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Effects of mixture of Nanoparticles under different Salinity in the clams *Ruditapes philippinarum* and *Ruditapes decussatus*

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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General abstract

Few studies have been done to determine the toxicity of Nanoparticles (NPs) on marine ecosystems and its effect on the biota. NPs interaction with Salinity, a major environmental factor, needs particular attention. Organisms in the Venice lagoon, the sampling site of this study, are subject to a wide range of salinity, yet little is known about the combined effect of an environmental stressor and NPs. Thus, this work aimed to evaluate the toxicological effects of salinity/NPs interaction. The study was conducted in two species: *R. decussatus*, native clams from the lagoon, and *R. philippinarum*, a clam deliberately introduced that has supplanted the native species. The two clams were exposed for 7 days to nZnO, nTiO2 and fullerene C60 (1 μg/L), at three salinity levels (18, 28, 38), with tissue collections after 1, 3 and 7 days. Biomarkers were evaluated in hemolymph, gills and digestive gland; RNA/DNA ratio and proteins were measured in foot tissue. *R. decussatus* had a significant increase in micronuclei, nuclear abnormalities, CAT and SOD at higher salinity, whereas LDH and NRU showed significant increases at low salinity. GST showed PCC showed increases at high salinity. RNA/DNA ratio did not show significant differences and the proteins presented significant decreases in medium and high salinity. *R. philippinarum* showed significant differences in micronuclei and nuclear abnormalities at high salinity and LDH and NRU with differences at lower salinity. CAT showed different values at low and high; SOD showed differences in the higher salinities. GST, LPO and PCC levels showed differences in the lower salinities. RNA/DNA ratio and proteins presented no differences. The results support the premise that *R. decussatus* is less resistant than the invasive species. This work is among the first to try to determine if the combination salinity/NPs have a synergistic effect on aquatic organisms.

Key words: *Ruditapes decussatus, Ruditapes philippinarum*, salinity, nanoparticles mixture, biomarkers, RNA/DNA ratios

Resumo geral

Os impatos antropogénicos nos ecossistemas marinhos são cada vez maiores, entre eles destacam-se os associados á alteração na qualidade e quantidade das águas de drenagem continental. Ao nível da qualidade da água, as atividades industriais em crescimento, como as associadas á nanotecnologia, responsáveis pela criação e manipulação de materiais dentro da nanoescala, têm consequências para o biota que vive nestes meios. As propriedades físicas e químicas das nanopartículas (NPs) diferenciam-se substancialmente da forma bruta desse mesmo material, e permitem uma crescente aplicação em diversos setores industriais e comerciais como a medicina, farmácia, cosmética, produção de energia, eletrónica e ambiente. O crescente desenvolvimento desta indústria é tal que se espera uma produção de 58000 toneladas de NPs até ao ano 2020. Com este número exorbitante de produção é inevitável que muitas NPs cheguem ao ambiente e aos ecossistemas aquáticos, levando a uma interação com o biota. Devido às inúmeras fontes de NPs (fábricas, uso de produtos, processos de combustão, etc…) é ainda impossível determinar exatamente a quantidade de NPs que pode ser encontrada no ambiente. Para ultrapassar a falta de informação quantitativa das NPs estimações obtidas por modelação são usadas em estudos. Contudo é imperativo o desenvolvimento de métodos para contabilizar e caracterizar as NPs para uma melhor avaliação de risco do ambiente. Poucos estudos foram feitos ainda para averiguar a toxicidade destas NPs, pois o que torna as NPs tão desejadas, como as diferentes propriedades que têm quando são reduzidas à nanoescala, também as tornam imprevisíveis quando em contacto com o ambiente e o biota. Com uma grande concentração a entrar nos ecossistemas a informação do efeito da interação destas com o biota é ainda escasso. Por outro lado, a variação da quantidade de água doce que atinge estas zonas de transição, associado às atividades antropogénicas interfere também com as comunidades. A salinidade é um importante fator ambiental que caracteriza um corpo de água, tem efeitos nos processos fisiológicos, como sobrevivência, conteúdo de água dos tecidos e tem outros efeitos subletais. Na lagoa de Veneza, o local de amostragem deste estudo, a variação é grande. Esta variação ocorre durante o ano mas também dentro da lagoa a salinidade diverge entre áreas. Por esta razão os organismos aquáticos ficam sujeitos a vários níveis de salinidade. A lagoa está atualmente bastante poluída, com contaminações vindas das diversas áreas industriais. *Ruditapes decussatus*, amêijoa nativa da lagoa de Veneza, foi quase completamente substituída pela espécie *Ruditapes philippinarum*, que foi introduzida propositadamente numa altura em que a

produção de organismos nativos estava irregular. Agora *R. decussatus* ocupa espaços bastante restritos e com densidades mais baixas que a espécie invasora. Poucos estudos foram feitos para averiguar o efeito combinado de fatores de stress ambiental com as NPs. Com as alterações globais é necessário averiguar o potencial efeito que a diferença nos fatores ambientais terá nos organismos e subsequentemente no ecossistema. Deste modo, esta dissertação teve como objetivo avaliar os efeitos toxicológicos da combinação de diferentes níveis de salinidade com uma mistura de NPs. Este estudo foi realizado em duas espécies de amêijoa, *R. decussatus* e *R. philippinarum*, uma nativa e outra invasora, para determinar se algum efeito sinergético ocorre e se existe alguma diferença de sensibilidades entre elas. Para compreender se algum efeito combinado existe, as duas amêijoas foram expostas a concentrações ambientalmente relevantes (1µg/L) de nZnO, nTiO2 e fulereno C60, a três níveis de salinidade: 18, 28 e 38. As amêijoas foram mantidas em tanques de vidro de 35 L, alimentadas diariamente e com mudas de água e fornecimento de NPs diárias. Para cada nível de salinidade havia um controlo, ou seja sem fornecimento de NPs, e cada tanque teve uma réplica. A exposição durou 7 dias mas recolhas de tecidos foram feitas depois de 1, 3 e 7 dias de exposição. Foi utilizada uma bateria de biomarcadores na hemolinfa, brânquias e glândula digestiva das amêijoas, nomeadamente biomarcadores de stress oxidativo (enzimas antioxidantes superóxido dismutase, catalase e glutationa S-transferase, peroxidação lipídica e conteúdo de proteína carbonilada), de citotoxicidade (lactato desidrogenase e capacidade pinocitótica) e de genotoxicidade (micronúcleo e aberrações nucleares). Para determinar a condição fisiológica das amêijoas também o conteúdo de RNA, DNA e conteúdo de proteínas foi determinado, em tecido do pé. *R. decussatus* apresentou aumento significativo em micronúcleos e aberrações nuclear na maior salinidade. Já a enzima LDH e capacidade pinocitótica apresentaram aumentos significativos a baixa salinidade. Nos biomarcadores de stress oxidativo CAT e SOD apresentaram valores altos nas salinidades mais altas, enquanto GST apresentou valores não muito claros. Os níveis de peroxidação lipídica apresentaram diferenças na salinidade mais baixa e mais alta, e proteínas carboniladas com aumentos na salinidade alta. No rácio RNA/DNA *R. decussatus* apresentou valores mais baixos em salinidades mais altas, mas as diferenças não eram significativas. O conteúdo proteico apresentou decréscimos significativos nas salinidades média e alta. *R. philippinarum* apresentou aumentos significativos de micronúcleos e aberrações nucleares na salinidade alta, e citotoxicidade alta na salinidade mais baixa. As enzimas antioxidantes mostraram resultados muito diferentes entre enzimas e tecidos. CAT mostrou valores diferentes a salinidade baixa e alta, SOD apresentou diferenças nas salinidades mais altas e GST apresentou diferenças nas

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Palavras-chave: *Ruditapes decussatus*, *Ruditapes philippinarum*, salinidade, mistura de nanopartículas, biomarcadores, rácio RNA/DNA

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Table 3.2. Two-way ANOVA table for nuclear abnormalities frequency in haemocytes, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **43**

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Table 3.4. Two-way ANOVA table for pinocytotic activity of haemocytes, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **45**

Table 3.5. Two-way ANOVA table for catalase activity in gills tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **46**

Table 3.6. Two-way ANOVA table for catalase activity in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **47**

Table 3.7. Two-way ANOVA table for superoxide dismutase activity in gills tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values

show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **48**

Table 3.8. Two-way ANOVA table for superoxide dismutase activity in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **49**

Table 3.9. Two-way ANOVA table for glutathione S-transferase activity in gills tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **51**

Table 3.10. Two-way ANOVA table for glutathione S-transferase activity in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **52**

Table 3.11. Two-way ANOVA table for glutathione S-transferase activity in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **53**

Table 3.12. Two-way ANOVA table for TBARS levels in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **54**

Table 3.13. Two-way ANOVA table for protein carbonyl content in gills tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **55**

Table 3.14. Two-way ANOVA table for protein carbonyl content in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **56**

Table 3.15. Two-way ANOVA table for micronucleus frequency in haemocytes, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **58**

Table 3.16. Two-way ANOVA table for nuclear abnormalities frequency in haemocytes, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **59**

Table 3.17. Two-way ANOVA table for cytotoxicity activity in haemocytes, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **60**

Table 3.18. Two-way ANOVA table for neutral red uptake of haemocytes, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **61**

Table 3.19. Two-way ANOVA table for catalase activity in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **62**

Table 3.20. Two-way ANOVA table for catalase activity in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **63**

Table 3.21. Two-way ANOVA table for superoxide dismutase activity in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **65**

Table 3.22. Two-way ANOVA table for superoxide dismutase activity in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **66**

Table 3.23. Two-way ANOVA table for glutathione S-transferase activity in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **67**

Table 3.24. Two-way ANOVA table for glutathione S-transferase activity in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **68**

Table 3.25. Two-way ANOVA table for lipid peroxidation in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **69**

Table 3.26. Two-way ANOVA table for lipid peroxidation in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **70**

Table 3.27. Two-way ANOVA table for protein carbonyl content in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values

show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **72**

Table 3.28. Two-way ANOVA table for protein carbonyl content in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **73**

Table 3.29. ANOVA table for RNA/DNA ratios in foot tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **74**

Table 3.30. ANOVA table for protein content in foot tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square). **76**

List of abbreviations

BSA – Bovine Serum Albumin
CAT – Catalase
CDNB - 1-chloro-2,4-dinitrobenzene
DNA - Deoxyribonucleic acid
DNPH – Dinitrophenylhydrazide
DW – Dry weight
EDTA – Ethylenediaminetetraacetic acid
ENPs – Engineered nanoparticles
GSH – Glutathione
GST – Glutathione S-transferase
HCl – Hydrochloric acid
MDA – Malondialdehyde
MN – Micronucleus
NA – Nuclear Aberrations
nAg – Silver nanoparticle
nAu – Gold nanoparticle
nCdTe – Cadmium telluride nanoparticle
nCuO – Copper oxide nanoparticle
NPs – Nanoparticles
NR – Neutral Red
nTiO2 – Titanium dioxide nanoparticle
nZnO – Zinc oxide nanoparticle
LDH – Lactate dehydrogenase
LPO – Lipid peroxidation
OD – Optical density
PCC – Protein Carbonyl Content
RNA – Ribonucleic acid
ROS – Reactive oxygen species
SN – Supernatant
SOD – Superoxide dismutase
STB – Sarcosine Tris
TBARS – Thiobarbituric acid
TCA – Trichloroacetic acid

1 Introduction

1.1 Nanoparticles

Richard Feynman, a physicist, was one of the first scientists to think small, and when he gave a lecture in 1959, he opened the door to a new field and countless possibilities. Nanotechnology is the science responsible for the study, fabrication and manipulation of structures, devices, materials or particles in the size range from 1 to 100 nanometers (or contain at least one component in said range). This size range is known as the nanoscale.

Nanoparticles (NPs) exist since the very beginning of the Earth's history. For example, the formation of natural NPs was found in sediments at the Cretaceous-Tertiary boundary (Verma *et al*., 2002).

The formation of NPs can have both natural and anthropogenic sources. Volcanic eruptions and forests fires both contribute for the formations of atmospheric NPs, as a natural source (Farré *et al*., 2009). Aquatic colloids, the fine fraction of desert sand, oil fumes, and certain atmospheric dusts also represent NPs produced naturally (Ostiguy *et al*., 2006). Even some plants have the ability to synthesize NPs which are used to reduce the uptake of metals in polluted soils (Bernhardt *et al*., 2010).

Anthropogenic NPs are produced in human activities, such as welding, metal smelting, automobile exhaust, and industrial processes, whose effects have been studied over the years (Farré *et al*., 2009).

Engineered nanoparticles (ENPs) are nanoparticles intentionally created. In nanotechnology, the areas of application of ENPs are diverse. They comprehend: optical, magnetic, thermal, mechanical, electronic, energy, biomedical, environmental, surfaces, and personal care (Farré *et al*., 2009). Nanotechnology is a high growing science and with the evolution of the industry the demand for ENPs highly increased. It is reported that around 2004 the production of ENPs was about 2,000 tons and it is expected to rise up to 58,000 tons, between 2011 and 2020 (Maynard, 2006).

1.1.1 Properties

When a particle is reduced to the nanoscale the physical and chemical properties will differ substantially from larger forms of the same material. These different properties can include melting point, color, electrical conductivity, magnetic permeability, and chemical

reactivity, and may be the reason the material was engineered in the first place (Boverhof and David, 2010). The cause for these differences in properties can be due to the smaller size, unique shape or arrangement, or the increased surface area of the material (Boverhof and David, 2010). The small size brings higher surface area/volume ratio, allowing higher number of atoms at the NPs surface. With these properties in mind, nanotechnology has the possibility to engineer a new dimension of materials.

1.1.2 Classification

The ever growing of the nanotechnology area demands for constant innovation and a wide range of ENPs are being created, with different composition, shape and size. The task of classifying these materials is challenging, and so ENPs should be categorized into a series of classes and not just one big group (Ju-Nam and Lead, 2008).

There are several ways to classify ENPs but the most used is by chemical composition and properties. Five main groups can be formed, classifying by chemical composition: carbon based NPs, metal-containing NPs, quantum dots, zero-valent metals and dendrymers (Bhatt and Tripathi, 2011).

Other systems are also used in literature, like classifying NPs by dimension, morphology, composition, uniformity, and agglomeration (Buzea *et al*., 2007).

1.1.3 Reaching the environment

The exorbitant amount of ENPs produced will inevitable reach the soil and waterways, at considerable quantities, leading to a contamination of the environment. Even though organisms have been dealing with natural NPs since the beginning of life, it is not known how they will handle high concentrations of engineered nanomaterials released into the environment. If natural NPs can be toxic under certain circumstances then other forms of NPs can present a serious threat to living organisms.

There are many ways for the NPs to reach the aquatic ecosystems. They can enter the environment by the production facilities and wastewater treatment plants (Nowack and Bucheli, 2007) and atmospheric emissions (combustion processes). Some will enter by the use of the product and by some erosion of the product itself (e.g. personal care products and paint) (Biswas and Wu, 2005). Independently of the initial source, aquatic ecosystems are the

probable destiny of the NPs and an interaction with the biota is inevitable (Klaine *et al.*, 2008).

With so many routes of exposure, uptake, distribution, and degradation of NPs it is difficult to predict the amount that can be found in the environment. Since the NPs are disperse through air, soil and water, analytical methods to quantify them are still in development (Scown *et al*., 2010).

To overcome the lack of quantitative knowledge of NPs in the environment generalized estimations of the potential environmental concentrations are used, obtained by modeling and estimates of production (Mueller and Nowack, 2008). For example, predicted modeled concentration of TiO2 and ZnO in water were 0.7–24.5 µg/L and 76 µg/L respectively, while organic nanoparticles like fullerenes round around 0.31 µg/L (Dunphy Guzman *et al*., 2006; Shenhar and Rotello, 2003). The considerable difference between inorganic and organic NPs is most likely due to the usage volume and industrial applications (da Silva *et al.*, 2011). Even with the modulated quantities, it is imperative the development of appropriated methods for detecting, characterizing and quantifying NPs, for a more efficient risk assessment in the environment.

1.1.4 NPs behavior and toxicity

Once reached the aquatic environment, the NPs interact with the water components, like colloids and organic matter, depending on the physical and chemical properties of the water (pH or ionic strength). The NPs fate and behavior will be determined by these interactions. Also, size, shape, chemical composition, surface charge, coating and particles state of the NPs can alter its behavior in water bodies (Corsi *et al.*, 2014).

The NPs tend to aggregate in water, and changes to this aggregation may occur due to interaction with natural colloids and affect the transport in the water column. The tendency for aggregation can prevent interaction with pelagic species but can lead to sedimentation where may be available for benthic organisms. Also, NPs stabilization in the water column is possible, suggesting a potential uptake by pelagic species and transport of the contaminants within the water body (Baalousha *et al*., 2008).

NPs may enter and affect the organism in diverse ways, though the exact method is specific to the particle (Bhatt and Tripathi, 2011). Upon entering the organism, NPs can block essential pores and membrane functions or go inside the cell via diffusion through pores, endocytosis or ion transport systems (Baker *et al*., 2013). Already inside the cell, NPs can

interfere with electron transport processes or enable the production of reactive oxygen species (ROS), which may lead to damage in the nucleic acid or disruption of cell membranes (Baker *et al*., 2013).

1.1.5 Bivalves and NPs

Bivalve organisms have been for long recognized as excellent indicators of contamination of the environment. They possess several characteristics which make them extensively used as sentinel species. Bivalves are filter feeders and able to accumulate water particles, which makes them good indicators of the health of the surrounding environment. They are found in high densities worldwide and can be easily collected. These organisms can withstand large changes of environmental stressors, like salinity and temperature, and resist a large variety of contaminants. Furthermore, there is extensive background information on the biological responses to extensive variety of environmental conditions (Canesi *et al*., 2012).

1.2 Salinity

There are many physical and chemical properties that characterize a water body. Salinity is an environmental factor of great importance. It is considered a limiting factor influencing the distribution of aquatic organisms. Salinity can affect the physiological processes of organisms, such as survival, hemolymph osmolarity, and tissue water content and have other sublethal effects (McFarland *et al*., 2013).

Seawater salinity is variable and depends on season and geographic location. In the Venice Lagoon salinity varies greatly, either according to the seasons or within the lagoon. A 9 year study over the lagoon (Zirino *et al*., 2014) showed evidence of three separate ecosystems due to strong salinity differences. Also, a heavy freshwater input during spring and autumn can result in a salinity difference of 10 to 20 in a great area of the lagoon.

The Hydrobiological Station of Chioggia, Italy, collects data of innumerous environmental parameters, like water temperature, salinity, pH, dissolved oxygen and air temperature. The records are public and it is possible to see the salinity variations from 1973 to this date. Taking the records of the year 2014 (Figure 1.1) it was possible to see the extent of the salinity variation by checking the daily measurements of February. The smallest month of the year showed salinities between 18 and 33 (Umberto D'Ancona Hydrobological Station, Chioggia Venice).

