Universidade de Aveiro Departamento de Química
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Rui Jorge Rodrigues Monteiro

Comportamento do titânio em ecossistemas marinhos e impacto em organismos-alvo

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica da Doutora Eduarda Pereira, Professora Associada ao Departamento de Química da Universidade de Aveiro, do Dr. Carlos Vale, Investigador Principal no Centro Interdisciplinar de Investigação Marinha e Ambiental, e da Dra. Rosa Freitas, Investigadora Doutorada (Nível 1) do Departamento de Biologia da Universidade de Aveiro.

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POCH

Dedico este trabalho à minha família e à minha namorada por todo o apoio e paciência ao longo deste projeto.

o júri

presidente

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palavras-chave

Titânio, $nTiO_2$, ecotoxicidade, ecossistema marinhos, alterações ambientais

resumo

As nanopartículas de dióxido de titânio tornaram-se num dos nanomateriais mais difundidos no mercado, prevendo-se que quantidades crescentes cheguem às áreas estuarinas e costeiras. Até agora, pouca atenção foi dada ao comportamento do titânio nesses ambientes, provavelmente devido às baixas concentrações resultantes da sua baixa solubilidade. O presente trabalho pretende melhorar o atual conhecimento sobre a dinâmica do titânio nos sistemas costeiros e os seus possíveis impactos nos organismos.

Ensaios laboratoriais foram realizados em água do mar sintética fortificada com 5, 50 e 100 µg L^{-1}, estudando-se o comportamento na água do aquário e possíveis efeitos tóxicos de Ti^{4+} e TiO_2 em organismos-alvo, mexilhão *Mytilus galloprovincialis* e ameijoa *Ruditapes philippinarum*. Variações de salinidade (20, 30 e 40 PSU), pH (7,6 e 8,0) e temperatura (17 °C e 21 °C) foram tidas em consideração na avaliação de potenciais efeitos tóxicos combinados em águas de transição. Nos ensaios, a concentração de Ti diminuiu abruptamente em 24 h, o que confirmou o baixo tempo de permanência desse elemento na fração dissolvida. A introdução de ar ou N_2 revelou a importância do oxigénio na conversão do Ti^{4+} na sua forma de óxido insolúvel. De acordo com o curto período de disponibilidade na fase dissolvida, os organismos também demonstraram concentrações residuais de Ti nos tecidos, 2 - 4 µg g^{-1}. Apesar das quantidades reduzidas de Ti nos tecidos, foram observadas alterações bioquímicas, o que implica que o Ti foi brevemente introduzido e rapidamente eliminado pelos organismos. Diversas evidências de respostas bioquímicas foram encontradas devido ao impacto do Ti(IV). Alterações das taxas metabólicas (cadeia de transporte de eletrões – ETS) e aumento nos indicadores de dano (maior peroxidação lipídica – LPO, e menor glutationa reduzida – GSH) foi generalizado e dependente da dosagem. As defesas antioxidantes e biotransformantes foram ativadas (destaque para glutationa peroxidase – GPx e glutationa s-transferases – GSTs), prevenindo danos fatais e permitindo uma recuperação quase completa quando os organismos foram colocados em depuração. No geral, ensaios controlados em laboratório mostraram que o Ti(IV) causou impactos para além da bioacumulação nas duas espécies modelo, no entanto, os indicadores de dano foram sub-letais e recuperáveis.

Alterações de salinidade, temperatura e pH combinadas com a exposição a Ti(IV) traduziu-se em diferentes efeitos iterativos nas respostas bioquímicas das ameijoas. A análise por PCO evidenciou que as mudanças de salinidade ofuscaram os efeitos do Ti(IV), o decréscimo de pH tem efeitos reduzidos sobre a toxicidade e o aumento da temperatura evidenciou bioquímica alterada, mas sem aumento de danos. As vulnerabilidades das ameijoas em relação à exposição ao Ti(IV) aparentam ser mais baixas ou comparáveis às observadas nas mudanças cíclicas das propriedades físico-químicas em sistemas estuarinos.

keywords **Titanium, $nTiO_2$, ecotoxicity, marine ecosystem, environmental changes**

abstract

Titanium dioxide nanoparticles became one of the most widespread nanomaterials in the market, and increasing amounts are predicted to ultimately reach estuarine and coastal areas. Until now, little attention was paid to the behavior of titanium in these environments, presumably due to the low concentrations resulted from its low solubility. The present work intends to improve the current knowledge on the dynamics of titanium in the coastal systems, and its potential impacts on organisms.

