear markers. Marine Genomics, 8, 49–58.

DeVries A. L. (1971). Glycoproteins as biological antifreeze agents in antarctic fishes. Science (New York, N.Y.), 172(3988), 1152–1155.

DeVries A. L., Wohlschlag D. E. (1969). Freezing Resistance in Some Antarctic Fishes. Science, 163(3871), 1073–1075.

Di Giulio R.T. (1991). Indices of oxidative stress as biomarkers for environmental contamination. In: Mayes MA, Barron MG (eds) Aquatic toxicology and risk assessment: vol 14 ASTM STP 1124. American Society for Testing and Materials, Philadelphia, PA, pp 15—3.

Di Prisco G. (1998). I pesci - evoluzione e adattamento alle basse temperature. Collana *In Antartide., PNRA,* Museo Nazionale dell'Antartide Felice Ippolito. Istituto di Biochimica delle Proteine ed Enzimologia – CNR.

Du J., Cai J., Wang S., You H. (2017). Oxidative stress and apotosis to zebrafish (Danio rerio) embryos exposed to perfluorooctane sulfonate (PFOS) and ZnO

nanoparticles. International Journal of Occupational Medicine and Environmental Health, 30(2), 213–229.

Eastman J. T. (2000). Antarctic notothenioid fishes as subjects for research in evolutionary biology. Antarctic Science, 12(3), 276–287.

Eastman J. T., DeVries A. L. (1985). Adaptations for cryopelagic life in the antarctic notothenioid fish Pagothenia borchgrevinki. Polar Biology, 4(1), 45–52.

Eastman J. T., DeVries A. L. (1985). Adaptations for cryopelagic life in the antarctic notothenioid fish Pagothenia borchgrevinki. Polar Biology, 4(1), 45–52.

Eastman J. T., DeVries A. L. (1985). Adaptations for cryopelagic life in the antarctic notothenioid fish Pagothenia borchgrevinki. Polar Biology, 4(1), 45–52.

Eastman J. T., Devries A. L. (1997). Biology and phenotypic plasticity of the Antarctic nototheniid fish Trematomus newnesi in McMurdo Sound. Antarctic Science, 9(1), 27–35.

Eastman J.T., DeVries A.L. (1987). I pesci dell'Antartide. Le Scienze, n°221, pag. 68-75.

Eastman J.T., Eakin R.R. (2000). An updated species list for notothenioid fish (Perciformes; Notothenioidei), with comments on Antarctic species.

Fedon L. (2019). Analisi di contaminanti organici in campioni di firn provenienti da una carota di ghiaccio prelevata a Dome C, plateau antartico: valutazione di un trend temporale di distribuzione di composti di origine antropogenica. Tesi di laurea, Corso di Laurea Magistrale in Scienze Ambientali, Università Ca' Foscari Venezia.

Ferreira M. F., Lo Nostro F. L., Fernández D. A., Genovese G. (2021). Endocrine disruption in the sub Antarctic fish Patagonotothen tessellata (Perciformes, Notothenidae) from Beagle Channel associated to anthropogenic impact. Marine Environmental Research, 171, 105478.

Fields L. G., DeVries A. L. (2015). Variation in blood serum antifreeze activity of Antarctic Trematomus fishes across habitat temperature and depth. Comparative

Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 185, 43–50.

Florindo F., Boschi E. (2009). Il collasso dell'Antartide. Editoriale Darwin, 32: 52-57.

Gao K., Miao X., Fu J., Chen Y., Li H., Pan W., Fu J., Zhang Q., Zhang A., Jiang G. (2020). Occurrence and trophic transfer of per- and polyfluoroalkyl substances in an Antarctic ecosystem. Environmental Pollution, 257, 113383.

Giangiacomo L., D'Avino R., di Prisco G., Chiancone E. (2001). Hemoglobin of the Antarctic Fishes Trematomus bernacchii and Trematomus newnesi: Structural Basis for the Increased Stability of the Liganded Tetramer Relative to Human Hemoglobin. Biochemistry, 40(10), 3062–3068.