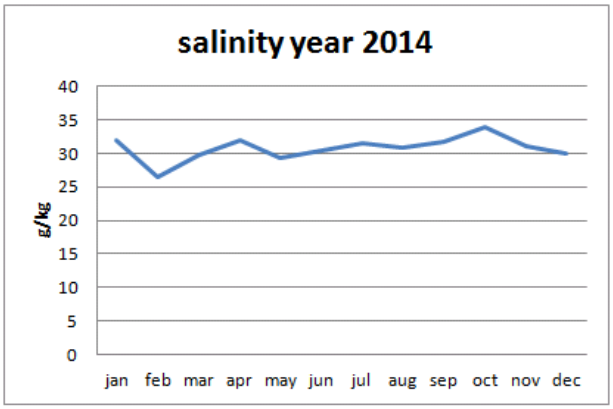

Figure 1.1. Salinity variations during the year 2014. (Umberto D'Ancona Hydrobological Station, Chioggia Venice)

1.2.1 Salinity and NPs

Despite the crescent attention that NPs are receiving from the science community, eventual changes undergone by NPs, physically and chemically, when interaction with the environment occurs and how these changes will affect the interaction with the biota are not yet understood. Little information is now rising about the combined effects of contaminants and different salinities.

A study showed that when Atlantic killifish (*Fundulus heteroclitus*) was exposed to silver NPs higher salinity led to less adsorption of nAg on the chorion of the embryos leading to lower mortality (Auffan *et al.*, 2014). Other study showed that freshwater has higher dissolution and lower sedimentation rate of nZnO than seawater (Majedi *et al.*, 2013). These properties make the NPs more available to the benthic communities rather than the pelagic ones in higher salinities. However, a report of increase growth inhibition with increasing salinity on a marine microalga (Macken *et al.*, 2012) suggests that increasing salinity may act as a stressor for *Ceramium tenuicorne*.

1.3 Ruditapes philippinarum

Ruditapes philippinarum (Adams and Reeve, 1850) has the common name of Manila Clam and is a species of high commercial value. It has a solid broadly oval shell with radiating ribs and concentric grooves making the posterior area markedly decussate. It presents three cardinal teeth in each valve, but not laterally. The pallial sinus is relatively deep however not extending beyond the center of the shell, and displays smooth margins. The shell is variable in external color from white to yellow or brown, often with curved radiating darker

bands or dark blotches. Internally shows a white polished surface and can have a purple area below the umbones. The siphons are shorter than most clams present and are mostly fused together only separating by the tip (Poppe and Goto, 1993).

Figure 1.2. *Ruditapes philippinarum* (FAO.org)

R. philippinarum can reach up to 8 cm of length and buries itself in shallow areas like bays and estuaries in the middle to low intertidal zone, in mud, sandy mud and among buried cobbles. It is an euryhaline organism and can withstand salinities in the range of 15-38 and temperatures of 13-21 ºC. The clam is strictly gonochoric and requires temperatures above 14 ºC to spawn (Carlton, 1979).

R. philippinarum is native to the Indian-Pacific region, having natural populations distributed in the Philippines, the South China and East China Seas, Yellow Sea, Sea of Japan, the Sea of Okhotsk, and around the Southern Kuril Islands (Scarlato, 1981).

Due to commercial purposes, this clam was introduced in France, Brittany, in the Adriatic Sea, in the Hawaiian Islands and along the Pacific coast of northern America (Poppe and Goto, 1993).

1.4 Ruditapes decussatus

Ruditapes decussatus (Linnaeus, 1758), with the common name of Grooved carpet shell, has a broadly oval to quadrate shell and an external color that can be cream, yellowish, or light brown. Most of the clams show darker markings. The pattern of the shell consists of concentric grooves and bold radiating ridges and quite distinct crisscross (decussate) markings present posteriorly. The growth stages can be seen very clearly, and each valve presents three cardinal teeth (Figueras, 1956). The two siphons are separated, which is the main way for differentiate *R. decussatus* from *R. philippinarum*.

Figure 1.3. *Ruditapes decussatus* (FAO.org)

The clam can grow up to 7.5 cm in length and buries itself 15 to 20 cm in sand and silty mud from the middle of the intertidal zone to a depth of a few meters (Figueras, 1956).

It is strictly gonochoristic, having its reproduction period during the summer due to higher temperatures. Grooved carpet shell clam can bear quite well the variations of chemical

and physical factors of water, such as temperature, salinity, dissolved oxygen and turbidity. The clam has some natural predators, in particular crabs, some fish species (e.g. *Sparus aurata*), gastropods (e.g. *Rapana venosa*), and birds (e.g. *herons and gulls*). In addition, other forms of competition can affect the stability of clam production. Marine organisms, such as other species of Bivalves, Hydroids, Bryozoans, Serpulids, being filter feeders can compete for food availability and others resources (Paesanti and Pellizzato, 1994).

The grooved carpet shell has a native distribution that extends from Norway to Somalia, along the Iberian Peninsula, and into the Mediterranean Sea (Parache, 1982).

1.4.1 Venice Lagoon

R. philippinarum and *R. decussatus* both inhabit the Venice Lagoon. With a surface area of 549 Km^2 and an average depth of 0.6 m, the lagoon consists in a network of canals, mud flats, tidal marshes and islets. The connection to the Adriatic Sea is made by three canals, on which exchanges water and sediments (Bellucci et al., 2002).

The contamination of the lagoon began around 1920, after the First World War, upon the construction of the first industrial area, and accelerated in 1930 with the assembly of the second industrial zone (Pavoni *et al*., 1992). Over the years, the industrial activity has been responsible for the discharge of pollutants and nutrients into the lagoon, which lead to an eutrophication and dystrophic crises of the ecosystem (Libralato *et al*. 2004).

The anthropogenic introduction in the Venice Lagoon of *R. philippinarum*, due to irregular incomes of the native organisms, lead this species to supplant the native clam *R. decussatus*, occupying now almost entirely its ecological niche and forcing it to inhabit very limited areas (e.g. Auby, 1993).

1.5 Biomarkers

In ecotoxicological studies the main issue concerns the assessment of exposure and the effect of polluting compounds on natural communities. When a change in a biological community occurs it means that the ecosystem is already altered, sometimes irreversible, and it does not work as a preventive tool. Thus, the need of more sensitive early-warning indicators of sub-lethal ecological effects in order to allow taking preventive measures before the ecological damage occurs (Martinez-Haro *et al.*, 2015).

Biomarkers refer to a measurable indicator of biological state or condition. In ecotoxicology, biomarkers are used to indicate an exposure to or the effect of xenobiotics which are present in the environment and in organisms. When a contaminant first infects an organism the primarily effects are felt at the biochemical and biomolecular level (enzymes, DNA), only later can be noticed at higher levels of organization (Figure 1.4). So a biomarker usually is a cellular, tissue, body fluid, physiological or biochemical change that normally can be quantified (van der Oost *et al.*, 2003).

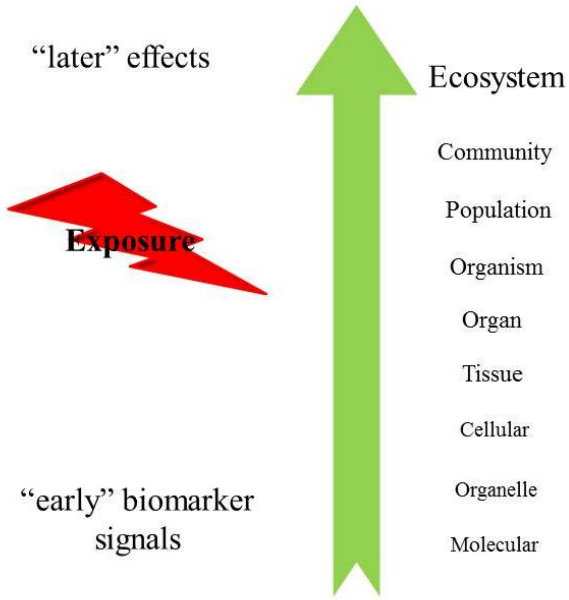

Figure 1.4. Sequential order of responses to pollutant stress in a biological organism (adapted from Bayne *et al.*, 1985).

Early detection of contamination can prevent further damage and set measures designed to lessen the impact of the affected environment.

1.5.1 Classification

Biomarkers can be separated in two different categories, according to the type of response (although not all biomarkers fall precisely in one of these two categories (De Lafontaine *et al.*, 2000)):

- Biomarkers of exposure,
- Biomarkers of effect.

Biomarkers of exposure indicate exposure to a chemical compound or to a class of compounds, however it do not provide any indication of the actual toxicological effects on the organism. Biomarkers of effect indicate both exposure and the effect of a toxic compound, including alterations at physiological, biochemical or genetic level, which can be measured in the tissues of an organism, and can be recognized and associated to a possible decline of health (Bayne, 1986).

1.5.2 Biomarkers typology

A biomarker used in environmental monitoring can be divided in categories according to the response at the level of biological organization (Fossi, 1998):

- DNA damage;
- Changes in protein;
- Changes in metabolic products;
- Immune system disorders;
- Histopathological abnormalities.

Some environmental contaminants have the ability to damage DNA, causing a series of alterations which cascade into changing the genetic material (double helix breaking, fragmentation of chromosomes) until a mutation occurs which can change the gene functionality (Shugart, 1995). The contact with an environmental pollutant can, also, originate induction or inhibition of a protein activity in an organism. The mechanisms involved can be protective, in order to proceed in the xenobiotic detoxification, some of defense against heavy metals (metallothioneins), and others of inhibition (McCarthy and Shugart, 1990). Some classes of pollutants can interfere with the normal metabolism of endogenous compounds and cause accumulation of intermediate products. Contaminants can also affect the immune system. Since it has the ability to neutralize foreign materials and to defend the body from pathogens it is a good indicator of an organism health. Phagocytosis activity and cytotoxic activity are examples of the biomarkers used. At last, histopathological abnormalities can occur, since the final stage of the toxic effect of many pollutant compounds can involve some target organs (especially the liver). It assesses the response to acute and chronic effects induced by contaminants.

1.5.3 Advantages and limitations of Biomarkers

Environmental monitoring or chemical toxicology investigations, in many cases, are not sufficient for environmental quality evaluation studies because are not representative of the evolution of contamination, even though they provide very precisely in terms of quality and quantity.

Biomarkers in environmental monitoring programs can provide additional information to the previous studies and offer an integrated response to the overall exposure of the species to contaminants, taking into account the different routes of intake, both of exposures over time within a particular area (Van der Oost *et al*., 1996). These tests require less time and effort, are more sensitive than chemical analysis, and can provide more realistic information from the biologic perspective of the contaminant effect on the organism (and extrapolate to the community) (Van der Oost *et al.,* 1996). Also, biomarkers can be used to investigate the efficiency of recovery interventions of a damaged ecosystem (by following the return of the biological responses to the basal values predetermined).

However, there are some limitations, mainly related to the variability of the biological model. Biotic factors such as sex, reproductive stage, age and diet, and abiotic factors like temperature, pH, food availability, oxygen, can affect the biochemical responses (Hyne and Maher, 2003). This variability can lead to a misinterpretation of the results and reach wrongful conclusions, when the biochemical or physiological alteration registered may be due to some change in environmental parameters or a normal fluctuation of vital basal signs of the organism (Figure 1.5). Thus, it is extremely important to have a deep knowledge of the biological model and the biotic and abiotic factors that can affect it (Vidal *et al*., 2002).

It is also important that several biomarkers are analyzed so that the health of the organisms are based in the alteration of several parameters, instead of just one or two that could give deceptive information and lead to wrong conclusions (Koukouzika and Dimitriadis, 2005). Another limitation in the use of biomarkers is the difficulty in finding a relationship between the immediate response of the individual and ecological effect in the long term.

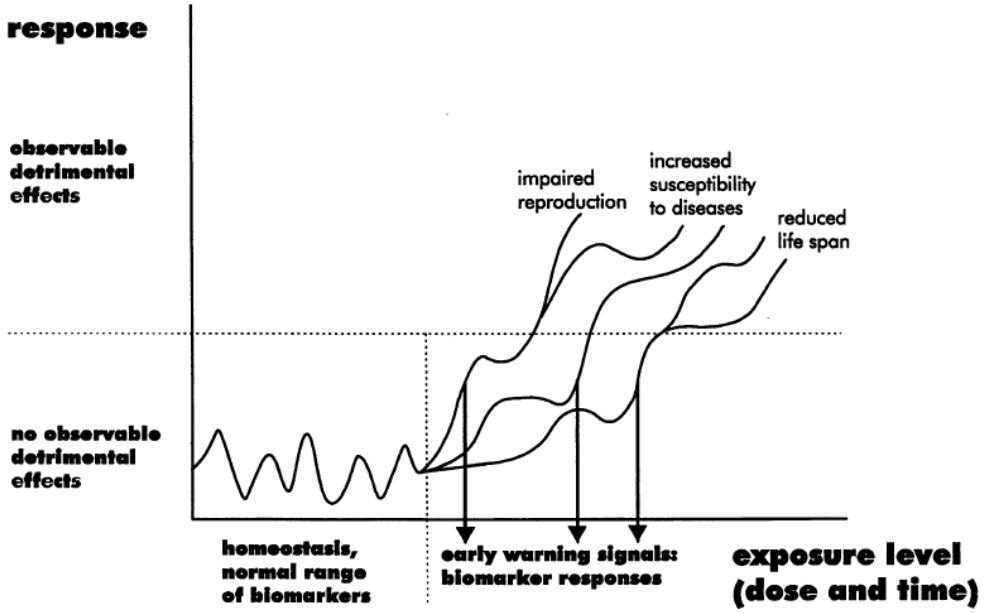

Figure 1.5. Organism responses to the damaging effects of contaminant exposure (adapted from McCarthy *et al.*, 1991).

1.6 Aims

The aquatic ecosystems are constantly subjected to anthropogenic impacts. With the growth of nanotechnology industry, the release of NMs and other by-products into the marine environment is inevitable. It is important to investigate the potential adverse effects of NPs in aquatic organisms and the associated risks to the aquatic environment, when combined with other abiotic factors, especially salinity, a key factor in transition systems. Many anthropogenic activities interfere with the discharge of freshwater, leading to an alteration of the water salinity. A large percentage of information about biota, such as bivalves as a bioindicator species exists, both in the field and in the laboratory, but it is not enough to understand the impact that the combination of contaminant and abiotic factors can cause.

The main objective of this work was to study the effect of a mixture of three well spread and commercially available NPs, nZnO, $nTiO_2$ and fullerene C60, in two species of clams, one invasive (*R. philippinarum*) and one native (*R. decussatus*) in the Venice Lagoon, at different salinity levels. The effect of the NPs mixture at different salinity levels was evaluated through the use of conventional biomarkers: antioxidant enzymes, LPO and DNA damage. Furthermore, the physiological condition of the clams was evaluated by measuring the RNA/DNA ratio, after a seven day exposure to the NPs and different salinity levels.

2 Methodology

2.1 Sampling and acclimation

Specimens of *R. philippinarum* and *R. decussatus* were collected in the Venice Lagoon (Italy), inside a licensed area for clam culture.

The clams were acclimatized in large, sandy bottom aquaria, for 10 days, with aerated seawater at a constant temperature (17 ºC), with daily feeding of microalgae (*Isochrysis sp.*). The 6 aquaria were divided in three groups, in order to acclimatize the clams to a different salinity: 18, 28 and 38. The water was exchanged daily, and the salinity, initially the same for all aquaria, was gradually changed to reach the target value.

2.2 Nanoparticle exposure test

The organisms were exposed for 7 days to a NP mixture of zinc oxide (nZnO), titanium dioxide ($nTiO_2$) and fullerene C60; 1µg/L of each nanoparticle. The nanoparticle mixture was prepared in Milli-Q water and sonicated (Braun Labsonic U sonifier at 50% duty cycles) for 30 min, at 4 °C. For each experimental condition a group of untreated clams was kept in clean sea water, functioning as control; also, two replicate tanks were prepared, for each condition of treated and untreated clams. The clams were maintained in 35 L glass tanks (without any sediment), at least 40 individuals, with aerated seawater at 3 different salinity regime (18, 28 and 38), previously acclimated. During the exposure the clams were fed with microalgae, water exchange and nanoparticle mixture supply was performed daily. Dead organisms were removed immediately, to avoid water deterioration. In order to prevent NPs sedimentation and promote aeration a movement pump (Hydor, Koralia nano 900, USA) was positioned inside the aquaria. Previously in the laboratory, inquiries were made whether the pump would alter the behavior of the clams and it was found that it does not.

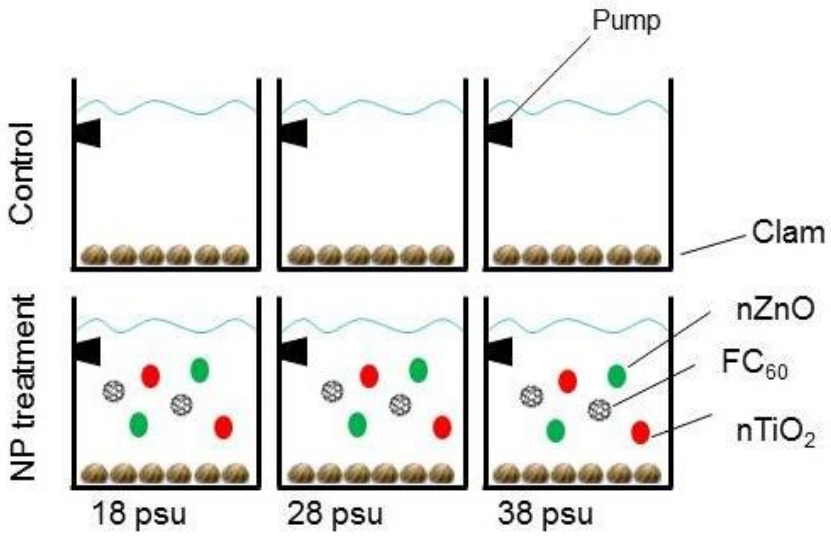

Figure 2.1. Experimental design.

2.3 Haemolymph and tissue collection

Haemolymph, and gills and digestive gland tissue from both species were collected after 1, 3 and 7 days of exposure (T1, T3 and T7). For each experimental condition, 5 pools (from 5 different clams) of each tissue were collected.

Gills and digestive gland were frozen in liquid nitrogen and stored at -20 ºC, until analysis.

Haemolymph was collected from the anterior adductor muscle with the aid of a 1 mL plastic syringe and stored at 4 ºC, for immediately analysis.