Controlled laboratory experiments were performed in synthetic seawater spiked at 5, 50 and 100 µg L^{-1}, and behavior in the aquarium water and potential toxic effects of Ti^{4+} and TiO_2 on the key-organisms, mussel *Mytilus galloprovincialis* and clam *Ruditapes philippinarum*, were studied. Changes on salinity (20, 30 and 40 PSU), pH (7.6 and 8.0) and temperature (17 °C and 21 °C), were also factored when evaluating combined potential toxic effects in transitional waters. In the experiments, Ti concentration decreased abruptly within 24 h, which confirmed the low residence time of this element in solution. Pumping air or N_2 revealed the importance of oxygen in the conversion of Ti^{4+} into its insoluble oxide form. In line with the low timeframe of availability in the dissolved phase, organisms also demonstrated low residual concentrations of Ti in tissues, 2 – 4 µg g^{-1}. Despite the reduced quantities of Ti in tissues, biochemical alterations were observed, which implies that Ti was briefly uptaked and quickly eliminated from the organisms. Several evidences of biochemical responses were found the impact of Ti(IV). Altered metabolic rates (electron transport chain – ETS) and increased damage indicators (higher lipid peroxidation – LPO, and lower reduced glutathione – GSH) were pervasive and dose dependent. Antioxidant and biotransformation mechanisms were activated (highlight for glutathione peroxidases – GPx and glutathione s-transferases – GSTs), preventing fatal damage and allowed for almost complete recovery when organisms were under depuration. Overall, laboratorial controlled assays showed that Ti(IV) caused impacts beyond accumulation on both model species, however damage indicators were found to be sublethal and recoverable.

Changes of salinity, temperature and pH combined with Ti(IV) exposure translated into different iterative effects on the clams' biochemical responses. PCO analysis evidenced that salinity shifts overshadowed Ti(IV) effects, lowered pH had minor effects on toxicity, and temperature rise evidenced altered biochemistry but without increased damage. Vulnerabilities of clams towards Ti(IV) exposure seems to be lower or comparable to those observed under the cyclic changes of physicochemical properties in estuarine systems.

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Chapter 1

Introduction

1. Introduction

1.1. Coastal and estuarine ecosystems

The ocean can be seen as an interconnected massive water body and its movement is driven by solar energy and influenced by the planet's rotation (Colling 2001; Gill 2016). Solar energy influences atmospheric temperature creating winds, which ultimately introduce movement in the oceans' surface layer. Furthermore, the oceans thermal capacity creates vertical (depth) and horizontal (longitudinal) temperature and salinity gradients, which translate into density variations and the formation of underwater currents.

Coastal areas are dominated by the oceans' physicochemical properties, and thus its dynamics are tightly linked to both near-shore atmospheric conditions as well as water movement (currents and tides) (Melet et al. 2016). Wave patterns as well as upwelling are an integral part of coastal zones, and of considerable importance due to their effect on vertical mass transport from the benthos to the surface (MC et al. 2011; Walter et al. 2016). Upwelling in particular, has been critical for coastal area productivity by increasing nutrients and carbon content in surface waters (Khangaonkar et al. 2012; Allen and Wolfe 2013), while also increasing trace metals content (Sunda 2012; Sánchez-Quiles et al. 2017) which can both act as micronutrients as well as contaminants (Rjeibi et al. 2015).

Estuarine areas, as an transitional system, are heavily influenced by the flow dynamics of both freshwater and saltwater systems, becoming one of the most productive aquatic habitats (McLusky et al. 2004). Classification of these complex systems can be done by water balance, geomorphology, vertical stratification and hydrodynamics (Valle-Levinson 2010). Its physicochemical characteristics such as salinity, temperature, dissolved oxygen and particulate matter dynamically fluctuate with time and space (Navarro et al. 2011; Matte et al. 2014).

The freshwater systems represent a major source of dissolved and particulate matter that arrive at estuarine areas. This dissolved and colloidal fraction can be of both inorganic (e.g. clays, carbonates) or organic (e.g. humic and fulvic acids) nature (Laing et al. 2009). Transport process is affected by different factors, e.g. physicochemical weathering and biological activity, thus influencing the changes and nature of particulate matter load out (Souza et al. 2010). Anthropogenic pressure has also greatly altered this balanced, be it by adding increased or new substances into the riverine systems (Kennish 2017), or by altering the physical structure of water courses (e.g. dams) and its constituents (Richter and Thomas 2007).

When entering the estuarine areas, particulates tend to flocculate, aggregate and eventually sediment, creating a high turbidity zone located at initial mixing zone (Wai et al. 2004). Further transport of suspended matter is considered to be mostly dependent on tidal forcing and vertical profiles (Garel et al. 2009). Some estuaries (e.g. tidal estuaries) have also been characterized by the high temporal retention of both water and particles (Middelburg and Herman 2007). Furthermore, tidal impact on resuspension of sediments giving another degree of complexity when considering the content of suspended matter (Wu et al. 2005).

1.2. Changes in environmental conditions

1.2.1. Ocean

As a major component of Earth's system, covering near 72 % of the planets' surface, the ocean thermal inertia and heat sinking capacity has been playing a major role on climate regulation (Barnett et al. 2001). However, anthropogenic activity has been slowly altering this dynamic equilibrium.

Global warming is perhaps the most mediatic climate change factor, attributed to the emission of greenhouse gases (CO_2, CH_4, NO_x, CFCs, among others) into the atmosphere (Ehhalt et al. 2001; Salawitch 2018). This increase of the planets' surface temperature, by c.a. 1 ºC, has been reflected in oceans' surface water, with observer temperature rise of 0.5 ºC, with predictions of further intensification by the end of the century (IPCC 2014). Thermal influences in both land and sea surface have also been noted to impact upwelling systems (Behrenfeld et al. 2006; Cheung et al. 2010).