Hu X.-Z., Hu D.-C. (2009). Effects of perfluorooctanoate and perfluorooctane sulfonate exposure on hepatoma Hep G2 cells. Archives of Toxicology, 83(9), 851–861.

Irianni A. et al. (2014). Italia in Antartide. ENEA UTA (Unità Tecnica Antartide), Centro Ricerche Casacci.

Jakubowski M. (1971). Morphological features of the lateral-line organs in members of the genus Notothenia Rich. and other genera of the family Nototheniidae (Pisces). J Ichthyol, 11, 493-499.

Kehrer J. P. (2000). The Haber–Weiss reaction and mechanisms of toxicity. Toxicology, 149(1), 43–50.

Knox G.A., (1994). The biology of the Southern Ocean. Cambridge University Press. Cambridge, UK.

Ku T., Zhou M., Hou Y., Xie Y., Li G., Sang N. (2021). Tebuconazole induces liver injury coupled with ROS-mediated hepatic metabolism disorder. Ecotoxicology and Environmental Safety, 220, 112309.

Kuhn K. L., Near T. J. (2009). Phylogeny of Trematomus (Notothenioidei: Nototheniidae) inferred from mitochondrial and nuclear gene sequences. Antarctic Science, 21(6), 565–570.

Kuhn K. L., Near T. J. (2009). Phylogeny of Trematomus (Notothenioidei: Nototheniidae) inferred from mitochondrial and nuclear gene sequences. Antarctic Science, 21(6), 565–570.

Lautrédou A.-C., Hinsinger D. D., Gallut C., Cheng C.-H. C., Berkani M., Ozouf-Costaz C., Cruaud C., Lecointre G., Dettai A. (2012). Phylogenetic footprints of an Antarctic radiation: The Trematominae (Notothenioidei, Teleostei). Molecular Phylogenetics and Evolution, 65(1), 87–101.

Lavut A., Raveh D. (2012). Sequestration of Highly Expressed mRNAs in Cytoplasmic Granules, P-Bodies, and Stress Granules Enhances Cell Viability. PLoS Genetics, 8(2), e1002527.

Liu C. (2014). Bioaccumulation and ecotoxicity study of perfluorinated chemicals. Nanyang Technological University.

Lovén J., Orlando D. A., Sigova A. A., Lin C. Y., Rahl P. B., Burge C. B., Levens D. L., Lee T. I., Young R. A. (2012). Revisiting Global Gene Expression Analysis. Cell, 151(3), 476–482.

Lushchak V. I., Bagnyukova T. V. (2006). Effects of different environmental oxygen levels on free radical processes in fish. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 144(3), 283–289.

Lushchak V. I., Bagnyukova T. V. (2006). Effects of different environmental oxygen levels on free radical processes in fish. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 144(3), 283–289.

Mahé K., Elleboode R., Loots C., Koubbi P. (2016). Growth of an Inshore Antarctic fish, Trematomus newnesi (Nototheniidae), off Adelie Land. Polar Science, 10(2), 167–172.

Margis R., Dunand C., Teixeira F. K., Margis-Pinheiro M. (2008). Glutathione peroxidase family – an evolutionary overview. The FEBS Journal, 275(15), 3959–3970.

Marrone A., La Russa D., Brunelli E., Santovito G., La Russa M. F., Barca D., Pellegrino D. (2021). Antarctic Fish as a Global Pollution Sensor: Metals Biomonitoring in a Twelve-Year Period. *Frontiers in Molecular Biosciences*, *8*, 794946.

Maschietto V, (2008). Caratterizzazione delle sequenze geniche della glutatione perossidasi e della superossido dismutasi in alcuni teleostei antartici. Tesi di Laurea, Laurea Magistrale in Biologia evoluzionistica, Università degli Studi di Padova.