At the 7 day of exposure, 10 samples of foot tissue of the clams, from each condition, were collected and stored with 1 mL of RNA*later*®, to stabilize and protect cellular RNA, for further RNA/DNA ratio and protein content analysis.

2.4 Haemolymph assays

2.4.1 Micronucleus test

The micronucleus test (MN) was performed to determine if any numerical or structural chromosomal damage occurred (according to Pavlica *et al.* 2000).

An aliquot of 150 µL of haemolymph was placed in a slide and left 15 min in a humidified chamber at room temperature, in order to hemocytes to settle. After, the slides were fixed with a glutaraldehyde (25% solution) solution (1% in PBS) for 5 min. Then, the

slides were rinsed with PBS, stained with bisbenzimide 33258 (Hoechst) (final concentration of 1 mg/mL) for 5 min, and washed and mounted in glycerol–McIlvaine buffer (1:1). The slides were then store in the dark, at 4 ºC. The examination of the slides was done by a fluorescent microscope (Leica 5000B) equipped with submerged lens at 100×magnification. The examination was always made by the same observer and following a pre-arranged pathway to reduce subjectivity. Three hundred cells per slide (intact and non-overlapping hemocytes) were counted in five slides for each condition, making a total of 1500 cells per treatment.

The identification of the micronucleus was done according to Kirsch-Volders *et al*. (2000). The micronucleus frequency (MN‰) was calculated.

2.4.2 Cytotoxicity assay

Cytotoxicity was evaluated measuring the lactate dehydrogenase (LDH) activity in cell-free haemolymph, using a colorimetric assay. LDH is a stable cytoplasmic enzyme that can be found inside the cells and its presence in the haemolymph indicates cell damage or destruction.

A commercial kit (Cytotoxicity Detection Kit, Roche) was used to assess cell damage on a 600 µL aliquot of haemolymph, after centrifuged at 800xg for 10 min to obtain cell-free haemolymph. 500 µL of reactive solution from the kit was added to the aliquot and was incubated for 30 min in the dark, at room temperature.

The absorbance at 490 nm was recorded using a spectrophotometer (Beckman 730). The results were expressed as the optical density (OD) at 490nm.

2.4.3 Neutral Red Uptake

The cationic probe neutral red (NR) was used to assess the pinocytotic ability of the haemocytes (Cajaraville *et al*., 1996; Matozzo *et al*., 2002).

An aliquot of 500 µL of haemolymph was centrifuged at 2,300xg for 10 min; the haemocytes were resuspended with NR dye (8 mg/L solution in FSW, Merck) and incubated at 20 ºC for 30 min, in the dark. After, the haemocytes were centrifuged at 2,300xg for 10 min and resuspended in Milli-Q water. The samples were then sonicated for 30 s (Braun Labsonic U sonifier at 50% duty cycles) and centrifuged at 13,400xg for 15 min, on which the supernatante was collected for the NR uptake assay. The absorbance was recorded at 550 nm

with a spectrophotometer (Beckman 730). The results were expressed as the optical density per millilitre of haemolymph (OD/ mL haemolymph).

2.5 Gills and digestive gland assays

2.5.1 Tissue homogenization

The polls of gills and digestive gland were individually homogenized, at 4 ºC, with buffer (four volumes of 50 mM Tris – HCl, pH 7.4, containing 0.15 M KCl, 0.5 M sucrose, Protease Inhibitor Cocktail, Sigma – Aldrich), using an Ultra-Turrax homogeniser (model T8 basic, IKA). Then, the samples were centrifuged at 13,400xg for 40 min at 4 °C. The supernatants (SN) were collected for analysis. The protein concentration for all supernatants was quantified according to Bradford (1976) using bovine serum albumin (BSA) as standard.

2.5.2 Catalase activity

The catalase (CAT) activity in the gills and digestive gland was determined following the method of Aebi (1984). The decreases in absorbance of a 50-mM H_2O_2 solution (ε = -0.0436 mM^{-1} cm^{-1}) in 50 mM phosphate buffer (pH 7.8), using 10 µL of sample SN, were continuously recorded at 240 nm, at 10 s intervals for 30 s.

The results were expressed in U/mg protein (one unit of CAT being the amount of enzyme that catalyzed the dismutation of 1 µmol of H_2O_2/min).

2.5.3 Superoxide dismutase assay

The superoxide dismutase (SOD) activity in both gills and digestive gland was measured using the xanthine oxidase/cytochrome *c* method, based on the study by Crapo *et al.* (1978). The cytochrome *c* reduction by superoxide anion generated by xanthine oxidase/hypoxanthine reaction was detected at 550 nm at room temperature.

The final reaction mixture contained 46.5 mM KH_2PO_4/K_2HPO_4 (pH 8.6), 0.1 mM EDTA, 195 mM hypoxanthine, 16 mM cytochrome *c*, and 2.5 mU xanthine oxidase.

The results were expressed in U/mg protein, being one unit SOD the amount of sample 50% inhibition in the assay conditions.

2.5.4 Glutathione S-transferase activity assay

The glutathione S-transferase (GST) activity was measured in the gills and digestive gland, according to the method described by Habig *et al.* (1974).

The reaction mixture contained 20 mM of 1-chloro-2,4-dinitrobenzene (CDNB), 0.1 M reduced glutathione (GSH) (Sigma Aldrich, Milano, Italy) and 0.1 M phosphate buffer (pH 6.5), which were incubated at 37 ºC for 15 min as individual solutions, then mixed with 10 µL of SN.

The increase in absorbance was measured spectrophotometrically, at 340 nm. The results were expressed as nmol/min/mg protein.

2.5.5 Lipid peroxidation assay

The lipid peroxidation (LPO) was quantified in SN of gills and digestive gland using the malondialdehyde (MDA) assay, based on the study of Buege and Aust (1978).

MDA is formed during oxidative degeneration as a product of free oxygen radicals, making it an indicator of lipid peroxidation.

Absorbance was read at 532 nm spectrophotometrically. The results were expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg protein. TBARS, considered as ‘‘MDA-like peroxide products’’, were quantified by reference to MDA absorbance ($\varepsilon = 156 \times 10^3\ M^{-1}\ cm^{-1}$) (Damiens *et al*., 2007). Results were not expressed as MDA levels, because TBA can react with a range of chemical compounds (Csallany *et al.*, 1984).

2.5.6 Protein carbonyl content

The protein carbonyl content (PCC) was measured based on the reaction with 2,4-dinitrophenylhydrazide (DNPH), spectrophotometrically (Dalle Donne *et al.*, 2003; Mecocci *et al.*, 1999).

To the SN of gills and digestive glands a solution of DNPH (10 mM in 2 N HCl) was added; a blank for each sample was prepared by adding to the SN 2 N HCl (without DNPH). The samples incubated for 1h in the dark, at room temperature, and were vortexed every 10 min. Then, a volume of 30% of trichloroacetic acid (TCA) was added and the samples were kept at 4 ºC for 15 min. After, the samples were centrifuged at 10,000xg for 15 min at 4 ºC, the supernatant was disposed and the pellets were washed three times with ethanol-ethyl acetate mixture (1:1), in order to remove the remaining DNPH and lipid contaminants. After

wash, a 6 M guanidine solution was added to the pellet, and kept at 37 °C for 30 min in a water bath with mixer.

The carbonyl content was calculated from the absorbance measurement at 370 nm, using a molar absorption coefficient of 22,000 mol/cm and expressed as nmol/mg protein.

2.6 Foot tissue assays

2.6.1 Protein content

Protein content was measured by the bicinchoninic acid method (Smith *et al*., 1985).

An aliquot of 7 µL of chemical and mechanically homogenized sample was transferred into a 96-well plate prior to the addition of 60 µL of deionized water and 133 µL of working reagent (1:50 copper sulphate/bicinchoninic acid). The plate was incubated at 37 ºC for 30 min before being analyzed at 562 nm using a spectrofluorometer with microplate reader (Biotek Synergy HT). Concentrations were determined by standard curves of known protein concentrations, using bovine serum albumin (BSA) as standard.

2.6.2 RNA/DNA ratios

RNA and DNA were measured with the microplate fluorescent assay (Wagner *et al.*, 1998).

Sarcosine-Tris (STB) (0,5%) was added to the foot tissue of clams, according to the weight of the sample. The sample was homogenized with a sonicator, giving 3 pulses of 50 A during 1 minute. After, the sample was vortexed for 30 minutes and centrifuged for 15 minutes at 12000 rpm, between 0 and 4 ºC. An aliquot of 40 µL of sample was mixed with 130 µL and dyed with GelRed before florescence reading. Total fluorescence was first read then samples were incubated with ribonuclease at 37 °C for 30 min, at 365 nm excitation wavelength and 590 nm emission wavelength, in a spectrofluorometer with microplate reader (Biotek Synergy HT).

The DNA fluorescence was assumed to be the result of RNase incubation after the first reading, since the enzyme breaks RNA; and the RNA fluorescence was calculated as the difference between the first and the second reading. Concentrations were determined by standard curves of known DNA and RNA concentrations, read at the same time as the samples, within the appropriated range of values. The ratio of the slopes of the standard curves of the DNA and the RNA was 3.17±1.29.

2.7 Statistical analysis

For all data, normal distribution was assessed (Shapiro-Wilk's test), as the homogeneity of variance (Bartlett's test). Two-way ANOVA test was performed to compare treatments, followed by a post-hoc test, Fisher LSD, in biomarker results. For RNA/DNA ratios one-way ANOVA followed by post-hoc Tukey's test was performed. The STATISTICA 10 software package (StatSoft, Tulsa, OK) was used for statistical analyses. Results were expressed as means ± standard deviation.

3 Results

3.1 Ruditapes philippinarum

3.1.1 Micronucleus frequency

Two-way ANOVA test showed that concentration had a significant effect (p=0.000) on the formation of micronucleus in haemocytes, whereas no effect was recorded for both time and concentration/time interaction (Table 3.1).

Table 3.1. Two-way ANOVA table for micronucleus frequency in haemocytes, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	4363,457	1	4363,457	158,1387	0,000000
Concentration	1500,247	5	300,049	10,8743	0,000000
Time	26,914	2	13,457	0,4877	0,616053
Concentration*Time	367,160	10	36,716	1,3306	0,231037
Error	1986,667	72	27,593		

Pair-wise comparisons showed a significant increase (p=0.018) in micronucleus formation in haemocytes from clams kept at 38, at T1, with respect to the control at the same salinity (Figure 3.1).

Within the control groups, data showed a significant high level of micronucleus frequency in clams at 38, at T1 and T7 (p=0.048 and p=0.018, respectively), in relation to controls of lower salinities.

Clams from treated groups showed an increasing micronucleus frequency with the increasing of the salinity levels.

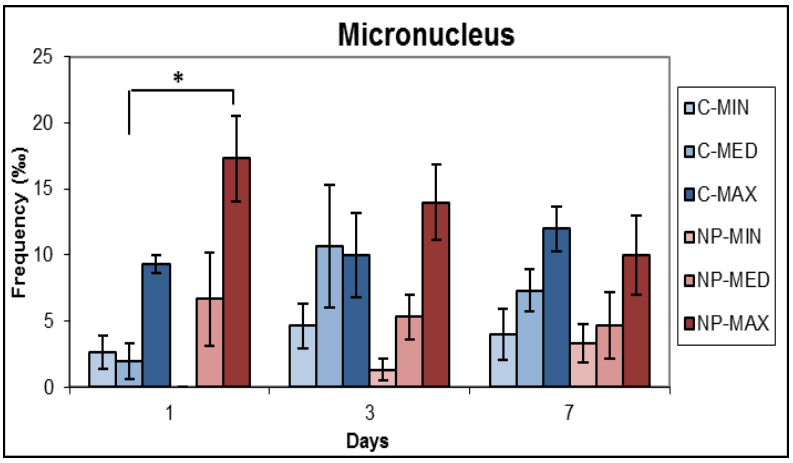

Figure 3.1. Effects of nanoparticle mixture and different salinity levels on micronucleus formation in haemocytes in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.2 Nuclear abnormalities frequency

Significant effect was determined in concentration and time variables (p=0.000) on the formation of nuclear abnormalities in haemocytes, as well as concentration/time interaction (p=0.005) (Table 3.2).

Table 3.2. Two-way ANOVA table for nuclear abnormalities frequency in haemocytes, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	3737,778	1	3737,778	273,9910	0,000000
Concentration	773,333	5	154,667	11,3376	0,000000
Time	275,556	2	137,778	10,0995	0,000136
Concentration*Time	386,667	10	38,667	2,8344	0,005027
Error	982,222	72	13,642		

Post-hoc test showed a significant increase (p=0.025) in the frequency of nuclear abnormalities in haemocytes of clams kept at 38, after seven days of exposure to a mixture of nanoparticles, with respect to controls at the same salinity (Figure 3.2).

Clams from both treated and untreated (control) groups showed an increasing frequency of nuclear abnormalities with the increase of salinity levels, being significant and more pronounced after seven days of exposure (C-MAX p=0.002; NP-MED p=0.005; NP-MAX p=0.012).

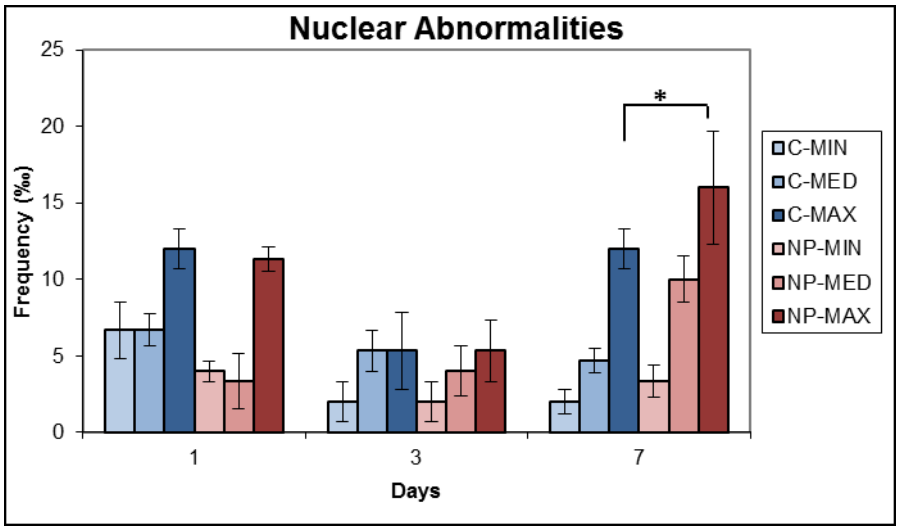

Figure 3.2. Effects of nanoparticle mixture and different salinity levels on nuclear abnormalities in haemocytes in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.3 Cytotoxicity activity

Two-way ANOVA test showed that both parameters concentration and time had a significant effect (p=0.000 and p=0.005, respectively) on the enzyme lactate dehydrogenase levels, as did the concentration/time interaction (p=0.032) (Table 3.3).

Table 3.3. Two-way ANOVA table for the cytotoxicity activity in haemocytes, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	1,969880	1	1,969880	621,6661	0,000000
Concentration	0,321052	5	0,064210	20,2639	0,000000
Time	0,036100	2	0,018050	5,6963	0,005051
Concentration*Time	0,067527	10	0,006753	2,1311	0,032550
Error	0,228147	72	0,003169		

Post-hoc test performed to the results shows no significant effect between clams treated with NPs mixture and the respect control group at the same salinity on the LDH enzyme (Figure 3.3).

Within the control groups, a significant higher value of LDH was recorded, in all measured days, in clams at 18, with respect to other two salinity levels (28 and 38) (T1 p=0.000; T3 p=0.037; T7 p=0.017). The same pattern was observed in the NP treated groups (T1 p=0.000; T3 p=0.020; T7 p=0.018).

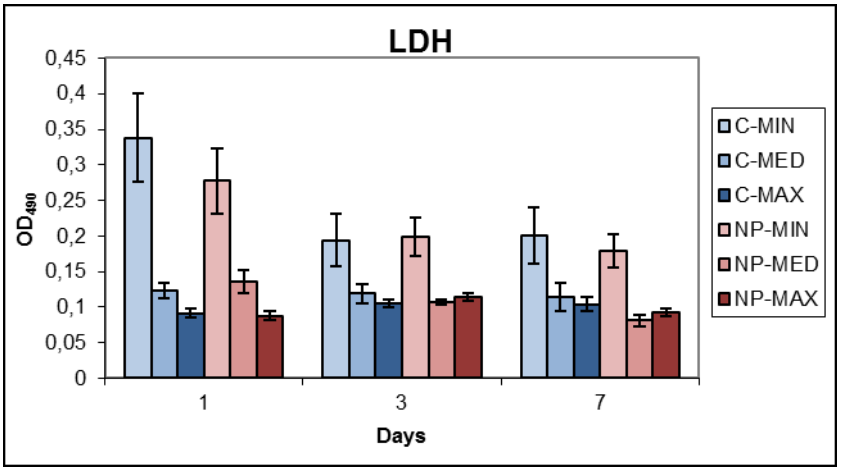

Figure 3.3. Effects of nanoparticle mixture and different salinity levels on lactate dehydrogenase in haemocytes in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38).

3.1.4 Neutral Red uptake

Time of exposure had a significant effect on the pinocytotic activity measured by the neutral red uptake assay in haemocytes (p=0.005), whereas concentration and concentration/time interaction had no significant effect (Table 3.4).

Table 3.4. Two-way ANOVA table for pinocytotic activity of haemocytes, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	0,418612	1	0,418612	488,4901	0,000000
Concentration	0,004677	5	0,000935	1,0916	0,372519
Time	0,009520	2	0,004760	5,5547	0,005709
Concentration*Time	0,016099	10	0,001610	1,8786	0,062295
Error	0,061700	72	0,000857		

Pair-wise comparisons determined that a significant increase in pinocytotic activity occurred (p=0.013), at T3, in clams kept at 18, with respect to controls at the same salinity (Figure 3.4).

Data showed a significant decrease in pinocytotic activity in NP-MIN and NP-MED (p=0.000 and p=0.015, respectively) from T3 to T7.

No significant differences within the control and treated group were found.