With steadily increasing temperature, the water vapor content in the atmosphere has been affected (Philipona et al. 2005). It has been estimated that most of water vapor comes from the seas (86 %) but, it is also in this same water mass that occurs most precipitation (78 %) (Marsh and Kaufman 2012). In terms of evaporation, it occurs mostly in the tropics induced by warm surface winds, while precipitation occurs at colder higher latitude areas (Durack et al. 2012; Marsh and Kaufman 2012). Within this contained systems, Durack et al. (2012) has described a positive feedback effect in which with increasing temperatures would make salinity shifts more pronounced.

Several greenhouse gases influence the increasing temperature of the planet, however it's carbon dioxide (CO_2) which has been of interest for the oceans' chemistry, particularly in its role as a carbon sink (Zehnder et al. 2018). The inorganic carbon equilibrium plays a pivotal role as pH buffer of the oceans, which had been stabilized at 8.25 (pre-industrial), but has been slowly decreasing by 0.1 as observed by Orr et al. (2005). Further predictions denote potential decreases of up to 0.4 (Caldeira and Wickett 2003), which shouldn't be underestimated as pH functions on a logarithmic scale. With the decreasing pH the carbonate system has been shown to be considerably affected (Barker and Ridgewll 2012) in detriment of lower carbonate (calcite and aragonite) saturations.

These change in physicochemical properties of seawater will invariably affect the marine biota. Temperature and salinity induced stratification as well as changes in upwelling have been linked to ocean productivity alterations, with large-scale redistribution of fishing areas (Cheung et al. 2010). Furthermore, albeit the primary production may be favored and lead to benefits for the pelagic consumers, benthic organisms may be affected (Rykaczewski and Dunne 2010). In later studies, it has been linked the increased CO_2 content to increased ocean productivity by stimulating primary production, however temperature rise negates it benefits by stalling secondary production (Goldenberg et al. 2017). Planktonic community structure was noted to be shifted under increased CO_2 pressure, and thus also proposed to be another influencing factor at a trophic level (Taucher et al. 2017). As final note, ocean acidification impact on the carbonate system puts additional pressure on organisms that form shells or exoskeletons, such as corals and bivalves, (Fabry et al. 2008; Doney et al. 2009; Liu and He 2012).

1.2.1. Transitional systems

When looking at transitional systems, such as estuaries and coastal lagoons, its noticeable that their physicochemical properties are highly dynamic (McLusky et al. 2004), in virtue of a natural multifaceted effect of water mass fluxes as well as weather conditions (Medina-Gómez and Herrera-Silveira 2003; Harley et al. 2006; Hennemann and Petrucio 2011).

Temperature fluctuation in transitional systems can be influenced by two factors, season and tides (Smith 1994; Vaz et al. 2005; Coelho et al. 2014b). Seasonal changes play a major role in the heat transference from the surface atmosphere to water bodies, while tides provide a cyclic change of temperature dominated by either the ocean or the freshwater sources. pH in estuarine regions tend to have a broader range and a lower limit when compared to oceans (Ringwood and Keppler 2002). Such pH variability is often connected to the influx of dissolved inorganic carbon from rivers, dilution effects from rain, and even bacterial activity altering the carbon content (Koné et al. 2009; Muduli et al. 2012; Srichandan et al. 2015). As the main driver for freshwater and saltwater mixing, tidal influence and river flows are thus the most influencing factors on estuaries and coastal lagoons salinity (Kjerfve 1994; Fichez et al. 2017). Furthermore, data modelling has further noticed the importance of neap/spring tides, as well a correlation between tide and proximity to freshwater sources, as important factors in the spatial and temporal variation of salinity in the hydrologic systems (Vaz et al. 2005).

Besides the normal fluctuations in physicochemical properties, estuarine systems are also prone to experience episodes of droughts and floods (Neto et al. 2010) which have been noted to be exacerbated by extreme weather conditions (Wetz and Yoskowitz 2013). Both droughts and floods have been closely related to not only salinity fluctuations of estuarine systems, but also the systems nutrient availability (Bruesewitz et al. 2013; Dittmann et al. 2015). Dittman et al. (2015), further denotes the complexity of extreme weather events impact on estuarine systems, as connections to freshwater/seawater sources may be interrupted or favored and henceforth completely changing the physicochemical parameters.

Organisms in transitional systems survive under in an habitat with constantly varying conditions, as such they’ve developed high resiliency (Elliott and Whitfield 2011) and have distributed according to their tolerance to such fluctuations (Whitfield et al. 2012). However, the dynamic of estuarine systems aligned with anthropogenic pressure, such as contamination, may increase risks for estuarine fauna (Carregosa et al. 2014; Verdelhos et al. 2015; Nardi et al. 2017; Moreira et al. 2018b). In the case of extreme weather events, its severity and the organisms adaptability were noted as key aspects when considering changes in community structure and recoverability (Baeta et al. 2011; Grilo et al. 2011; Boucek and Rehage 2014).

1.3. Contamination of aquatic systems

Contamination has been one of the focus of the scientific community in later decades, as the decline in water quality has been related to the presence of compounds or elements above the background levels. Contamination of aquatic systems has often been attributed to effluent discharges, either of domestic of industrial activity, which introduce many foreign substances into the environments eventually affecting current physicochemical and biological processes (Goel 2006; Sivakumar 2010).