Mazzarella L., D'Avino R., di Prisco G., Savino C., Vitagliano L., Moody P. C. E., Zagari A. (1999). Crystal structure of Trematomus newnesi haemoglobin re-opens the root effect question11Edited by K. Nagai. Journal of Molecular Biology, 287(5), 897–906.

Mccord J., Fridovich I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. The Journal of biological chemistry, 244, 6049–6055.

Meyer-Rochow V. B., Devine C. E. (1986). Ultrastructural observations and pH-measurements on red and white muscle from Antarctic fish. Polar Biology, 6(4), 241–246.

Meyer-Rochow V. B., Devine C. E. (1986). Ultrastructural observations and pH-measurements on red and white muscle from Antarctic fish. Polar Biology, 6(4), 241–246.

Near T. J., MacGuigan D. J., Parker E., Struthers C. D., Jones C. D., Dornburg A. (2018). Phylogenetic analysis of Antarctic notothenioids illuminates the utility of RADseq for resolving Cenozoic adaptive radiations. Molecular Phylogenetics and Evolution, 129, 268–279.

Near T. J., Pesavento J. J., Cheng C.-H. C. (2004). Phylogenetic investigations of Antarctic notothenioid fishes (Perciformes: Notothenioidei) using complete gene

sequences of the mitochondrial encoded 16S rRNA. Molecular Phylogenetics and Evolution, 32(3), 881–891.

Nichols J. T., La Monte F. R., Siple P., Sterrett J. M. (1936). Pagothenia, a new Antarctic fish. American Museum novitates; no. 839.

Nicorelli E., Gerdol M., Buonocore F., Pallavicini A., Scapigliati G., Guidolin L., Irato P., Corrà F., Santovito G. (2018). First evidence of T cell restricted intracellular antigen (TIA) protein gene expression in Antarctic fish.

Noori S. (2012). An Overview of Oxidative Stress and Antioxidant Defensive System. Journal of Clinical & Cellular Immunology, 01(08).

O'Brien K. M., Oldham C. A., Sarrimanolis J., Fish A., Castellini L., Vance J., Lekanof H., Crockett E. L. (2022). Warm acclimation alters antioxidant defences but not metabolic capacities in the Antarctic fish, Notothenia coriiceps. Conservation Physiology, 10(1), coac054.

Olech M., Kwiatek W. M., Dutkiewicz E. M. (1998). Lead pollution in the Antarctic region. X-Ray Spectrometry, 27(4), 232–235.

Olszewska M., Bujarski J. J., Kurpisz M. (2012). P-bodies and their functions during mRNA cell cycle: Mini-review. Cell Biochemistry and Function, 30(3), 177–182.

Perry J. J. P., Shin D. S., Getzoff E. D., Tainer J. A. (2010). The structural biochemistry of the superoxide dismutases. Biochimica et biophysica acta, 1804(2), 245–262.

Pfaffl M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research, 29(9), e45.

Phleger C. F., Nichols P. D., Erb E., Williams R. (1999). Lipids of the notothenioid fishes Trematomus spp. And Pagothenia borchgrevinki from East Antarctica. Polar Biology, 22(4), 241–247.

Piva E., Schumann S., Dotteschini S., Brocca G., Radaelli G., Marion A., Irato P., Bertotto D., Santovito G. (2022). Antioxidant Responses Induced by PFAS Exposure in Freshwater Fish in the Veneto Region. Antioxidants, 11(6), Art. 6.

Ploch S. A., Lee Y.-P., MacLean E., Di Giulio R. T. (1999). Oxidative stress in liver of brown bullhead and channel catfish following exposure to tert-butyl hydroperoxide. Aquatic Toxicology, 46(3), 231–240.

Poli A., Fabbri E., Agnisola C., Calamita G., Santovito G., Verri T. (2014). Fisiologia Animale. *EdiSES s.r.l.*, Napoli.

Poli A., Fabbri E., Agnisola C., Calamita G., Santovito G., Verri T. (2018). Fisiologia animale. *EdiSES*.