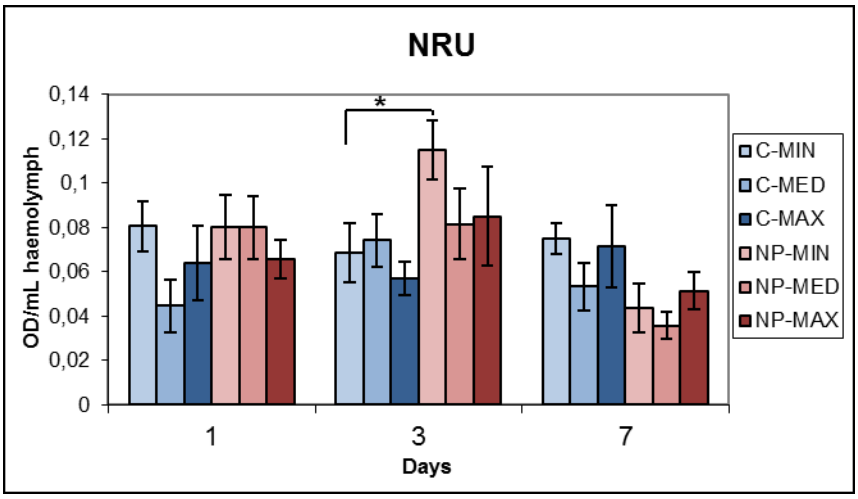

Figure 3.4. Effects of nanoparticle mixture and different salinity levels on pinocytotic activity in haemocytes in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.5 Catalase activity

3.1.5.1 Gills

Two-way ANOVA analysis revealed statistical significant effects of time (p=0.000) on catalase activity in the gills tissue, whereas no effect of both concentration and concentration/time interaction were recorded (Table 3.5).

Table 3.5. Two-way ANOVA table for catalase activity in gills tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	22864,33	1	22864,33	2458,962	0,000000
Concentration	95,97	5	19,19	2,064	0,079820
Time	258,84	2	129,42	13,919	0,000008
Concentration*Time	148,15	10	14,81	1,593	0,126017
Error	669,48	72	9,30		

Post-hoc test showed at T1 a significant increase (p=0.004) of CAT activity, in clams kept at 28, with respect to control at the same salinity; however at T7 a significant decrease of CAT was registered in clams at 18 and 38 (p=0.046 and p=0.012, respectively), regarding to controls at the same salinity (Figure 3.5).

Data also determined a significant increase of CAT activity in the C-MIN and C-MAX group, from T3 to T7 (p=0.000 and p=0.003).

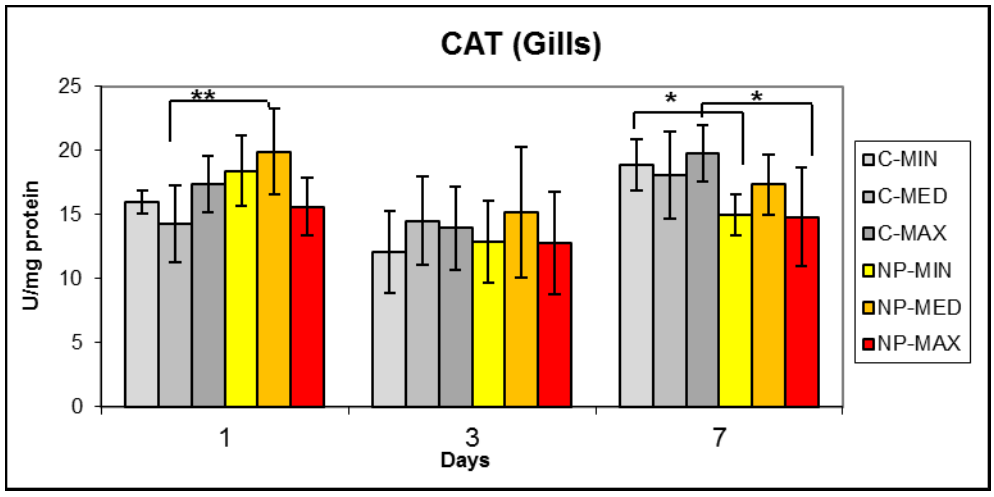

Figure 3.5. Effects of nanoparticle mixture and different salinity levels on catalase activity in gills tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.5.2 Digestive Gland

In the digestive gland tissue, two-way ANOVA test demonstrated that time (p=0.004), concentration (p=0.010) and concentration/time interaction (p=0.002) significantly influenced catalase activity (Table 3.6).

Table 3.6. Two-way ANOVA table for catalase activity in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	28142,47	1	28142,47	3163,144	0,000000
Concentration	144,90	5	28,98	3,257	0,010446
Time	101,71	2	50,86	5,716	0,004965
Concentration*Time	274,57	10	27,46	3,086	0,002559
Error	640,58	72	8,90		

NP-MAX clams significantly increased CAT activity in the digestive gland tissue (p=0.025) at T1, as did the NP-MIN clams at T7 (p=0.000) (Figure 3.6), when compared with control groups at the same salinity level.

Within the treated group, at T1, NP-MAX clams showed a significantly high value of CAT activity (p=0.014) than those from NP-MIN. At T7, a significantly low value of CAT activity was registered in NP-MED clams, regarding other salinities (p=0.000).

Data also showed a significant increase in the NP-MIN and NP-MAX group between T3 and T7 (p=0.000 and p=0.002 respectively).

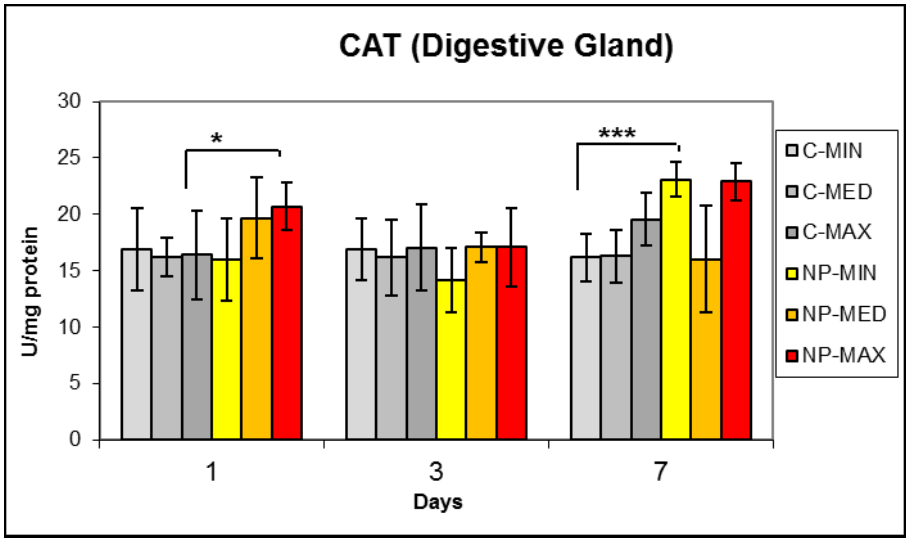

Figure 3.6. Effects of nanoparticle mixture and different salinity levels on catalase activity in digestive gland tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.6 Superoxide dismutase activity

3.1.6.1 Gills

In gills, two-way ANOVA test demonstrated that both time (p=0.024) and concentration (p=0.025) significantly influenced SOD activity (Table 3.7), while the concentration/time interaction had no significant effect.

Table 3.7. Two-way ANOVA table for superoxide dismutase activity in gills tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	102308,1	1	102308,1	7457,979	0,000000
Concentration	188,0	5	37,6	2,740	0,025349
Time	107,6	2	53,8	3,920	0,024208
Concentration*Time	208,7	10	20,9	1,522	0,149428
Error	987,7	72	13,7		

At T1 a significant decrease on SOD activity in gills tissue of clams at 18 and 28 (p=0.009 and p=0.029, respectively) was detected. At T7, SOD activity decreased significantly in exposed clams at 28 (p=0.013), whereas at 38 increased significantly (p=0.045), regarding the controls at the same salinity level (Figure 3.7).

A significantly high value of SOD activity was registered within the treated group, at T1, in clams at 38 regarding those at 28 (p=0.041); at T7, a significantly lower value of SOD activity was registered in clams from the control group at 38 regarding clams at 28 (p=0.006).

A significant increase of SOD activity was recorded in clams treated with NP at 18 and 28 between T1 and T3 (p=0.011 and p=0.003, respectively).

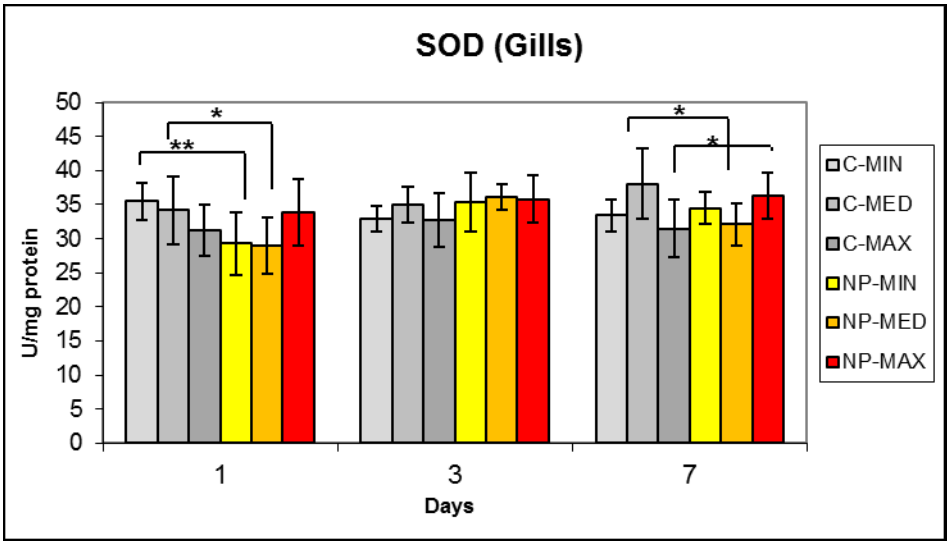

Figure 3.7. Effects of nanoparticle mixture and different salinity levels on superoxide dismutase activity in gills tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.6.2 Digestive gland

In the digestive gland tissue, statistical analysis demonstrated that time (p=0.000), concentration (p=0.000) and concentration/time interaction (p=0.001) had a significant effect in SOD activity (Table 3.8).

Table 3.8. Two-way ANOVA table for superoxide dismutase activity in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	82335,12	1	82335,12	13229,28	0,000000
Concentration	194,17	5	38,83	6,24	0,000073
Time	2533,18	2	1266,59	203,51	0,000000
Concentration*Time	201,90	10	20,19	3,24	0,001675
Error	448,11	72	6,22		

In the digestive gland tissue, a significant decrease in SOD activity was shown, at T1 in clams kept at 18 (p=0.047), at T3 in clams at 28 (p=0.000), and at T7 in clams at 38 (p<0.01), with respect to the controls at the same salinity levels (Figure 3.8).

Data showed that, at T3, SOD activity in cams at NP-MED was significantly lower than at NP-MIN (p=0.009) and NP-MAX (p=0.008); also, at T7 SOD activity was significantly lower at NP-MAX than in NP-MIN and NP-MED (p=0.000).

Data also showed a significant increase of SOD activity, from T1 to T3, in all concentration of both control and treated groups (C-MIN, C-MED, C-MAX, NP-MIN, NP-MED and NP-MAX p=0.000); moreover, a significant decrease followed T3 to T7 in all concentration of both groups, excepting NP-MED (C-MIN, C-MED, C-MAX and NP-MAX p=0.000; NP-MIN p=0.026).

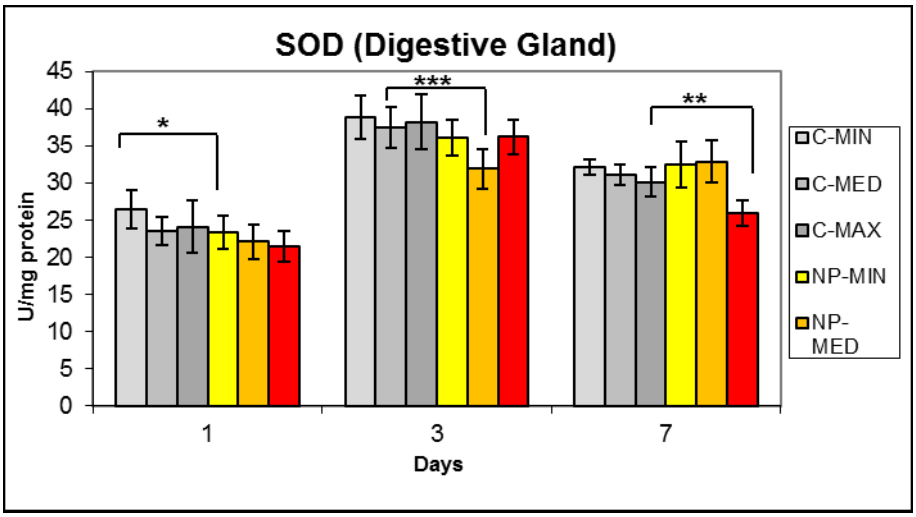

Figure 3.8. Effects of nanoparticle mixture and different salinity levels on superoxide dismutase activity in digestive gland tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.7 Glutathione S-transferase activity

3.1.7.1 Gills

The two-way ANOVA analysis revealed both time (p=0.002) and concentration (p=0.000) significantly influenced glutathione S-transferase activity in gills tissue (Table 3.9), while concentration/time interaction had no significant effect.

Table 3.9. Two-way ANOVA table for glutathione S-transferase activity in gills tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	115,5722	1	115,5722	424,4606	0,000000
Concentration	27,2445	5	5,4489	20,0121	0,000000
Time	3,6209	2	1,8104	6,6492	0,002239
Concentration*Time	3,6368	10	0,3637	1,3357	0,228488
Error	19,6042	72	0,2723		

Post-hoc test showed a significant decrease on glutathione S-transferase (GST) activity in gills tissue of clams kept at 18, after three days of exposure to NPs (p=0.023), and on the seventh day of exposure (p=0.001), regarding the control groups at the same salinity levels (Figure 3.9).

Data showed that GST activity, one day after exposure, in clams at 18 in control group are significantly higher than clams at 38 (p=0.000), and for both three and seven days after exposure, clams at 18 showed significantly higher levels of GST activity than clams at 28 and 38 (day 3, C-MED p=0.002, C-MAX p=0.000; day 7, C-MED and C-MAX p=0.000). The same pattern was observed for clams in the treated groups, with clams at 18 having significantly higher levels of GST activity than calms at 38 in all registered days (T1 p=0.027; T3 p=0.014; T7 p=0.001).

A significant decrease in GST activity was registered from day one to day three after exposure in clams at 28 in both control and treated groups (p=0.032 and p=0.018, respectively); in contrast, a significant increase was observed in clams at 18 on the control group from the third to the seventh day (p=0.046).

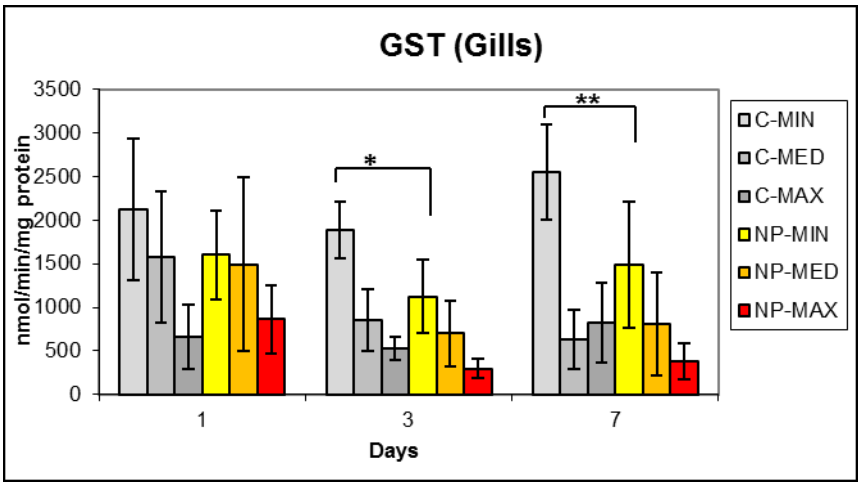

Figure 3.9. Effects of nanoparticle mixture and different salinity levels on glutathione S-transferase activity in gills tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.7.2 Digestive gland

Two-way ANOVA analysis revealed statistical significant effects of time (p=0.000) on glutathione S-transferase activity in the digestive gland tissue, whereas no effect of both concentration and concentration/time interaction were recorded (Table 3.10).

Table 3.10. Two-way ANOVA table for glutathione S-transferase activity in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	272,3344	1	272,3344	2477,229	0,000000
Concentration	1,0049	5	0,2010	1,828	0,118084
Time	10,6373	2	5,3186	48,380	0,000000
Concentration*Time	1,8571	10	0,1857	1,689	0,099862
Error	7,9153	72	0,1099		

A post-hoc test showed a significant increase in GST activity (p=0.032) in digestive gland tissue of NP-MED after seven day of exposure, with respect to control at the same salinity levels (Figure 3.10).

At the seventh day of exposure, clams at 18 within the control group had significantly higher levels of GST activity in comparison with clams at 28 and 38 (p=0.005). No significant alterations were recorded within the treated group.

A significant increase in GST activity was recorded from day one to day three in clams at 38 from control and treated groups (p=0.000 and p=0.014, respectively); the same increase occurred in clams at 18 in both control and treated groups and calms at 38 from treated group from the third to the seventh day (p=0.000, p=0.000 and p=0.023, respectively).

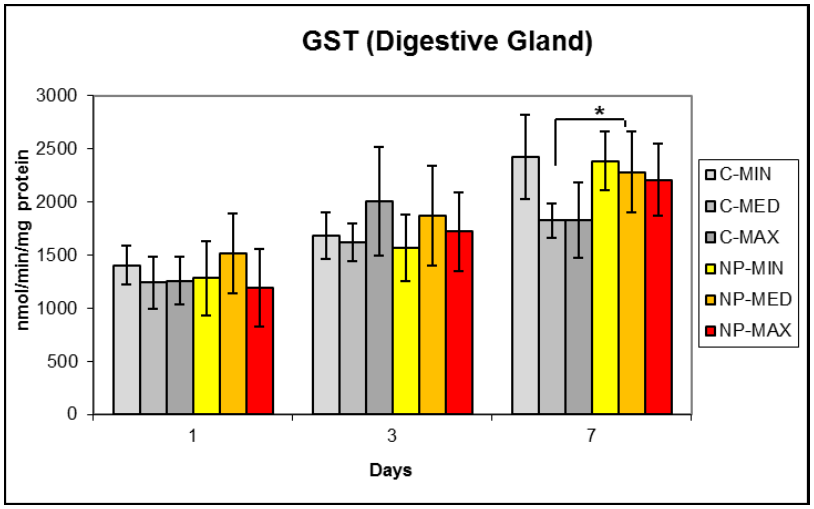

Figure 3.10. Effects of nanoparticle mixture and different salinity levels on glutathione S-transferase activity in digestive gland tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.8 Lipid peroxidation assay

3.1.8.1 Gills

LPO in gills was affected significantly by concentration (p=0.006), time (p=0.000), and concentration/time interaction (p=0.001) (Table 3.11).