Agriculture fertilizers are often required to increase the production yield; however, they're also easily leached into the surrounding aquatic basins increasing the nutrients sources and thus leading to eutrophication problems (Chislock et al. 2013). Pesticides and herbicides are also another factor to account, which for similar reasons can also reach the aquatic environment and with rather damaging effects to the biota (Renault 2011).

The mining industry, as well as other activities related to the explorations of underground resources, have also their fair share of responsibility in the current state of decrease in water quality. The extraction and processing of several ores has not only been a source of several metal contaminants, e.g. mercury, cadmium and arsenic, but also can form as byproducts, e.g. sulfuric acid, all of which can to pose threats to the environment (Tiwary 2001; Luís et al. 2009).

Petrochemistry shows identical issues, be in its procurement of oil in land or sea sources that lead to the release of several pollutants, but can also lead to thermal pollution, as a byproduct of energy production in thermoelectrical facilities (Sivakumar 2010). This localized heating can then lead to an increased bacterial activity, which can not only greatly decrease dissolved oxygen (Coutant and Brook 1970; Blumberg and Di Toro 1990) but also metabolize other contaminants making them even more toxic, as the formation of methyl-mercury for example (Ullrich et al. 2001; Merritt and Amirbahman 2009).

Contamination is thus one of the major anthropogenic pressures to aquatic medium, and while several contaminants have been studied (classic contaminants), others are only in its early steps of research (nanomaterials).

1.3.1. Classic contaminants

Currently there are listed various environmental contaminants. Some are organic contaminants, such has polychlorinated biphenyls (PCBs), pesticides, phthalates, volatile organic compounds (VOCs), other are inorganic, such as metals like cadmium, lead or mercury (ATSDR [I, II]). All these contaminants are under regulations, such as the European Water Framework [III] or European Marine Strategy [IV], which clearly states methods of analysis and parameter limits.

As an example, metal contaminants are usually present in very low concentrations in the environment and still have a major impact in the ecosystems (Selin 2009). Through the effects of accumulation by the organisms with time (bioaccumulation) and the incremental impact through food web (biomagnification), these types of contaminant pose a serious threat towards the ecosystem health and human safety, as it stands at the top of the food chain. Table 1 reports maximum discharge limits of potential toxic elements (PTEs) and environmental quality objectives for surface waters (EQS SW).

Table 1 – List of PTEs, ATSDR ranking, discharge limits and EQO SW

Potential Toxic Element	Ranking	Effluent limit (µg L^{-1})	EQO SW (µg L^{-1})
As	1	1 000	50
Cd	7	200	10
Cr total / Cr (VI)	78 / 16	2 000 / 100	50 / -
Cu	118	1 000	50
Hg	3	50	1
Ni	57	2 000	50
Pb	2	1 000	50
Se	146	-	-
Zn	75	-	500

Adapted from ATSDR [II] and DR 236/98 [V]

When a contaminant enters the environment it may or may not interact with the biota and it does not necessarily mean that the organism can uptake it (Semple et al. 2004; Herrmann et al. 2016). Various environmental parameters may interact with the uptake, such as temperature, organic matter, pH, salinity, as it influences both the speciation (Bui et al. 2016; Roig et al. 2016). In recent years the focus of research in aquatic systems has been shifting from metals to metal/metal oxide based engineered nanoparticles (Baker et al. 2014; Vale et al. 2016). According to both author, there's an increasing anthropogenic pressure and behavior in the medium as well as effects on biota.

1.3.2. Plastics

Awareness of plastic debris in the aquatic systems has increased in the last decade, with current researchers pointing to a universal presence of plastics in both freshwater and seawater compartments (Driedger et al. 2015; Xanthos and Walker 2017). The trend of plastic production has also been increasing, with estimates of 335 millions of tons in 2016 increasing to 359 millions of tons by 2018 (PlasticsEurope 2017; PlasticsEurope 2019), with major sources traced to Asia (51 %), North America (18%) Europe (17 %). Inputs from land to ocean were estimated at 4.8 to 12.7 million tons (Jambeck et al. 2015), while through riverine systems models projected 1.15 to 2.41 million tons per year (Lebreton et al. 2017). Due to their durability its lifetime expectancy is estimated at the hundreds of thousands of years (Wang et al. 2016a), which makes plastics an easily accumulated ocean debris (Barnes et al. 2009).

Barnes et al. (2009) noted that only 10 % of discarded waste was related to plastics, however plastics may reach up to 90-95 % of total marine litter (Moore 2008). Sources of these plastics are however not only attributed to direct discarded waste, with the inefficiency of waste water treatment plants being reported as not properly equipped (Driedger et al. 2015). Plastics in these effluents are however of reduced sizes (< 1 mm) often related to common consumer products, such as abrasive agents found in soaps, toothpastes and cosmetic products (Eriksen et al. 2013). Fibers have also been noted to be an important source of plastics in the aquatic medium, resultant of runoff from textile laundering (Eriksen et al. 2013), and have been detected in marine sediments (Browne et al. 2011).