Pozzobon E. (2019). Antartide: Cooperazione o Competizione Evoluzione di un continente e nuove sfide. Tesi di laurea, Laurea Magistrale in Relazioni Internazionali Comparate, Università Ca' Foscari Venezia.

Regoli F., Principato G. B., Bertoli E., Nigro M., Orlando E. (1997). Biochemical characterization of the antioxidant system in the scallop Adamussium colbecki, a sentinel organism for monitoring the Antarctic environment. Polar Biology, 17(3), 251–258.

Ren Q., Sun R.-R., Zhao X.-F., Wang J.-X. (2009). A selenium-dependent glutathione peroxidase (Se-GPx) and two glutathione S-transferases (GSTs) from Chinese shrimp (Fenneropenaeus chinensis). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 149(4), 613–623.

Rhee S. G., Woo H. A., Kil I. S., Bae S. H. (2012). Peroxiredoxin Functions as a Peroxidase and a Regulator and Sensor of Local Peroxides. Journal of Biological Chemistry, 287(7), 4403–4410.

Santovito G., Cassini A., Piccinni E. (2006). Cu,Zn superoxide dismutase from Trematomus bernacchii: Functional conservation and erratic molecular evolution in Antarctic teleosts. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 143(4), 444–454.

Santovito G., Cassini A., Piccinni E. (2006). Cu,Zn superoxide dismutase from Trematomus bernacchii: Functional conservation and erratic molecular evolution in Antarctic teleosts. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 143(4), 444–454.

Santovito G., Piccinni E., Boldrin F., Irato P. (2012). Comparative study on metal homeostasis and detoxification in two Antarctic teleosts. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 155(4), 580–586.

Santovito G., Piccinni E., Cassini A., Irato P., Albergoni V. (2005). Antioxidant responses of the Mediterranean mussel, Mytilus galloprovincialis, to environmental variability of dissolved oxygen. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 140(3), 321–329.

Sattin G., Bakiu R., Tolomeo A. M., Carraro A., Coppola D., Ferro D., Patarnello T., Santovito G. (2015). Characterization and expression of a new cytoplasmic glutathione peroxidase 1 gene in the Antarctic fish Trematomus bernacchii. Hydrobiologia, 761(1), 363–372.

Sattin G., Bakiu R., Tolomeo A. M., Carraro A., Coppola D., Ferro D., Patarnello T., Santovito G. (2015). Characterization and expression of a new cytoplasmic glutathione peroxidase 1 gene in the Antarctic fish Trematomus bernacchii. Hydrobiologia, 761(1), 363–372.

Sattin G., Santovito G., Cassini A. (2008). Physiological antioxidant responses against high environmental oxygen concentration: Glutathione peroxidase from the Antarctic teleost Trematomus eulepidotus. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 151(1, Supplement), S27.

Storey K. B. (1996). Oxidative stress: Animal adaptations in nature. Brazilian Journal of Medical and Biological Research, 29(12), 1715–1733.

Suh K. S., Choi E. M., Kim Y. J., Hong S. M., Park S. Y., Rhee S. Y., Oh S., Kim S. W., Pak Y. K., Choe W., Chon S. (2017). Perfluorooctanoic acid induces

oxidative damage and mitochondrial dysfunction in pancreatic β-cells. Molecular Medicine Reports, 15(6), 3871–3878.

Teh S. J., Adams S. M., Hinton D. E. (1997). Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquatic Toxicology, 37(1), 51–70.

Thannickal V. J., Fanburg B. L. (2000). Reactive oxygen species in cell signaling. American Journal of Physiology-Lung Cellular and Molecular Physiology, 279(6), L1005–L1028.

Tolomeo A. M., Carraro A., Bakiu R., Toppo S., Garofalo F., Pellegrino D., Gerdol M., Ferro D., Place S. P., Santovito G. (2019). Molecular characterization of novel mitochondrial peroxiredoxins from the Antarctic emerald rockcod and their gene expression in response to environmental warming. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 225, 108580.