Table 3.11. Two-way ANOVA table for lipid peroxidation in gills tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	1,893657	1	1,893657	6340,224	0,000000
Concentration	0,005260	5	0,001052	3,522	0,006635
Time	0,004912	2	0,002456	8,223	0,000608
Concentration*Time	0,009543	10	0,000954	3,195	0,001910
Error	0,021504	72	0,000299		

Pair-wise comparisons revealed that at T3 a significant increase (p=0.037) of TBARS levels was registered in clams exposed to mixture and kept at 18, and at T7 a significant increase was registered in clams kept at 18 and 28 (p=0.000 and p=0.002, respectively) compared to control groups at the same salinity levels (Figure 3.11).

At T7 clams at NP-MIN group showed higher TBARS values than NP-MED and NP-MAX condition (p=0.005).

A significant decrease in TBARS levels was detected in clams kept at C-MIN and C-MAX from T1 to T3 (p=0.000 and p=0.018, respectively). In contrast, a significant increase was recorded in clams kept at NP-MIN group from T3 to T7 (p=0.001).

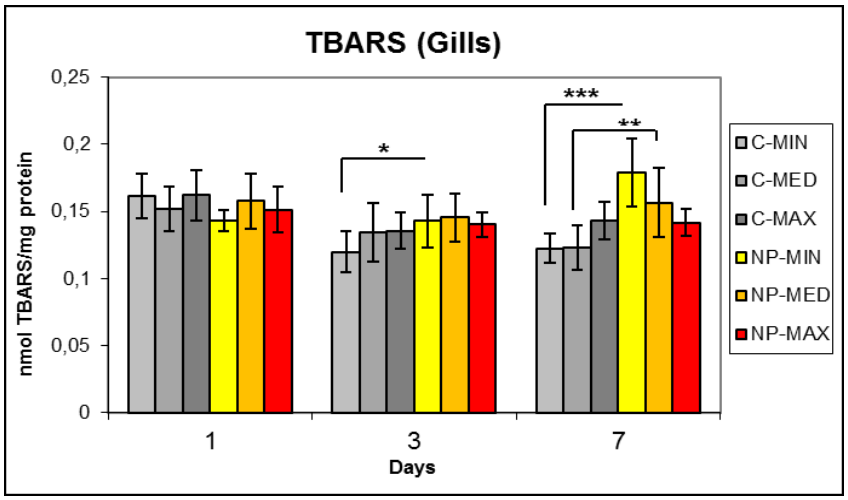

Figure 3.11. Effects of nanoparticle mixture and different salinity levels on lipid peroxidation in gills tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.8.2 Digestive gland

A two-way ANOVA analysis showed significant effect of concentration, time, and concentration/time interaction (p=0.000) on the TBARS levels in the gills tissue of the clams (Table 3.12).

Table 3.12. Two-way ANOVA table for lipid peroxidation in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	1,602151	1	1,602151	6871,166	0,000000
Concentration	0,008354	5	0,001671	7,166	0,000017
Time	0,008136	2	0,004068	17,446	0,000001
Concentration*Time	0,011404	10	0,001140	4,891	0,000023
Error	0,016788	72	0,000233		

Post-hoc test demonstrated a significant increase of TBARS levels in the clams exposed to a mixture of nanoparticles kept at 18 and 28 (p=0.014 and p=0.034, respectively) at T7, with respect to control groups at the same salinity levels (Figure 3.12).

A significantly high value of TBARS levels was found at T7 in clams kept at 38 in both control and treated groups, regarding clams at 18 and 28 (p=0.000; p=0.000 and p=0.00, respectively).

Data also showed that a significant decrease in TBARS levels occurred in clams kept at C-MIN (p=0.002), C-MAX (p=0.016) NP-MIN (p=0.006) and NP-MAX (p=0.008), from T1 to T3; followed by a significant increase from T3 to T7 in clams kept at C-MAX (p=0.000), and NP-MIN (p=0.002) and NP-MAX (p=0.000).

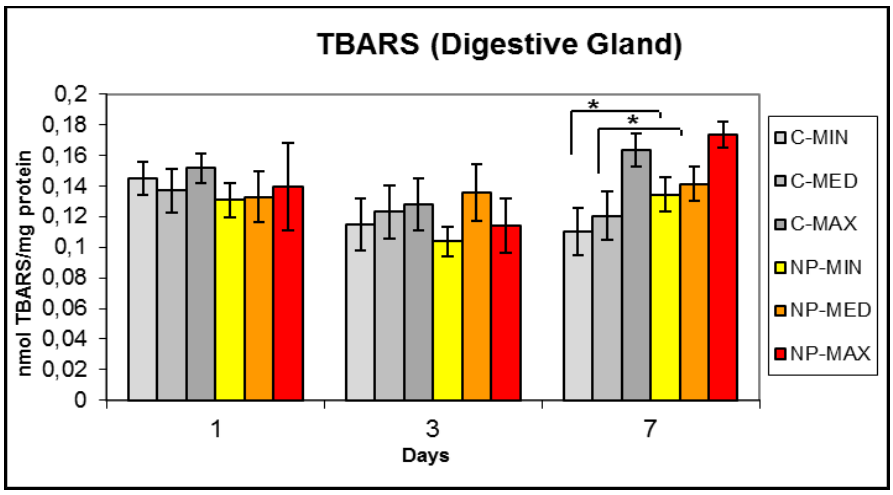

Figure 3.12. Effects of nanoparticle mixture and different salinity levels on lipid peroxidation in digestive gland tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.9 Protein Carbonyl content

3.1.9.1 Gills

Two-way ANOVA test showed that the interaction concentration/time had a significant effect on PCC (p=0.000), as well as time and concentration (p=0.000), on gills tissue (Table 3.13).

Table 3.13. Two-way ANOVA table for protein carbonyl content in gills tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	18057,46	1	18057,46	2242,430	0,000000
Concentration	395,55	5	79,11	9,824	0,000000
Time	214,60	2	107,30	13,325	0,000012
Concentration*Time	417,44	10	41,74	5,184	0,000011
Error	579,79	72	8,05		

The post-hoc test determined a significant increase in PCC in treated clams kept at 18 and 28 (p=0.000 and p=0.010, respectively) at T1, compared to controls. At T3, pair-wise comparison showed also a significant increase of PCC in clams kept at 18 (p=0.000), with respect to control groups at the same salinity levels (Figure 3.13).

Clams kept at NP-MIN, at T3, showed significantly higher values of PCC than clams at NP-MED and NP-MAX (p=0.000).

Data also demonstrated a significant increase in PCC values, from T1 to T3, in clams from C-MED (p=0.015) and NP-MIN (p=0.000) groups. In contrast, a significant decrease in PCC levels occurred in clams from NP-MIN (p=0.000) and NP-MED (p=0.003) groups, from T3 to T7.

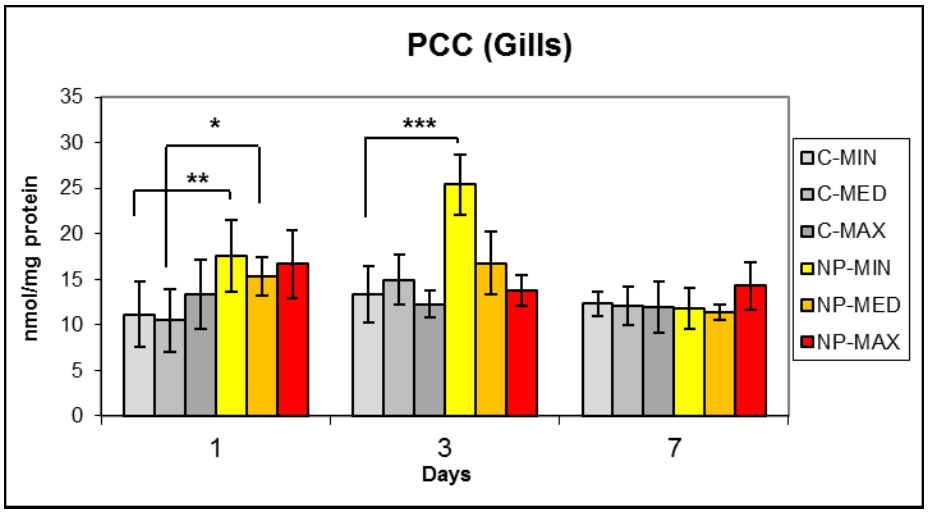

Figure 3.13. Effects of nanoparticle mixture and different salinity levels on protein carbonyl content in gills tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.9.2 Digestive gland

Two-way ANOVA analysis determined that time of exposure had a significant effect on PCC (p=0.000), whereas concentration and concentration/time interaction had no significant effect on the clams (Table 3.14).

Table 3.14. Two-way ANOVA table for protein carbonyl content in digestive gland tissue, on *R. philippinarum*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	30625,07	1	30625,07	3632,372	0,000000
Concentration	84,87	5	16,97	2,013	0,086911
Time	410,83	2	205,42	24,364	0,000000
Concentration*Time	71,80	10	7,18	0,852	0,581517
Error	607,04	72	8,43		

A pair-wise comparison showed no significant differences on PCC in digestive gland tissue between treated and untreated clams (Figure 3.14).

A significantly high level of PCC was recorded in clams in NP-MAX, at T3, regarding NP-MIN and NP-MED (p=0.004 and p=0.022).

Data also determined a significant decrease, from T3 to T7, in clams at C-MIN (p=0.005), C-MED (p=0.001), C-MAX (p=0.000), and NP-MAX (p=0.004).

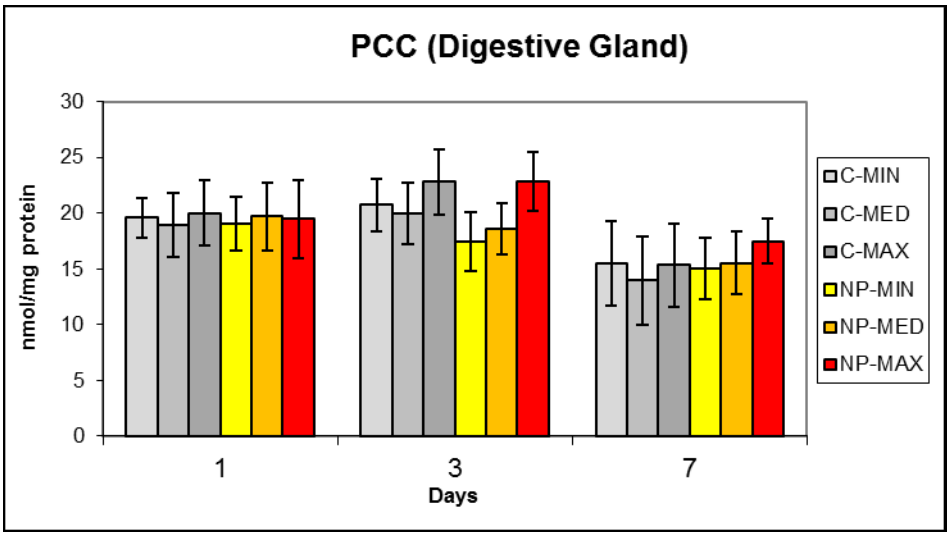

Figure 3.14. Effects of nanoparticle mixture and different salinity levels on protein carbonyl content in digestive gland tissue, in *R. philippinarum*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.1.10 Protein content

ANOVA test demonstrated that concentration did not had an effect on the protein content of *R. philippinarum* (p=0.143). The protein content values were lower in the treated groups but no significant differences were found (Figure 3.15).

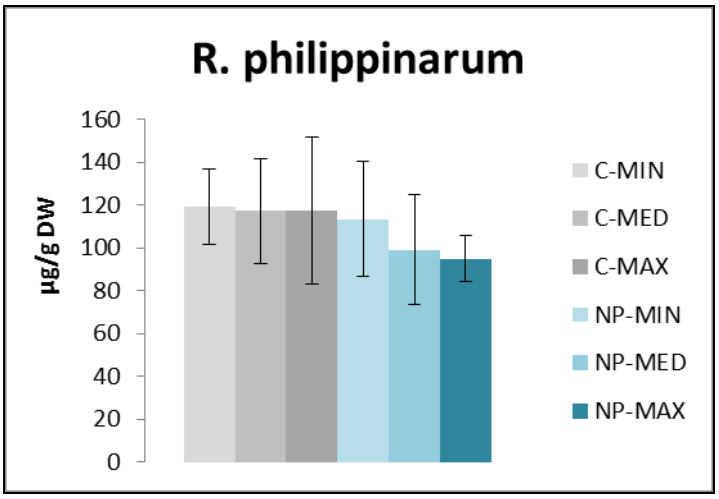

Figure 3.15. Effects of nanoparticle mixture and different salinity levels on protein content in foot tissue, in *R. philippinarum*, after 7 days of exposure. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38).

3.2 Ruditapes decussatus

3.2.1 Micronucleus frequency

Two-way ANOVA test determined that concentration had a significant effect on the formation of micronucleus in haemocytes (p=0.000), whereas time and concentration/time interaction had no significant effect (table 3.15).

Table 3.15. Two-way ANOVA table for micronucleus frequency in haemocytes, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	2086,420	1	2086,420	118,5965	0,000000
Concentration	592,099	5	118,420	6,7312	0,000034
Time	34,321	2	17,160	0,9754	0,381953
Concentration*Time	287,160	10	28,716	1,6323	0,114720
Error	1266,667	72	17,593		

A post-hoc test performed on the results shows a significant increase (p=0.048) in the micronucleus frequency in haemocytes of clams kept at 38, after three days of exposure to a mixture of nanoparticles, with respect to controls at the same salinity (figure 3.16).

At T1, a significant low value of micronucleus was recorded in clams at 18 in both control (C-MED p=0.026; C-MAX p=0.048) and treated groups (NP-MED p=0.026; NP-MAX p=0.000), comparing to higher salinities.

Data showed a significant decrease in micronucleus in NP-MAX from T3 to T7 (p=0.048).

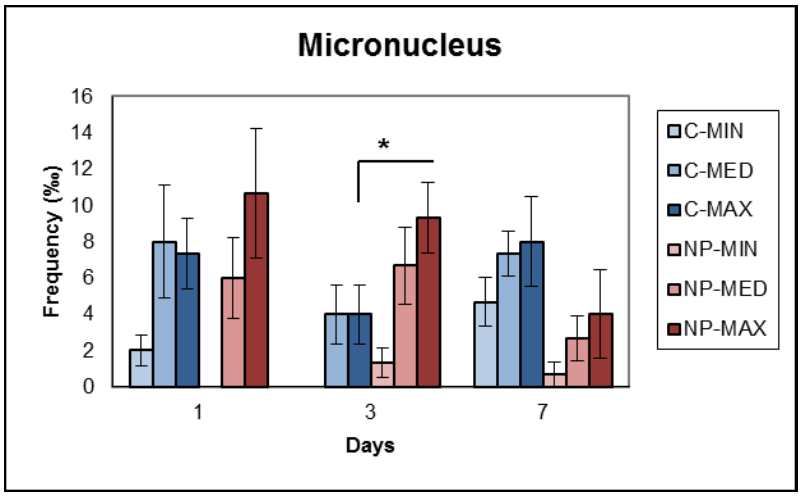

Figure 3.16. Effects of nanoparticle mixture and different salinity levels on micronucleus formation in haemocytes in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.2 Nuclear abnormalities frequency

Two-way ANOVA analysis showed that salinity concentration had a significant effect (p=0.000) on the frequency of nuclear abnormalities in haemocytes, while time and concentration/time interaction had no significant effect (Table 3.16).

Table 3.16. Two-way ANOVA table for nuclear abnormalities frequency in haemocytes, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	3443,086	1	3443,086	235,3502	0,000000
Concentration	759,136	5	151,827	10,3781	0,000000
Time	1,728	2	0,864	0,0591	0,942685
Concentration*Time	220,494	10	22,049	1,5072	0,154558
Error	1053,333	72	14,630		

Pair-wise comparisons between results shows a significant decrease (p=0.030) in nuclear abnormalities in clams kept at 38, after three days of exposure of a mixture of nanoparticles, with respect to control at the same salinity (Figure 3.17).

A significant low value was recorded at T1 in clams from C-MIN group comparing with C-MED (p=0.015) and C-MAX (p=0.003). The same pattern was observed for C-MIN (C-MED and C-MAX p=0.000) and for NP-MIN (NP-MED p=0.007 and NP-MAX p=0.030) at T3, and at T7 for NP-MIN (NP-MED p=0.015 and NP-MAX p=0.030).

A significant increase in nuclear abnormalities was registered in C-MAX from T1 to T3 (p=0.030), followed by a significant decrease from T3 to T7 (p=0.007).

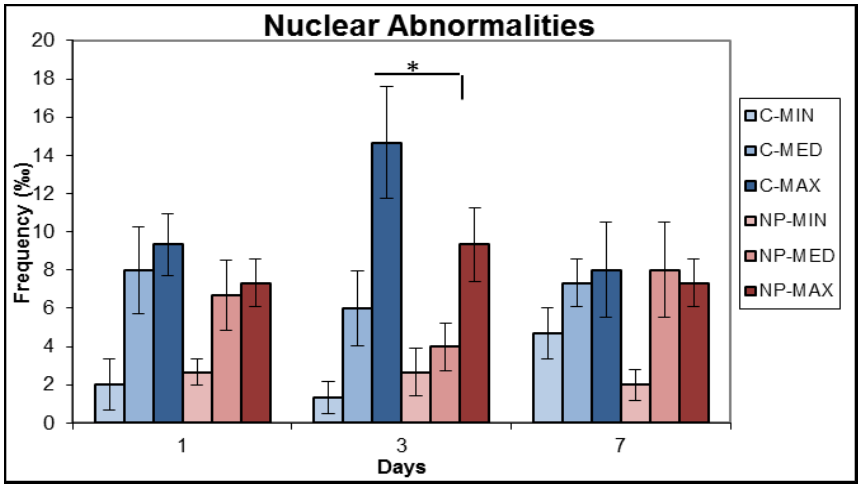

Figure 3.17. Effects of nanoparticle mixture and different salinity levels on nuclear abnormalities on haemocytes in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.3 Cytotoxicity activity

Two-way ANOVA test showed that both concentration and time had a significant effect (p=0.000) on the enzyme lactate dehydrogenase levels, as did the concentration/time interaction (p=0.000) (Table 3.17).

Table 3.17. Two-way ANOVA table for cytotoxicity activity in haemocytes, on *R. decussatus,* exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	13,29842	1	13,29842	1315,593	0,000000
Concentration	2,76254	5	0,55251	54,659	0,000000
Time	0,70394	2	0,35197	34,820	0,000000
Concentration*Time	0,50869	10	0,05087	5,032	0,000016
Error	0,72780	72	0,01011		

Pair-wise comparisons showed that, at T3, LDH levels significantly increased in clams kept at 18 and 28 (p=0.004 and p=0.000, respectively), compared with controls at the same salinities. At T7, clams from all three salinity concentrations showed significantly increased levels of LDH (18 and 38 p=0.000; 28 p=0.039 (Figure 3.18).