Debris classification is often done relating to its size (Table 2), with size, density and. buoyancy being critical parameters for plastic dynamics in the oceans, i.e. vertical transport and migration (Wang et al. 2016a; Avio et al. 2017). Microplastics, the most intensive research focus, have also been classified as primary or secondary, the former being directly introduced into the systems while the latter being resultant of weathering breakdown processes (Cauwenberghe et al. 2015; Andrady 2017).

Table 2 – Size classification of plastic debris

Classification		Size
Macroplastic		> 2.5 cm
Mesoplastic		0.5 – 2.5 cm
Microplastic	Large	1 – 5 mm
	Small	1 – 1000 µm
Nanoplastic		< 1 µm

Adapted from Cauwenberghe (2015)

The presence of plastics in the oceans has been correlated to diverse impacts on the marine biota. For larger plastics, entanglement has been observed on several aquatic species but also on preying birds (Monteiro et al. 2018). Smaller plastics threat is however related to ingestion, which may not only directly act as a contaminant but also add trojan horse effects for other organic, inorganic, and nanomaterials (Wang et al. 2016a; Andrady 2017).

1.3.3. Nanomaterials

Most nanomaterials in the environment have natural origin, be it biogenic, geogenic, atmospheric or pyrogenic. Volcanic eruptions, forest fires, erosion, and animal skin shedding are most common natural sources (Buzea et al. 2007; Nowack and Bucheli 2007). Even comparing to the current anthropogenic origin of the nanomaterials, e.g. fossil fuel combustion, the human impact is expected to be not far from 10% of the total nanoparticulate matter in the aerosols (Buzea et al. 2007). Table 3 illustrates the types of nanomaterials and their sources.

Table 3 – Nanomaterials according to sources

Source	Nature	Formation	Type	Examples
Natural	Carbon	Biogenic	Organic colloids Organism	Humic Acids Virus
		Geogenic	Soot	Fullerenes
		Atmospheric	Aerosols	Organic acid
		Pyrogenic	Soot	CNTs Fullerenes
	Inorganic	Biogenic	Oxides Metals	Magnetite AgNPs, AuNPs
		Geogenic	Oxides Clays	FeONPs Allophane
		Atmospheric	Aerosols	Sea salt
Anthropogenic	Carbon	By-product	Combustion residues	CNTs
		Engineered	Soot	Fullerenes CNTs
			Polymeric NPs	Dendrimers
	Inorganic	By-product	Combustion residues	Metal NPs
		Engineered	Oxides Metals Salts Aluminosilicates	TiO_2, SiO_2 AgNPs, AuNPs Metal-phosphates Zeolites, ceramics

(Legend: CNTs – Carbon Nanotubes; NPs – Nanoparticles) Adapted from Nowack and Bucheli (2007)

Despite most of nanoparticles being of natural origin the anthropogenic input should not be discarded. The worldwide use of nanomaterials was foreseen to grow from 225 060 tons in 2014 to 584 984 tons in 2019 (McWilliams 2017). Real values are however expected to be higher, since the lack of legislation doesn't condemn manufacturers to provide real values, which hinders the works of prediction of environmental effects (Piccinno et al. 2012; Gottschalk et al. 2015).

After released onto the environment, most of nanomaterials are either highly reactive or fairly unstable (Rauscher et al. 2014). As such, when exposed to environmental conditions, they may either directly act as contaminants or, as through secondary reagents, originate a new toxic or otherwise harmful agent (Vale et al. 2014; Fu et al. 2014).

It's also commonly accepted that the water systems are the ultimate end-point of the nanomaterials (Selck et al. 2016; Vale et al. 2016). Since a significant portion of the anthropogenic waste is already released towards the aquatic system, while airborne nanoparticles either end up

depositing in the same domain or in soils. These nanoparticles present in the soil may still be highly mobile, mostly due to their size, and leach into nearby hydrographic network. Both physical and chemically factors lead to a series of possible interactions that will change nanoparticles in aquatic systems (Vale et al. 2016). The nature of nanoparticles as well as the constituents of the water are a critical factor on the environmental risk (Domingos et al. 2013b).

Regarding most metal and metal-oxide nanoparticles have been known to form partially soluble metal oxides, as in the case of nCuO or nZnO (Heinlaan et al. 2008; Domingos et al. 2013b), some undergo oxidation processes, such as nCuO or nAg (Lok et al. 2007; Ma et al. 2014), while other may be complexed, CdTe/CdS quantum dots (Domingos et al. 2013a). Dissolution leads to the release of the corresponding ions into the water column, effectively increasing the toxicity effects (Vale et al. 2016). Aggregation is also a common occurrence, as the nanoparticles lose their protective coating or stabilizing agents, and can be bound to either organic or inorganic matter (Studart et al. 2007; Bhatt and Tripathi 2011). The former can act as stabilizing agents, maintaining a dissolved state while bonded or, it can drive to its flocculation, and subsequent sedimentation (Baalousha et al. 2008; Domingos et al. 2010). These interactions are difficult to study but, due to the high concentration of organic matter in aquatic systems, when compared to the concentration of nanoparticles, it's suspected to play an important role on aquatic nanoparticle physicochemical changes (Vale et al. 2016). Nanoparticles may also aggregate to each other or other inorganic particulates in suspension, which often leads to their sedimentation, though interaction with organic matter is more likely (Baker et al. 2014). River systems, for example, contain large amounts of organic matter which often strongly binds to nanoparticles, ultimately decreasing their sedimentation rate (Baker et al. 2014; Vale et al. 2016). On the other hand, the high ionic strength of seawater tends to lead nanoparticles into deposition, although it has been verified that depends on a complex relation between the concentration of organic matter, ionic strength and nanoparticle concentration (Baker et al. 2014). Furthermore, studies also indicate that the concentration of hardness ions, such as Ca^{2+} and Mg^{2+}, may play a predominant role on destabilizing nanoparticles inducing a faster aggregation (Domingos et al. 2015).