Turner J., Bindschadler R., Convey P., Di Prisco G., Fahrbach E., Gutt J., Hodgson D., Mayewski P. A., Summerhayes C., SCAR (2009). Antarctic Climate Change and the Environment.

Vacchi M. (1996). I pesci e la pesca. Collana *In Antartide., PNRA,* Museo Nazionale dell'Antartide Felice Ippolito. Istituto Centrale per la Ricerca Applicata al Mare – ICRAM.

Valko M., Leibfritz D., Moncol J., Cronin M. T. D., Mazur M., Telser J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology, 39(1), 44–84.

Wells R. M. G., Ashby M. D., Duncan S. J., Macdonald J. A. (1980). Comparative study of the erythrocytes and haemoglobins in nototheniid fishes from Antarctica. Journal of Fish Biology, 17(5), 517-527.

Westerlund S., Öhman P. (1991). Cadmium, copper, cobalt, nickel, lead, and zinc in the water column of the Weddell Sea, Antarctica. Geochimica et Cosmochimica Acta, 55(8), 2127–2146.

Wilhelm Filho D. (2007). Reactive oxygen species, antioxidants and fish mitochondria. Frontiers in Bioscience, 12, 1229–1237.

Willmer P., Stone G., Johnston I. (2003). Fisiologia ambientale degli animali. Editore: Zanichelli.

World Health Organization, & Safety, I. P. on C. (1993). Biomarkers and risk assessment: Concepts and principles. World Health Organization.

Wuerges J., Lee J.-W., Yim Y.-I., Yim H.-S., Kang S.-O., Carugo K. D. (2004). Crystal structure of nickel-containing superoxide dismutase reveals another type of active site. Proceedings of the National Academy of Sciences, 101(23), 8569–8574.

Zelko I. N., Mariani T. J., Folz R. J. (2002). Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radical Biology and Medicine, 33(3), 337–349.

Zitka O., Skalickova S., Gumulec J., Masarik M., Adam V., Hubalek J., Trnkova L., Kruseova J., Eckschlager T., Kizek R. (2012). Redox status expressed as GSH:GSSG ratio as a marker for oxidative stress in paediatric tumour patients. Oncology Letters, 4(6), 1247–1253.

Zuo L., Zhou T., Pannell B. K., Ziegler A. C., Best T. M. (2015). Biological and physiological role of reactive oxygen species – the good, the bad and the ugly. Acta Physiologica, 214(3), 329–348.

8. WEBSITE REFERENCES

Dewitt H.H., Heemstra P.C., Gon O. (1990). Nototheniidae. p. 279-331. In O. Gon and P.C. Heemstra (eds.) Fishes of the Southern Ocean. J.L.B. Smith Institute of Ichthyology, Grahamstown, South Africa. (Ref. 5179). https://www.fishbase.se/summary/trematomus-newnesi.

Environmental Protection Agency, EPA (2022). What are PFAS? https://www.epa.ie/our-services/monitoring--assessment/waste/chemicals/pfas/

Froese, R. and D. Pauly Editors, WoRMS Editorial Board (2022). World Register of Marine Species. https://www.marinespecies.org

Italia in Antartide XXXVII SPEDIZIONE 2021/2022. Il PNRA. https://www.italiantartide.it/il-prna/

OligoAnalyzer™ Tool, IDT Integrated DNA Technologies. https://eu.idtdna.com/calc/analyzer.

Primer3web, version 4.1.0. https://primer3.ut.ee/.

PROGRAMMA NAZIONALE DI RICERCHE IN ANTARTIDE, PNRA (2019). Chi siamo. Ricerca. Spedizioni. Stazione Mario Zucchelli. Antartide. Atmosfera e Clima. Cambiamento globale. Biodiversità e adattamento. https://www.pnra.aq/

Secretariat of the Stockholm Convention (2022). Listing of POPs in the Stockholm Convention. http://chm.pops.int/TheConvention/ThePOPs/AllPOPs/tabid/2509/Default.aspx