Within the control and treated group significant differences were detected in all measured days in clams at 18 showing values higher than the other groups. AT T1, C-MIN showed higher levels than C-MED and C-MAX (p=0.000), as did NP-MIN with NP-MED (p=0.008) and NP-MAX (p=0.000). At T3 the same pattern was registered with C-MIN differentiating from C-MED (p=0.003) and C-MAX (p=0.000), NP-MIN differentiating from NP-MED and NP-MAX (p=0.000), and NP-MED from NP-MAX (p=0.000). At T7, C-MIN differed from C-MED and C-MAX (p=0.000), and NP-MIN differed from NP-MED and NP-MAX (p=0.000).

A significant increase in LDH levels was recorded in C-MIN (p=0.012), NP-MIN (p=0.000) and NP-MED (p=0.000) groups from T1 to T3. A significant increase was also registered in NP-MAX (p=0.000) from T3 to T7.

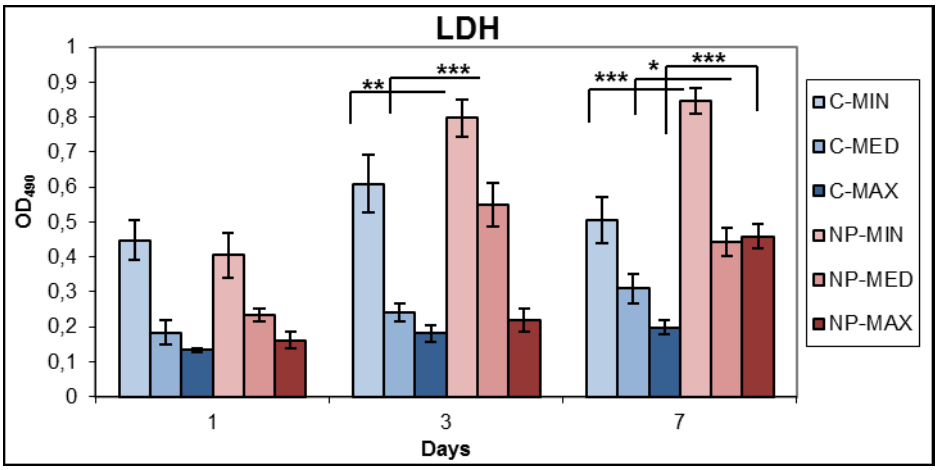

Figure 3.18. Effects of nanoparticle mixture and different salinity levels on lactate dehydrogenase on haemocytes in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.4 Neutral Red uptake

Two-way ANOVA analysis determined that only concentration had a significant effect on the pinocytotic activity by haemocytes (p=0.001), whereas time and concentration/time interaction had no significant effect (Table 3.18).

Table 3.18. Two-way ANOVA table for neutral red uptake of haemocytes, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	0,462250	1	0,462250	738,9169	0,000000
Concentration	0,013615	5	0,002723	4,3529	0,001618
Time	0,001329	2	0,000665	1,0624	0,350970
Concentration*Time	0,001456	10	0,000146	0,2327	0,992050
Error	0,045042	72	0,000626		

Pair-wise comparison showed no significant difference between clams treated with a mixture of nanoparticles and the control groups at the same salinity, on pinocytotic activity (Figure 3.19).

At T1 C-MIN showed significant high values of NRU compared with C-MAX (p=0.015). At T7 C-MIN also showed significant high values when compared with C-MED (p=0.009) and C-MAX (p=0.005).

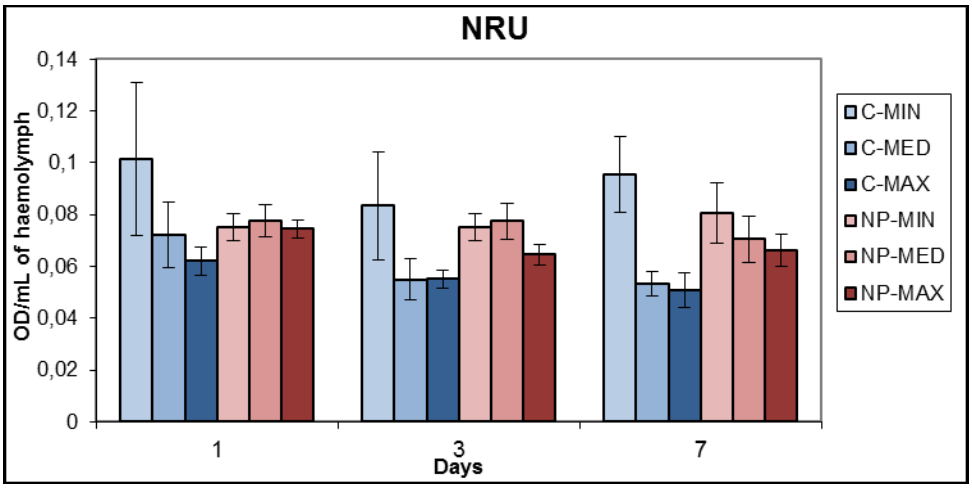

Figure 3.19. Effects of nanoparticle mixture and different salinity levels on pinocytotic activity in haemocytes in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; MIN=18; Salinity: MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.5 Catalase activity

3.2.5.1 Gills

Two-way ANOVA test showed that catalase activity in gills tissue of clams was significantly affected by both concentration and time (p=0.000), and by concentration/time interaction (p=0.000) (Table 3.19).

Table 3.19. Two-way ANOVA table for catalase activity in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	37778,15	1	37778,15	5423,415	0,000000
Concentration	311,80	5	62,36	8,952	0,000001
Time	1620,94	2	810,47	116,350	0,000000
Concentration*Time	595,27	10	59,53	8,546	0,000000
Error	501,53	72	6,97		

Post-hoc test shows a significant increase (p=0.000) on CAT activity in clams kept at 18, at T1. At T3, a significant decrease (p=0.000) in CAT activity was recorded in clams from 28. In the end of exposure, T7, a significant increase of CAT activity was recorded, in clams kept at 18 and 38 (p=0.000 and p=0.007, respectively), with respect to control groups at the same salinity (Figure 3.20).

At T1 C-MED showed significant high value of CAT activity comparing with C-MIN (p=0.008), the same was observed in NP-MIN and NP-MED when compared with NP-MAX

(p=0.000). At T3 C-MIN showed a significant low value comparing with C-MED (p=0.026) and C-MAX (p=0.010), in contrast NP-MAX showed a significant high value when compared with NP-MIN and NP-MED (p=0.000). At T7 clams at C-MIN group showed a significant low value comparing with C-MED (p=0.000) and C-MAX (p=0.001).

A significant decrease in CAT activity was recorded in the C-MIN (p=0.010), C-MED (p=0.003), NP-MIN (p=0.000) and NP-MED (p=0.000) groups from T1 to T3, followed by a significant increase from T3 to T7 in C-MIN, C-MED, C-MAX, NP-MIN, NP-MED and NP-MAX groups (p=0.000).

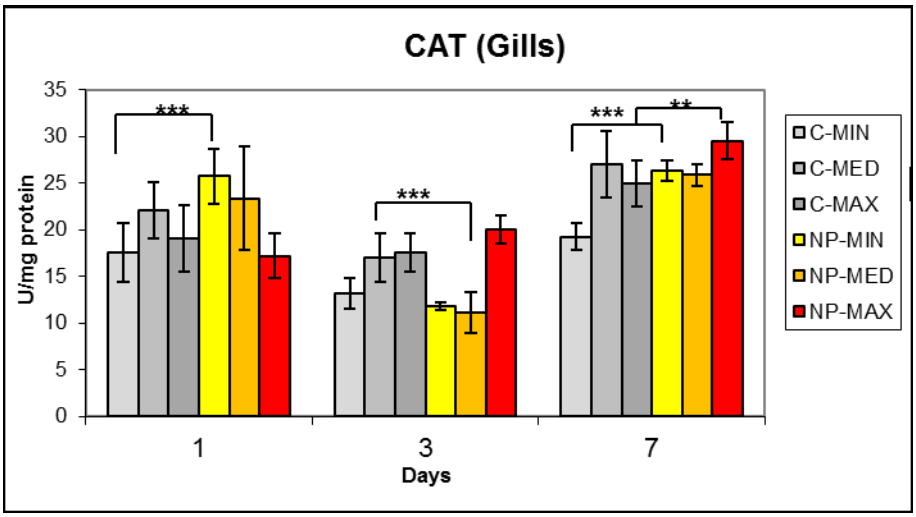

Figure 3.20. Effects of nanoparticle mixture and different salinity levels on catalase activity in gills tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.5.2 Digestive Gland

Two-way ANOVA test shows that concentration, time and concentration/time interaction had a significant effect (p=0.000) on catalase activity in digestive gland tissue (Table 3.20).

Table 3.20. Two-way ANOVA table for catalase activity in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	44814,35	1	44814,35	4073,291	0,000000
Concentration	399,95	5	79,99	7,271	0,000015
Time	1737,74	2	868,87	78,974	0,000000
Concentration*Time	763,56	10	76,36	6,940	0,000000
Error	792,14	72	11,00		

Pair-wise comparisons shows a significant increase in CAT activity in clams at 18 (p=0.026), at T1, whereas in clams at 28, a significant decrease (p=0.012) was recorded. At

T3, in clams at 28, a significant increase in CAT was recorded (p=0.000), and at T7 a significant increase was registered in clams at 28 and 38 (p=0.012 and p=0.000, respectively), with respect to control groups at the same salinity (Figure 3.21).

Within the control group, at T1 C-MED and C-MAX showed a significant high value when compared with C-MIN group (p=0.003 and p=0.005, respectively), at T3 C-MIN and C-MAX also showed a significant high value compared with C-MED (p=0.000 and p=0.003, respectively), and at T7 C-MAX had a significant high value compared with C-MIN group (p=0.003). Within the treated group both NP-MED and NP-MAX showed significant high values of CAT activity compared with NP-MIN (p=0.023 and p=0.000, respectively).

From T1 to T3 a significant increase in CAT activity was recorded in C-MIN and NP-MED (p=0.000) whereas C-MED showed a significant decrease (p=0.000). From T3 to T7 a significant increase was registered in C-MED, C-MAX, NP-MIN, NP-MED and NP-MAX (p=0.000).

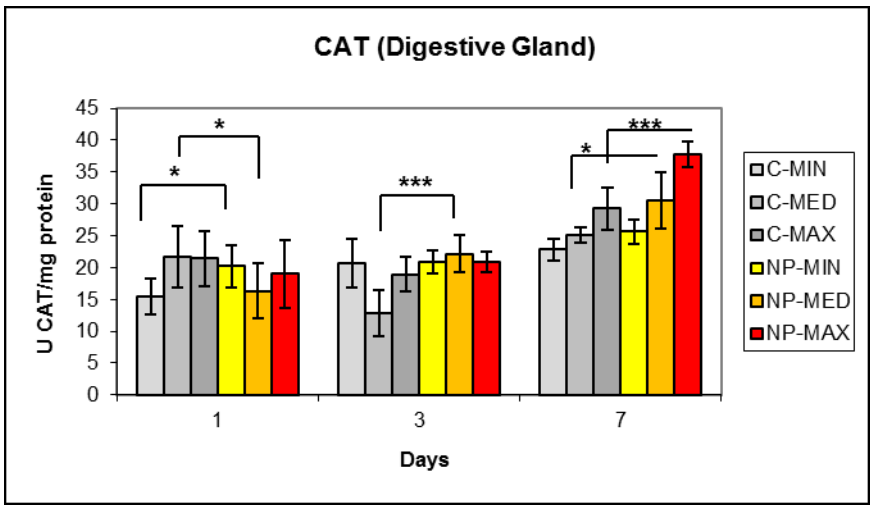

Figure 3.21. Effects of nanoparticle mixture and different salinity levels on catalase activity in digestive gland tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.6 Superoxide dismutase activity

3.2.6.1 Gills

Two-way ANOVA analysis determined that concentration, time and concentration/time interaction significantly (p=0.000) affected superoxide dismutase activity, in the gills tissue (Table 3.21).

Table 3.21. Two-way ANOVA table for superoxide dismutase activity in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	74148,99	1	74148,99	15938,60	0,000000
Concentration	311,30	5	62,26	13,38	0,000000
Time	3392,31	2	1696,16	364,60	0,000000
Concentration*Time	732,10	10	73,21	15,74	0,000000
Error	334,96	72	4,65		

Post-hoc test performed shows a significant decrease in SOD activity at T1 in clams kept at 18 (p=0.000), whereas clams kept at 38 significantly increased SOD activity (p=0.000). At T3, clams kept at 18 and 28 showed a significant decrease in SOD activity (p=0.000); and at T7 a significant increase in SOD activity was recorded in clams at 28 and 38 (p=0.000 and p=0.003, respectively), with respect to control groups at the same salinity (Figure 3.22).

Significantly high values of SOD were shown at T1 by C-MIN and C-MED compared to C-MAX (p=0.0002 and p=0.000 respectively), and by NP-MAX compared to NP-MIN and NP-MED (p=0.000), at T3 by C-MAX compared to C-MIN (p=0.044), and by NP-MAX when compared with NP-MIN (p=0.000) and NP-MED (p=0.037), at T7 by C-MAX compared to C-MIN (p=0.016) and by NP-MED and NP-MAX compared to NP-MIN (p=0.003 and p=0.000 respectively).

A significant decrease in SOD activity was observed from T1 to T3 in C-MIN, NP-MED and NP-MAX (p=0.000), and the significant decrease continued from T3 to T7 in clams from C-MIN, C-MED, C-MAX, NP-MIN, NP-MED and NP-MAX (p=0.000 for all except NP-MED p=0.011).

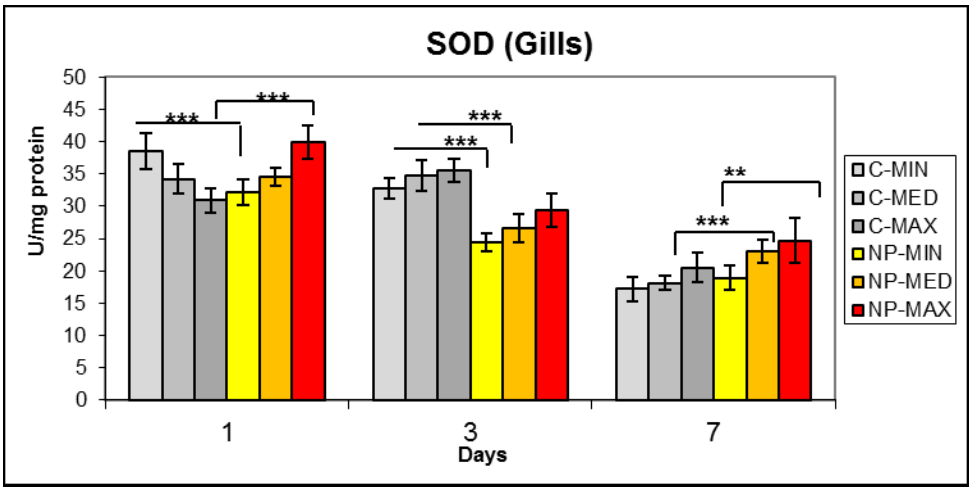

Figure 3.22. Effects of nanoparticle mixture and different salinity levels on superoxide dismutase activity in gills tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.6.2 Digestive gland

Two-way ANOVA showed that concentration, time, and concentration/time interaction had a significant effect (p=0.000) on the SOD activity in the digestive gland tissue of the clams (Table 3.22).

Table 3.22. Two-way ANOVA table for superoxide dismutase activity in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	79667,41	1	79667,41	16420,52	0,000000
Concentration	678,47	5	135,69	27,97	0,000000
Time	2030,82	2	1015,41	209,29	0,000000
Concentration*Time	296,09	10	29,61	6,10	0,000001
Error	349,32	72	4,85		

Pair-wise comparisons of data showed, at T1, a significant decrease in SOD activity in digestive gland tissue, in clams kept at 18 (p=0.001), at T3, clams kept at 38 showed a significant increase in SOD activity (p=0.000), and at T7, a significant increase of SOD was recorded in clams kept at 28 and 38 (p=0.006 and p=0.049, respectively), with respect to control groups at the same salinity (Figure 3.23).

Significantly high values of SOD were shown at T1 by clams at NP-MED and NP-MAX in comparison with NP-MIN (p=0.001 and p=0.000 respectively), at T3 by C-MED compared to C-MIN (p=0.014) and C-MAX (p=0.023) and by NP-MAX compared to NP-MIN and NP-MED (p=0.000), and at T7 by NP-MED and NP-MAX when compared with NP-MIN (p=0.001 and p=0.000 respectively).

A significant increase in SOD activity was register in NP-MIN and NP-MAX (p=0.001 and p=0.000 respectively) from T1 to T3, followed by a significant decrease from T3 to T7 in clams at C-MIN, C-MED, C-MAX, NP-MIN, NP-MED and NP-MAX (p=0.000).

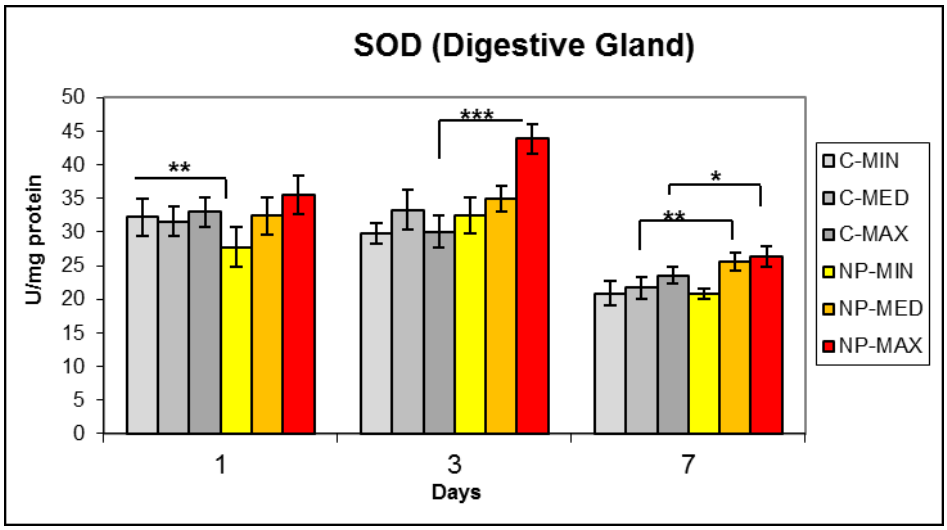

Figure 3.23. Effects of nanoparticle mixture and different salinity levels on superoxide dismutase activity in digestive gland tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.7 Glutathione S-transferase activity

3.2.7.1 Gills

Two-way ANOVA analysis showed that concentration, time, and concentration/time interaction had a significant effect (p=0.000) on the activity of GST in gills tissue (Table 3.23).