Despite all this knowledge, the current information regarding the dynamics of nanoparticles in water systems, and especially the marine system, is still lacking. This is mostly attributed to the fact that most works either work in very controlled environments, ultrapure or distilled water, or in artificial mediums. Some reasons for these types of work are related to the determination of basic interactions between the nanoparticles and the study subject systems (Rosenkranz et al. 2009; Asghari et al. 2012), manipulating other parameter to acquire new knowledge, only then moving onto more complex systems. Others focus their work solely in freshwater and waste water systems, usually in the subject of water remediation systems (Haham et al. 2015; Lamba et al. 2015). Furthermore, the issue of working in marine environments stems mostly for the analytical techniques used to obtain results (Rao et al. 2005). Usually salinity is a parameter that must be in a narrow working range, which will require sample dilution that will often make the analyte to be below the quantification limits, or further manipulation through pre-concentration techniques. From all these interactions, as well as from the direct effect that nanoparticles may have in aquatic environments, there is a need to understand both origin and fate of these types of materials.

1.4. Ecotoxicological evaluation

1.4.1. Bioaccumulation and oxidative stress biomarkers

Bioaccumulation is one of the most prominent effects when organisms are exposed to contaminants, characterized by a faster intake of these foreign substances in comparison to their metabolic elimination (Jitar et al. 2015). Due to trophic chain magnification, top tier predators are generally more vulnerable (Liu et al. 2019). Metal contaminants have been the focus of such studies, which still continues, now grouped with several more elements and currently known as toxic and potentially toxic elements (Reinfelder et al. 1998; Yang et al. 2013; Jebali et al. 2014). Tissue partitioning has also been used to understand uptake pathways, with gills and the digestive glands being commonly analyzed (Baker et al. 2014; Vale et al. 2016). Furthermore, the liver has often been used for measurements as it tendentially accumulates contaminants, while muscle tissue is directly related to trophic transference and risk assessment (Joo et al. 2013; Rajeshkumar and Li 2018).

Besides bioaccumulation, the organisms have triggered responses when exposed to foreign bodies. One of them is the oxidative stress, which is caused by an imbalance of the cell's reactive oxygen species (ROS), such has superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) or singlet oxygen ($^1O_2$), and the biologic mediating capacity (Regoli and Giuliani 2014).

In order to fight this abnormal status, the cell triggers their defense mechanisms. The primary defense system is mainly composed by superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione S-transferases (GSTs), while secondary defense system is composed by the reduced glutathione (GSH) (Stahl et al. 1998). They act in a coordinated manner, with SOD converting the superoxide radicals, into less reactive species, H_2O_2 (Fridovich 1995). The lesser reactive ROS can be taken care by CAT, ending up converter into water and molecular oxygen (Matés and Sánchez-Jiménez 1999), or by a cooperative work of GPx or GSTs and GSH, with similar result (Jornot et al. 1998). In the cell, most of the present glutathione content is reduced, making the cell environment highly reducing and thus acting as xenobiotic scavengers (Regoli and Giuliani 2014). As ROS are being scavenged the ratio of GSH and GSSG (oxidized glutathione) will lower, (Arora et al. 2012), until a point in which the cell becomes vulnerable to damage. Damage caused by the excess of ROS can be manifested in different forms, depending on the affected area. One of the main focus of studies is its oxidative interaction with lipids from the cellular membrane or proteins from the cell, known respectively as lipidic peroxidation (Halliwell and Gutteridge 2015) and protein carbonilation (Mesquita et al. 2014; Weng et al. 2017).

Metabolic and energetic biomarkers have also been used for a more complete impact analysis in marine organisms (Sokolova et al. 2012). Energy reserves, such as glycogen and total protein content, have been extensively used when fitness is being correlated to stressors such as contamination and environmental changes (Darriba et al. 2005; Pardeshi 2015). The metabolism, more specifically the mitochondria electron transport system (ETS), has also been used to assess the organisms activity and fitness (Fanslow et al. 2001; Gagné et al. 2007; Schmidlin et al. 2015).

It is in this conjugation of bioaccumulation and biochemical biomarkers analysis that the scientific community has been assessing the fitness of marine organisms and even using different biomonitor species to infer the viability of different ecosystems and their predicted changes (Freitas et al. 2015b; Moreira et al. 2018a; Henriques et al. 2019; Pinto et al. 2019).