Table 3.23. Two-way ANOVA table for glutathione S-transferase activity in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	110069023	1	110069023	1010,891	0,000000
Concentration	8642391	5	1728478	15,875	0,000000
Time	2269664	2	1134832	10,422	0,000106
Concentration*Time	4183732	10	418373	3,842	0,000341
Error	7839593	72	108883		

Post-hoc test showed, at T1, a significant decrease in GST activity in clams kept at 18 (p=0.011), whereas clams at 38 showed a significant decrease (p=0.000). At T7, clams kept at 18 registered a significant decrease in GST activity (p=0.000), with respect to control groups at the same salinity (Figure 3.24).

Significantly high values of GST were presented at T1 by C-MAX compared with C-MED and C-MAX (p=0.000), at T3 by C-MIN and C-MAX when compared to C-MED

(p=0.012 and p=0.007 respectively) and by NP-MAX when compared with NP-MED (p=0.021), and at T7 by NP-MED and NP-MAX compared to NP-MIN (p=0.000 and p=0.021).

A significant decrease in GST values was found in clams at C-MED and C-MAX from T1 to T3 (p=0.004 and p=0.000 respectively) followed by a significant increase from T3 to T7 by C-MED (p=0.005), NP-MED (p=0.007) and NP-MAX (p=0.011).

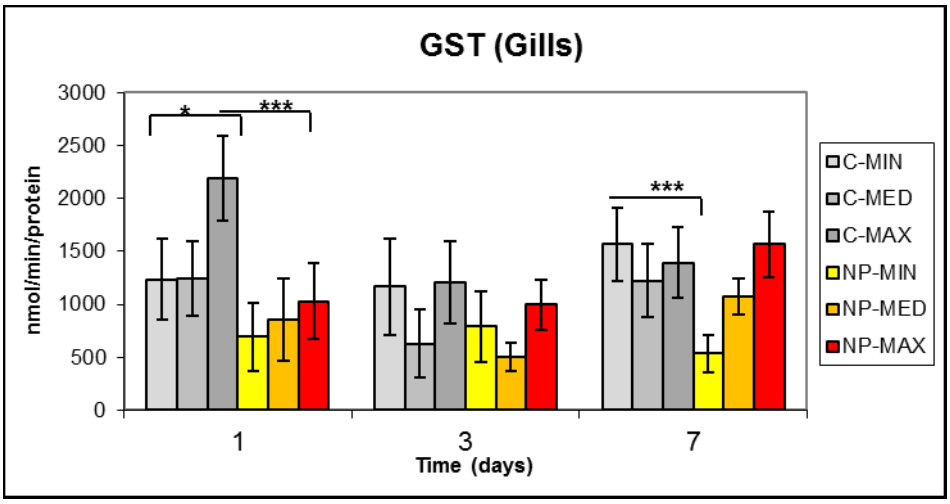

Figure 3.24. Effects of nanoparticle mixture and different salinity levels on glutathione S-transferase activity in gills tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.7.2 Digestive gland

Two-way ANOVA test determined that both time (p=0.000) and concentration (p=0.038), as well as concentration/time interaction (p=0.043) had a significant effect on the activity of GST enzyme in digestive gland tissue of clams (Table 3.24).

Table 3.24. Two-way ANOVA table for glutathione S-transferase activity in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	121590199	1	121590199	1612,938	0,000000
Concentration	938928	5	187786	2,491	0,038798
Time	3179040	2	1589520	21,086	0,000000
Concentration*Time	1520708	10	152071	2,017	0,043712
Error	5427669	72	75384		

Pair-wise comparisons showed no significant differences between controls and treated groups of clams (Figure 3.25).

A significant high value of GST was found in C-MIN when compared with C-MED and C-MAX (p=0.000 and p=0.005 respectively) and in NP-MIN when compared to NP-MAX (p=0.032).

A significant increase in GST activity was found from T1 to T3 in C-MED and C-MAX (p=0.013 and p=0.042 respectively), followed by a decrease in C-MED (p=0.020), C-MAX (p=0.000), NP-MED (p=0.003) and NP-MAX (p=0.026).

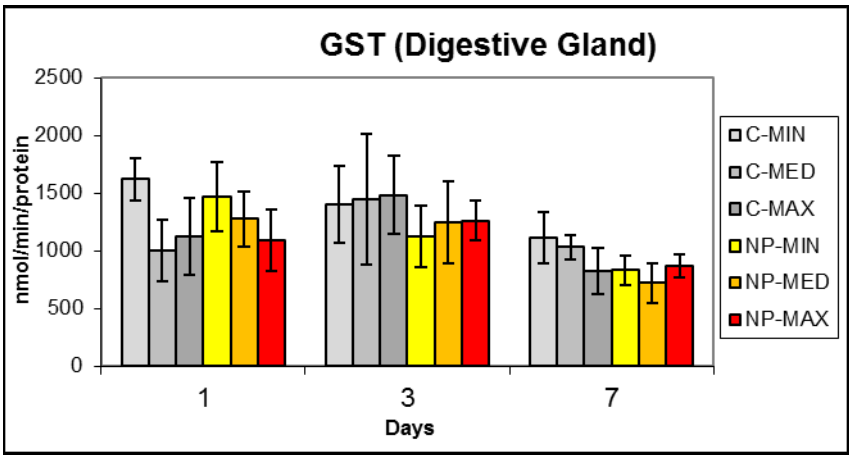

Figure 3.25. Effects of nanoparticle mixture and different salinity levels on glutathione S-transferase activity in digestive gland tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.8 Lipid peroxidation

3.2.8.1 Gills

Two-way ANOVA test showed that time, concentration and concentration/time interaction (p=0.000) had a significant effect on GST activity in digestive gland tissue of clams (Table 3.25).

Table 3.25. Two-way ANOVA table for lipid peroxidation in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	0,294850	1	0,294850	1430,266	0,000000
Concentration	0,027666	5	0,005533	26,841	0,000000
Time	0,015839	2	0,007920	38,416	0,000000
Concentration*Time	0,034237	10	0,003424	16,608	0,000000
Error	0,014843	72	0,000206		

Pair-wise comparisons showed a significant decrease in TBARS levels in clams kept 18, 28 and 38 (p=0.000) at T1, with respect to control groups at the same salinity (Figure 3.26).

At T1 C-MIN and C-MED showed significantly high values of TBARS when compared to C-MAX (p=0.040 and p=0.000 respectively), and at T3 the same pattern was observed in C-MIN and C-MED compared to C-MAX (p=0.043 and p=0.002). At T7 NP-MAX presented significantly high compared to NP-MED (p=0.008).

A significant decrease in TBARS levels was recorded from T1 to T3 in C-MIN, C-MED and C-MAX (p=0.000), followed by a significant increase in C-MIN, C-MAX, NP-MIN and NP-MAX (p=0.017, p=0.001, p=0.000 and p=0.000 respectively).

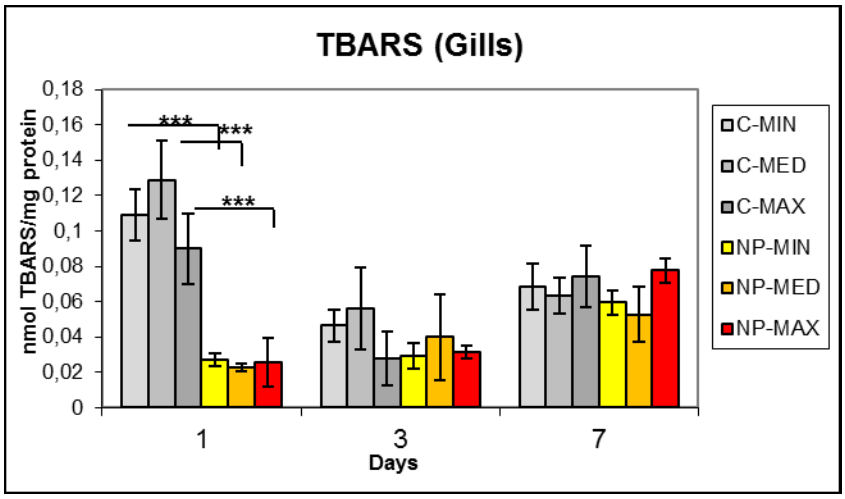

Figure 3.26. Effects of nanoparticle mixture and different salinity levels on TBARS levels in gills tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.8.2 Digestive gland

Two-way ANOVA test determined that concentration, time and the interaction concentration/time had a significant effect (p=0.000) on the TBARS levels (Table 3.26).

Table 3.26. Two-way ANOVA table for lipid peroxidation in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	2,919770	1	2,919770	6474,014	0,000000
Concentration	0,037889	5	0,007578	16,802	0,000000
Time	0,086256	2	0,043128	95,628	0,000000
Concentration*Time	0,024515	10	0,002452	5,436	0,000006
Error	0,032472	72	0,000451		

Pair-wise comparisons showed, at T3, a significant increase in TBARS levels, in clams kept at 28 (p=0.038). At T7, a significant increase of TBARS levels was recorded in clams kept at 18 and 38 (p=0.000), with respect to control groups at the same salinity (Figure 3.27).

Significantly high values of TBARS were presented at T1 in C-Max when compared to C-MED (p=0.047), at T3 by NP-MED and NP-MAX when compared to NP-MIN (p=0.002 p=0.004 respectively), and at T7 by NP-MIN and NP-MAX compared to NP-MED (p=0.040 and p=0.000 respectively).

A significant increase in TBARS levels was registered from T1 to T3 in clams from C-MIN (p=0.000), C-MED (p=0.000), C-MAX (p=0.000), NP-MED (p=0.000) and NP-MAX (p=0.003), followed by another increase in clams from NP-MIN and NP-MAX (p=0.000).

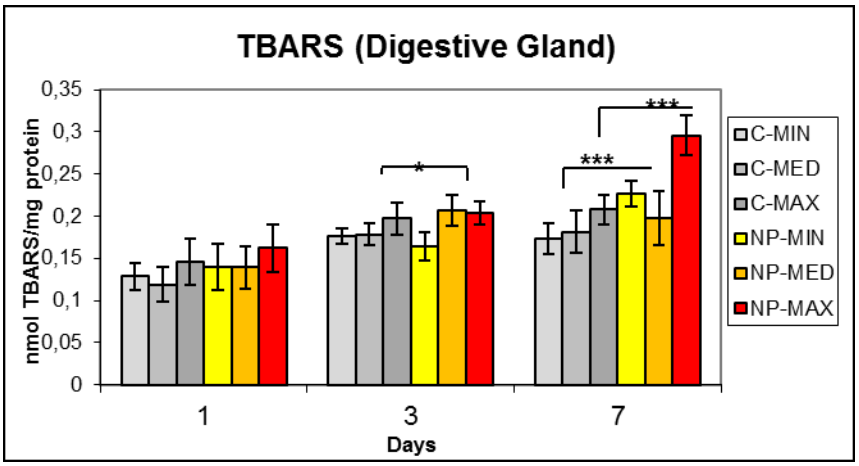

Figure 3.27. Effects of nanoparticle mixture and different salinity levels on TBARS levels in digestive gland tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.9 Protein carbonyl content

3.2.9.1 Gills

Two-way ANOVA test showed that both time and concentration (p=0.000), as well as concentration/time interaction (p=0.016), had a significant effect on the protein carbonyl content (Table 3.27).

Table 3.27. Two-way ANOVA table for protein carbonyl content in gills tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	38978,33	1	38978,33	3599,835	0,000000
Concentration	425,00	5	85,00	7,850	0,000006
Time	888,87	2	444,43	41,046	0,000000
Concentration*Time	259,42	10	25,94	2,396	0,016220
Error	779,60	72	10,83		

Post-hoc test showed a significant increase in PCC in gills tissue, at T3, in clams kept at 38 (p=0.000). At T7, a significant increase in PCC was recorded in clams at 38 (p=0.002), with respect to controls at the same salinity (Figure 3.28).

At T3 a significantly high value of PCC was found in C-MED when compared to C-MAX (p=0.010), and in NP-MAX when compared to NP-MIN and NP-MED (p=0.004 and p=0.000 respectively). At T7 clams from C-MED showed significantly high PCC levels when compared to C-MIN (p=0.003) and C-MAX (p=0.004).

A significant increase of PCC was found in C-MIN (p=0.000), C-MED (p=0.012), NP-MIN (p=0.000), NP-MED (p=0.000) and NP-MAX (p=0.000), followed by a significant decrease in C-MIN (p=0.007), NP-MIN (p=0.015) and NP-MAX (p=0.000).

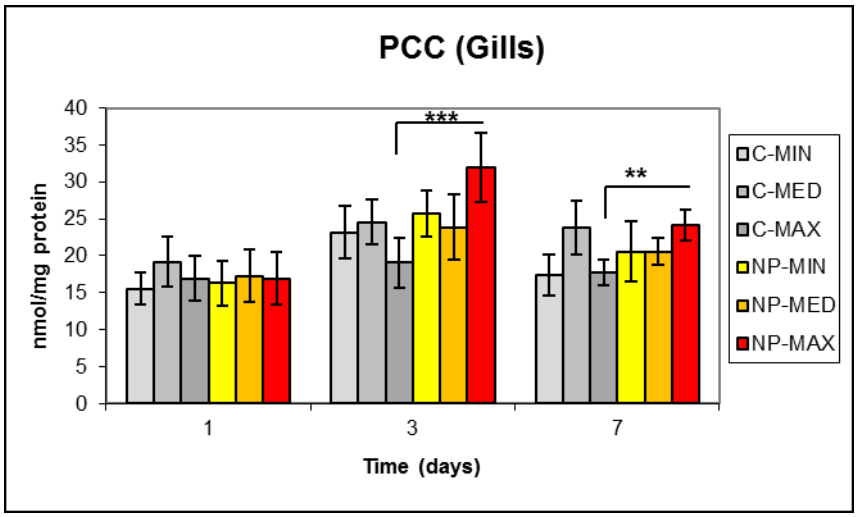

Figure 3.28. Effects of nanoparticle mixture and different salinity levels on protein carbonyl content in gills tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.9.2 Digestive gland

Two-way ANOVA analysis determined that time, concentration and the interaction concentration/time had a significant effect (p=0.000) on PCC in the digestive gland tissue in clams (Table 3.28).

Table 3.28. Two-way ANOVA table for protein carbonyl content in digestive gland tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	55167,39	1	55167,39	6857,382	0,000000
Concentration	496,20	5	99,24	12,336	0,000000
Time	5111,21	2	2555,60	317,665	0,000000
Concentration*Time	515,84	10	51,58	6,412	0,000001
Error	579,24	72	8,04		

Post-hoc test showed a significant increase of PCC, at T1, in clams kept at 18 and 28 (p=0.005 and p=0.035, respectively). At T3, a significant increase in PCC was recorded in clams kept at 28 and 38 (p=0.000), with respect to control groups at the same salinity (Figure 3.29).

Significant high values of PCC were found at T1 in C-MAX compared with C-MIN (p=0.005), at T3 in C-MIN compared with C-MED (p=0.049) and C-MAX (p=0.006) and in NP-MED and NP-MAX compared with NP-MIN (p=0.000), and at T7 in NP-MAX compared with NP-MIN (p=0.034) and NP-MED (p=0.000).

A significant increase in PCC levels was found in all groups from T1 to T3 (p=0.000), followed by a significant decrease also in all groups (p=0.000).

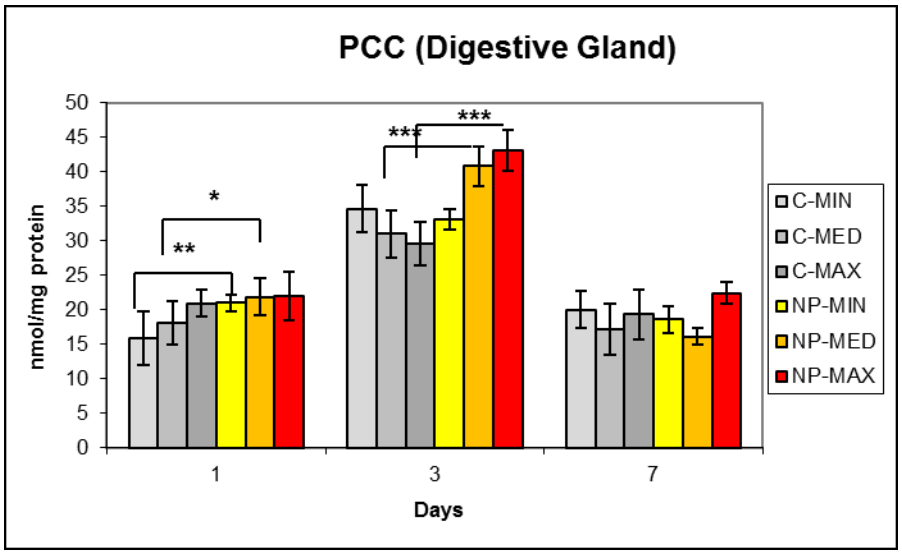

Figure 3.29. Effects of nanoparticle mixture and different salinity levels on protein carbonyl content in digestive gland tissue, in *R. decussatus*, exposed for 7 days. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.2.10 Protein content

ANOVA test (Figure 3.29) showed that concentration had a significant effect on the protein content in *R. decussatus* (p=0.000).

Table 3.29. One-way ANOVA table for protein content in foot tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	475709,0	1	475709,0	1840,686	0,000000
Concentration	20170,9	5	4034,2	15,610	0,000000
Error	9303,9	36	258,4		

Post-hoc test (Figure 3.30) showed significant decrease in protein content in clams kept at 28 and 38 (p=0.015 and p=0.000, respectively), with respect to the control at the same salinities

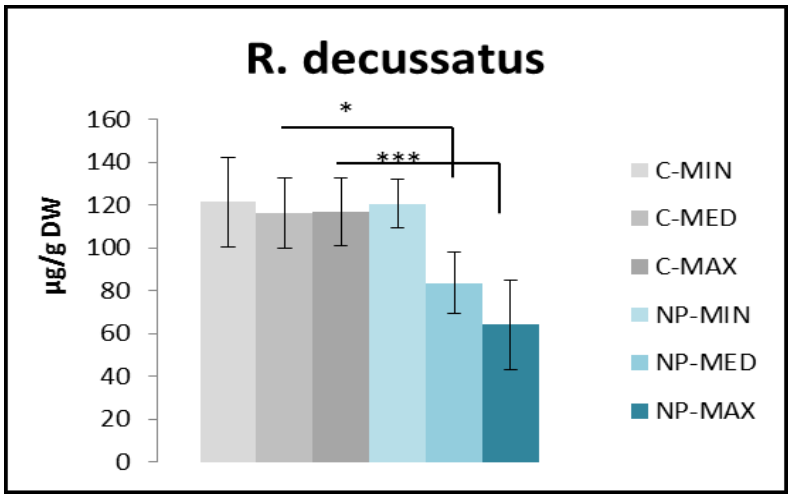

Figure 3.30. Effects of nanoparticle mixture and different salinity levels on protein content in foot tissue, in *R. decussatus*, after 7 days of exposure. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38). Asterisks show statistical significant differences compared with the control group (* = p<0.05; ** = p<0.01; *** = p<0.001).

3.3 RNA/DNA ratios

3.3.1 Ruditapes philippinarum

ANOVA analysis demonstrated that concentration did not had a significant effect on the RNA/DNA ratios of *R. philippinarum* (p=0.392), the values remained constant between control and treated groups.

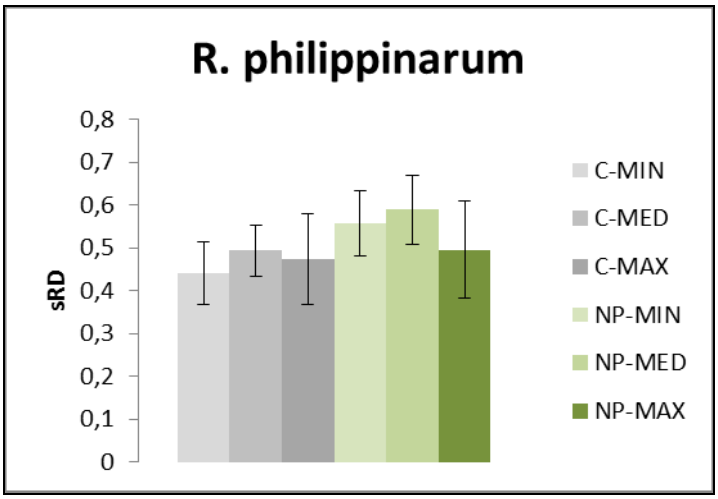

Figure 3.31. Effects of nanoparticle mixture and different salinity levels on RNA/DNA ratios in foot tissue, in *R. philippinarum*, after 7 days of exposure. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38).

3.3.2 Ruditapes decussatus

ANOVA test (Table 3.30) showed that concentration had a significant effect on the RNA/DNA ratios of the clam *R. decussatus* (p=0.010).

Table 3.30. One-way ANOVA table for RNA/DNA ratios in foot tissue, on *R. decussatus*, exposed to a mixture of NPs at different salinity levels, for 7 days (red values show statistically significant differences) (SS=Sum of squares; DF= Degrees of freedom; MS=Mean Square).

	SS	DF	MS	F	p
Intercept	8,836366	1	8,836366	412,7415	0,000000
Concentration	0,376017	5	0,075203	3,5127	0,010925
Error	0,770723	36	0,021409		

Controls groups at 28 and 38 showed lower values than control at 18, as did treated clams at 38 but pair-wise comparisons showed no significant differences between controls and treated groups of clams at the same salinities (Figure 3.32).

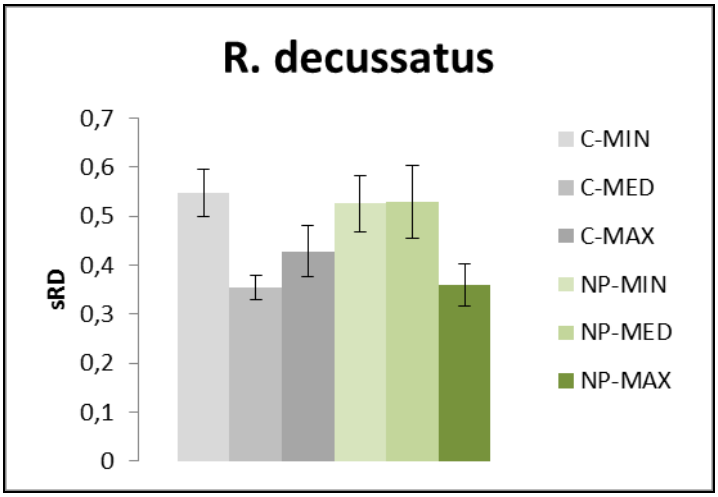

Figure 3.32. Effects of nanoparticle mixture and different salinity levels on RNA/DNA ratios in foot tissue, in *R. decussatus*, after 7 days of exposure. Values are means ± standard deviation (C=control; NP=nanoparticle mixture; Salinity: MIN=18; MED=28; MAX=38).

4 Discussion

In this study the combined effect of a mixture of NPs (ZnO, TiO_2 and fullerene C60) and different salinity levels (18, 28 and 38) on a battery of biomarkers of *R. philippinarum* and *R. decussatus* was evaluated. The organisms in the Venice lagoon are exposed to a large variation of salinity levels throughout the year, and with the NPs contamination it is pertinent to inquire if the combined effect can induce damage. *R. philippinarum* is an invasive species in the lagoon of Venice, and in several other locations, and has supplanted the native species that now inhabit very restricted areas. This study intends to compare these two species and understand if the damage level differs between them. A revision of the literature revealed that little work was done on the combined effect of contaminants and variations on the salinity.

4.1 Haemolymph

Many studies have demonstrated that both biotic and abiotic factors can alter haemocyte functional responses in bivalve molluscs. Hemolymph is widely used in toxicological investigations, and several studies suggest that haemocytes are important in internal defense of clams (Donaghy *et al.*, 2009).

The MN test is commonly performed in environmental studies in order to detect genotoxicity. In this work, MN detected significant damage in the genetic material of *R. philippinarum* at T1, in clams at 38, whereas in *R. decussatus* significant damage was detected at T3, also in clams at 38, regarding the untreated clams. *R. philippinarum* clams in the treated group at 38 showed significant damage in all measured days, regarding other salinities, while in *R. decussatus* showed only significant damage at T1. As with the pattern observed for MN, nuclear abnormalities (NA) in *R. philippinarum* clams at 28 and 38 in both treated and control groups showed significant damage at T1 and T3, while *R. decussatus* showed significant damage on the control and treated group at T3 and at T7 only on treated group. The results suggest that high salinity is responsible for DNA damage in both species, even though it appears more accentuated in *R. philippinarum*. Time had little effect on the MN of both clams but in NA on *R. philippinarum* a significant increase in frequency occurred in clams at high salinities from T3 to T7. DNA damage is only possible to see after the cell

has passed by at least one cycle of cellular division. Therefore low frequency is expected in acute assays, at least in the early days.

Little information exists on the influence of salinity on DNA damage in bivalves. Several studies show that bivalves exposed to NPs can suffer DNA damage. A study performed on the saltwater mussel *Mytilus galloprovincialis* when exposed to $nTiO_2$ showed significant increase in micronucleated cell frequency (Rocco *et al.*, 2015), and the saltwater clam *Scrobicularia plana* showed an increase in DNA damage in haemocytes, when exposed to nCuO (Buffet *et al.*, 2013).Also, the freshwater mussel *Elliptio complanata*, when exposed to nCdTe and nAg, showed increase DNA damage, in the gills and digestive gland (Gagnè *et al.*, 2008; Gagnè *et al.*, 2013). Exposure high salinities and NPs mixture increased the frequency in DNA damage in the clams' haemocytes suggesting a combined effect, more clear and accentuated in the invasive clam.

LDH is a stable cytoplasmic enzyme that can be released into the hemolymph due to cell damage. In this study, LDH was used to assess the cytotoxicity of the combination of a mixture of NPs and different salinity levels in *R. philippinarum* and *R. decussatus*. Both clams showed the same pattern with significant higher levels of LDH in clams kept at 18 in control and treated groups. *R. decussatus* showed significant differences between treated and control group, in clams at 18 and 28 after three days of exposure, and in clams at 38 after seven days of exposure.

Results suggest that NPs mixture caused destabilization of haemocyte membranes and induced a release of LDH, since all treated groups showed significant differences. Studies with the clam *S. plana* when exposed to nCuO showed no change in LDH levels (Buffet *et al*., 2014). Contrarily, in an exposure to nZnO, *S. plana*, showed increase in LDH values (Buffet *et al.*, 2012) when analyzed the entire organism. *R. philippinarum* results agreed with the results of the first study showing no differences between treated and control groups, suggesting that only lower salinity had an effect, inducing a release of LDH from the haemocytes, but with values considerably lower than the native clam. Contrarily, *R. decussatus* results suggest that the clams were affect by the NPs. The differences between *R. philippinarum* and *R. decussatus* support the premise that the former clam is somewhat more resistant to contamination than the latter clam.

Phagocytosis is the main internal defense mechanism against pathogens. The NRU assay was used in this work to evaluate pinocytotic capability in haemocyte. The uptake can occur

by pinocytosis or passive diffusion across the cell membrane. Any alteration, increase or decrease, can imply weakening of pinocytotic activity or damage of cellular membrane. No decrease or increase in pinocytotic activity was found between clam groups in both species. These results differ from others reports that indicate that phagocytosis have been reduced in bivalves submitted to salinity stress, like the clam *Macrocallista nimbosa* showed decrease in pinocytotic capability when exposed to low salinity (18) (Jauzein *et al*., 2013). Also, when exposed to NPs, the clam *R. philippinarum* showed an increase in the pinocytotic activity (Matozzo and Marin, 2005). The results of this work suggest that salinity and NPs mix combination did not affect pinocytotic activity in the clams.

4.2 Oxidative stress

When an organism is exposed to ROS-generating conditions, the antioxidant systems induce the activity of antioxidant enzymes, like CAT, SOD and GST. It can also happen that the exposure to contaminants causes inhibition of the antioxidant enzymes. If the defenses are overwhelmed then the organism is experiencing oxidative stress. In this study, antioxidant enzymes activity was analyzed in both gills and digestive gland. Gills of clams process the water that enters the organism being the first organ to contact with the contaminants. This tissue is particular representative and is used in environmental studies. After passing through the gills, the water is directed to the digestive gland where the contaminants have contact with the organ, making the digestive gland an important tissue to analyze.

R. philippinarum showed different patterns between gills and digestive gland results. Gills at T7 showed significant decrease in CAT activity at 18 and 38, whereas in digestive gland at T7 a significant increase at 18 was recorded suggesting an effect at lower salinities. *R. decussatus* showed a clear pattern and similar results between tissues. Gills tissue recorded significant CAT activity at T7 in clams at 18 and 38, and digestive gland tissue with significant values at T7 in clams at 28 and 38.

Several reports show increase of CAT when an organism is exposed to NPs. For example, *M. galloprovincialis* when exposed to nAg and nCuO (Gomes *et al.,* 2014; Gomes *et al.,* 2012) and *S. plana* when exposed to nZnO (Buffet *et al.,* 2012). The results showed that gills tissue was more sensitive in lower and higher salinities in both species, while digestive gland showed different results in the clams: with *R. philippinarum* showing more stable results

along the experiment than *R. decussatus*. Results suggest that the NPs mixture induce oxidative stress in the organisms, but with different effects on the tissue and on the clam. Comparing CAT activity values it is clear that *R. decussatus* was in higher oxidative stress than the invasive species.

SOD activity in *R. philippinarum* in the gills showed significant lower activity in 18 and 28 at T1 and 28 at T7, and significant higher activity at T7 in 38, and digestive gland showed significant low activity in all salinities but at different times, not presenting a clear pattern. *R. decussatus* tissues showed similar results, with significant high activity at T7 in clams at 28 and 38. With that knowledge and the significant difference between 38 and 18 in all recorded days, data suggests that the combined effect of salinity and NPs mix induced higher oxidative stress at higher salinities in this species.

Several studies show that SOD enzyme increases when an organism is exposed to NPs, for example: *M. galloprovincialis* with nAg (Gomes *et al.,* 2014), *S. plana* with nAu (Pan *et al.,* 2012) and *S. plana* with nCuO (Buffet *et al.,* 2011). *R. philippinarum* results suggest that after prolonged exposure the combination of high salinities and NPs mixture induced oxidative stress in this clam.

Gills tissue of both species showed a significant decrease in GST activity in clams at low salinity, in *R. philippinarum* after T3 and in *R. decussatus* at T1 and T7. *R. philippinarum* showed significant higher values in GST activity in clams at 18 (control and treated groups) in all days in gills, and at T7 in digestive gland, compared with other salinities. *R. decussatus* showed clams at higher salinity with significantly higher values at T3 and T7 in gills, and digestive gland showed no significant changes with the salinity variation. For GST, results suggest that gills tissue was more sensitive to exposure than digestive gland. The results of this study are contrary of others that report increase of GST when organisms are exposed to NPs (e.g. Buffet *et al*., 2011; Pan *et al*., 2012), which suggest that the combination low salinity and NPs mixture inhibits the production of the GST enzyme.

Some studies were performed with salinity. For example, a study was performed in the oyster *Ostrea edulis* and showed higher ROS production in higher salinities (Hauton *et al*., 2000). The results of the present work corroborate that information: *R. decussatus* showed increase CAT and SOD activity (antioxidant enzymes) at high salinity, but are contrary with the GST results, emphasizing that the combination had an inhibitory effect on the clams.

The results of the antioxidant enzymes of the clam *R. philippinarum* are in agreement with Wu *et al*. (2013), which found that low salinities decrease the activity of SOD and GST, in *R. philippinarum,* suggesting that hyposaline condition may induce oxidative stress.

LPO is the oxidative degradation of the lipid membrane performed by ROS. When LPO occurs it generates products like MDA and the TBARS assay measures those products. *R. philippinarum* TBARS levels were consistent in both gills and digestive gland tissue. In gills tissue, a significant increase in clams at 18 was shown at T3 until the end of the experiment, and in clams at 28 was shown at T7. *R. decussatus* TBARS levels in the gills tissue did not showed clear pattern, while digestive gland showed significant increases at 18 and 38. Several studies determined an increase in LPO when the organism was exposed to NPs (Gomes *et al*., 2012; McCarthy *et al*., 2013; Gomes *et al.,* 2014).

A study using metal NPs and different levels of salinity demonstrated that sheepshead minnow experienced higher oxidative stress at low levels of salinity (Ates *et al.,* 2014), in agreement with *R. philippinarum* results.

Another study demonstrated that lipid peroxidation would increase in calms kept outside of the optimal salinity (21-28) due to higher ROS production (Carregosa *et al*., 2014). These results are consistent with the ones in *R. decussatus,* in digestive gland tissue, where an increase of TBARS levels occurred in clams kept at 18 and 38.

ROS can induce damage in proteins and the result of this oxidation is the production of stable carbonyl groups. In this study PCC was used as a measure of oxidative injury. *R. philippinarum* showed significant increase in PCC levels in the gills tissue in clams kept at 18 and 28 at T1, and in clams at 18 at T3, but at T7 the values returned to control, whereas in the digestive gland tissue no difference between control and treated groups was demonstrated. These results are in agreement with the TBARS, suggesting oxidative stress in *R. philippinarum* when combining lower salinities and NPs mixture.

R. decussatus showed in gills tissue significant increase in clams at 38 at T3 and T7. In the digestive gland tissue at T3 a significant increase at 28 and 38 was followed by a returning of PCC values to the control levels, although when compared to lower salinities in the treated group clams at 38 showed significant higher levels. These results suggest that the combination of high salinity and NPs mix cause oxidative damage in *R. decussatus*, in contrast with the invasive clam results.

The results of the present study suggest that combining high salinities and NPs can cause oxidative stress and damage in *R. decussatus.* In *R. philippinarum* the combination can exert different modes of action on antioxidant defenses and damage, depending on the animal tissue considered.

It is difficult to compare results of this study to others, as information concerning influence of NPs, salinity and their interaction on DNA damage, oxidative stress of bivalve haemocytes, gills and digestive gland lack in the literature. This work was one of the first experiments combining NPs and salinity, thus it is essential to investigate further on this subject.

4.3 Protein content

Proteins play a crucial role in metabolic activities, and can change according to physiological status of the clam and artificial environmental stress (Mukadam and Kulkarni, 2014). *R. decussatus* showed significant lower values at the highest salinities, in comparison with the controls, and it is in agreement with the results of PCC at T7, which demonstrated more oxidized proteins at highest salinities. *R. philippinarum* protein content did not suffered significant change. The results suggest that the combination NPs mixture and different salinity levels affect the proteins content.

A significant decrease in protein content was found in a study performed on an estuarine clam, *Marcia opima*, when subjected to environmental stress (Mukadam and Kulkarni, 2014), and in a gastropod, *Thiara tuberculate*, due to mercury toxicity (Chaudhary and Kulkarni, 1998). The results of the present work suggest that *R. decussatus* was in more environmental stress at higher salinities than the invasive species was in the same conditions.

This study is also in agreement with others that point out *R. decussatus* as less resistant to physical stress than *R. philippinarum* (e.g Tanguy *et al.,* 2008).

4.4 RNA/DNA ratios

The RNA:DNA ratio is one of the most used biochemical indicator of physiological and nutritional condition (Chícharo and Chícharo, 2008). The premise of this technique is that DNA, which is an index of cell number or biomass, remains relatively constant during periods of starvation, and RNA, which is related to protein synthesis, oscillates in response to food availability and the demand for protein synthesis (Caldarone *et al.*, 2001), which makes

RNA:DNA directly related to nutritional condition and tissue growth. In this work, the RNA:DNA ratio of the clams was determined after the clams were exposed to different levels of salinity and NPs mixture, to evaluate the physiological conditions of the clams, seven days after exposure. Reduced ratio indicates low protein synthesis which could occur when organisms experience stress.

Results showed that *R. philippinarum* was not affected by the concentration, while *R. decussatus* did. The native clam demonstrated to be more sensitive to the combination of salinity and NPs mixture than the invasive species. These results are consistent with the protein content results for both species. The difference between these two species suggests that native species *R. decussatus* is more susceptible to contamination and so its physiological condition is more deteriorated than the invasive species. The results obtained in this work are consistent with studies showing *R. decussatus* RNA/DNA ratios significantly decrease when in physiological stress (Chícharo *et al.*, 2001).

5 Conclusions

The results of the present work show that the combination NPs mixture and different salinity levels can have an effect on *R. decussatus* and *R. philippinarum*. When exposed, *R. decussatus*, a native species of the Venice lagoon, was determined to be more sensitive to oxidative stress when combined NPs and higher salinities. *R. philippinarum* also showed oxidative stress with the combination NPs mixture and salinity but with different effects on the tissue and on the clam. Furthermore, when the physiological condition was evaluated, *R. decussatus* was found to have a more deteriorated condition than the invasive species, after seven days of exposure. The results of this work are agreeing with the premise that *R. philippinarum* clams are more resistant to environmental stress than the native species *R. decussatus*.

Further investigations on the combination of environmental stressors and NPs is needed since water bodies are constantly being contaminated and suffering variations on its abiotic factors, and it is crucial to understand if and what extent those combination can have an effect on the biota of the aquatic ecosystem.

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