1.4.2. Nanomaterial toxicity – physicochemical aspects

Engineered nanomaterials have very unique properties, typically attributed to their size, but it should not be forgotten other effects such as chemical nature, e.g. purity, crystallinity, electronic properties, surface structure, i.e. surface reactivity due to, or lack thereof, coating and functionalization, and shape (Nel et al. 2006; Loos 2014). What has been researched and discussed, is that all the properties that cause nanomaterials to be so attractive, are also the reason for its distinctive, and potentially negative, behavior in regards to its interactions with biota, e.g. increased tissue uptake (Nel et al. 2006; Buzea et al. 2007).

In general, the smaller the size the higher the surface area, and thus the potentially reactive surface groups are more pronounced. Furthermore, the smaller the size the higher mobility and reactivity, which is an effect that may also be seen in biological interactions (Sonavane et al. 2008). The shape is also a critical factor, as shown in works with silver nanomaterials and *E. coli* (Pal et al. 2007) or gold nanomaterials (Chithrani et al. 2006) in cells. Surface charge was also found to play a major role in biological systems (Lockman et al. 2004), due to their interference in cellular processes, which may prompt cellular death (Schaeublin et al. 2011).

It should be noted however that many of the studies that focus on effects are done *in vitro*. This leads to some problems when trying to reach final conclusion as several of the organisms' defense mechanisms are ignored, as well as the disregarded possible alterations on the nanomaterials pristine nature (Vale et al. 2016). Furthermore, the exposure conditions, mainly nanomaterial concentration, may be unrealistic, usually much higher than what can be found available in nature, just in order to trigger the observed damage (Arora et al. 2012; Baker et al. 2014).

1.4.3. Nanomaterial toxicity in marine organisms

From the many nanomaterials that reach the marine systems, metal and metal oxide nanoparticles have been under the investigation due to their widespread use and current economic importance (Baker et al. 2014; Selck et al. 2016). This anthropogenic pressure on marine system has been growing, with greater outputs being predicted (Piccinno et al. 2012; Gottschalk et al. 2015) and thus greater threat to marine organisms.

So far, most of the research on effects of nanoparticles in aquatic systems was done in freshwater species and reports showed non-lethal effects, such as reduced growth, reproduction, swimming, feeding as well as the presence of bioaccumulation effects (Minetto et al. 2016; Henriques et al. 2017a). Interest on marine systems has been low, although it's been picking up on later years, but due to the significant difference between freshwater and saltwater systems, in regards to both the physicochemical nature and properties of nanoparticles as well as the different involved organisms, it has been difficult to correlate the results already obtained (Baker et al. 2014; Minetto et al. 2016).

It's important to notice that when researching the nanoparticle effects on marine biota there are several types of organisms, which have very different characteristic exposure pathways, as seen in Table 4, thus providing new insights into the whole marine system dynamic.

Table 4 – Nanoparticle uptake pathways of different types of marine organisms

Organism	Uptake Pathway
Bacteria	Adsorption to cell membrane Possible accumulation inside cell
Algae	Adsorption to cell surface and absorption dissolved ions
Arthropod	Adsorption (NPs or dissolved ions) or through direct feed on NPs
Annelids	Ingestion of NPs contaminated sediment Ingestion (filtration) of NPs or NPs contaminated suspended matter
Bivalves	Ingestion (filtration) of NPs or NPs contaminated suspended matter
Fish	Gills Absorption of dissolved ions Trophic transference

Adapted from Baker et al. (2014)

1.4.4. Bivalves as biomonitors

Bivalves are recognized by their high capacity as filtration feeders, reaching up to almost 10 L h^{-1}, and thus have a high exposure towards particulate marine contaminants (Meyhöfer 1985). They've been used as biomonitors of several organic and inorganic contaminants (Richir and Gobert 2014; Olenycz et al. 2015). Furthermore, their wide geographical distribution and sedentary nature has been explored for reliable and comparable field tests results (Spada et al. 2013).

Several genera of bivalves have been researched, e.g. the *Mytilus*, *Donax*, *Scrobicularia*, *Ruditapes*, *Crassostrea*, among others, ranging from environmental monitoring conditions as well as targets for contamination (Silva et al. 2001; Beldi et al. 2006; Kljaković-Gašpić et al. 2010; Sacchi et al. 2013; Coelho et al. 2014a). The increasing release of nanomaterials onto the environment, has been reflected in the growing scientific concern of interaction between nanomaterials and bivalves (Tedesco et al. 2010; Pan et al. 2012; Barmo et al. 2013; Faggio et al. 2018).

The studies vary in focus depending on the aim being on the environmental evaluation of a specific area or in the study of single or multiple stressors. Ranging from abiotic parameter changes (salinity, temperature, pH), single or multiple contaminants, or a combination of previous parameters, the investigation has been getting more complex as new information is being unraveled (Carregosa et al. 2014; Regoli and Giuliani 2014; Hu et al. 2015; Coppola et al. 2017; Braga et al. 2018; Moreira et al. 2018b).

In this regard mussels and clams, more specifically the *Mytilus galloprovincialis* and *Ruditapes philippinarum*, are some of the forefront research species in regard to environmental research (Gomes et al. 2011; Moschino et al. 2011; Wang et al. 2011; Zhang et al. 2011). Despite dwelling in different habitats, with mussels generally fixed on rocky areas while the clams burrowing on the surface part of the sediment, both exhibit similar filter feeding behavior (CABI [VI, VII]). Furthermore, both species have shown great economic importance, with over 100 000 tons per year captured for each species (FAO [VIII, IX]), and thus its research has been intensified over the years, mostly to prevent and predict potential damage for consumers.

1.5. Titanium

1.5.1. History, properties and natural occurrence

Titanium was independently discovered in 1791 by William Gregor and in 1793 by Martin Klaproth, in England and Germany respectively. The British mineralogist found traces of an unidentified white ore in black sand (ilmenite), to which named gregorite when reporting to the scientific community of the time. The German chemist, in his works with rutile identified the substance as a new element, to which called titanium as a dual reference to Earth (Latin) and the Titans (Greek mythology). Only in 1797 did Klaproth concluded that his element was the same ore previously identified by Gregor, henceforth the latter being attributed the discovery and the former the naming of the element. Its isolation was only achieved in 1910 by Matthew Hunter but only after 1932 a new processing method, by William Kroll, allowed for large scale production, method which is still currently applied in an adapted form. (Krebs 2006; Roza 2008; Emsley 2011)

As the 22nd element of the periodic table, titanium (Ti) belongs to the transition metals block, also known as the d-group due its electronic configuration that also confers it a diverse oxidation state (-2, -1, +1, +2, +3, +4). Most commonly present is the +4 oxidation state and from its 5 main isotopes, ^{48}Ti is the most abundant (74 %). It has a very low solubility in water and shows high affinity for oxygen easily forming oxides, state in which it shows amphoteric nature. As a metal it presents a lustrous metallic color, density of 4.5 g cm^{-3}, melting point of 1668 ºC and resistance to atmospheric corrosion. Titanium (metal) also shows good thermal conductivity, and its low density and high tensile strength, when compared to steel. (Winter 2019)

By abundance, titanium ranks 9th in elements present in earth crust with 0.55 to 0.76 % of TiO_2 (Rudnick and Gao 2003) and its present mostly as metal oxides (TiO_2, $FeTiO_3$, $CaTiO_3$, $CaTiSiO_3$) in magmatic or sedimentary rocks formations (Salminen et al. 2006). Several minerals contain titanium, e.g. anatase, brookite, rutile and ilmenite, while only the latter two having commercial value for titanium refinement. Weathering mechanisms have been noted to be the natural source of titanium in aquatic systems, being reported that suspended particles in rivers may be composed up to 0.56 % of Ti (McLennan and Murray 1998) and sediments ranging from 0.016 to 4.99 % in TiO_2 (Salminen et al. 2006). In the water it was determined that the main species being its hydrated form ($TiO(OH)_2$), thus being mostly transported in its colloidal state (Turner et al. 1981; Orians et al. 1990). Its dissolved content tends to be very low, ranging from the 0.1-100 nM and 5-350 pM in rivers and oceans, respectively (Yan et al. 1991; Yokoi and van den Berg 1991; Skrabal 1995). Skrabal (1995) further attributes the reduction of dissolved Ti in seawater to coagulation of the metal oxides and subsequent aggregation and stabilization by organic material, similar to cases of iron and rare earth elements. In the oceans it has been observed a vertical distribution of dissolved Ti, increasing in content with depth, and an estimated 100-200 years of residence time in deep ocean (Orians et al. 1990).

The distribution of titanium in the different environmental compartments has been relatively stable, mostly attributed to its high weathering resistance and therefore low mobility (Brookins 1988). However, since its applications on different products increased and new anthropogenic sources have disrupted this equilibrium, it can be now viewed as an element of research interest (Robichaud et al. 2009; Botta et al. 2011).

1.5.2. Extraction, applications and anthropogenic sources

From its initial discovery titanium was viewed with interest due to its unusual behavior when trying to isolate from its ore. Usual smelting techniques, metal reduction by heating with carbon, converted titanium to its dioxide (TiO_2) and ultimately transformed it into a carbide (TiC) (Emsley 2011). Example for ilmenite in eq. 1-2.

$$FeTiO_3 + C \rightarrow Fe + TiO_2 + CO \quad \text{eq. 1}$$

$$TiO_2 + 3C \rightarrow TiC + 2CO \quad \text{eq. 2}$$

It was only in 1910 that the Hunter process allowed for the first successful isolation of metallic titanium, by reducing $TiCl_4$ with Na (eq. 3) (Roza 2008).

$$TiCl_4 + Na \rightarrow 4NaCl + Ti \quad \text{eq. 3}$$

This inefficient batch method was later (1940) replaced by the optimized Kroll process, which was the first industrial scale refinement of this metal (Greenwood and Earnshaw 2012). In this process, the ores containing titanium were converted to TiO_2 through carbon smelting and then to $TiCl_4$ under chlorine atmosphere (eq. 4 and 5; example for ilmenite). Using magnesium as a reducing agent, a titanium sponge was formed (eq. 6), i.e. porous metallic Ti that can be formed into ingots.

$$FeTiO_3 + C \rightarrow Fe + TiO_2 + CO \quad \text{eq. 4}$$

$$TiO_2 + 2C + 2Cl_2 \rightarrow TiCl_4 + 2CO \quad \text{eq. 5}$$

$$TiCl_4 + 2Mg \rightarrow Ti + 2MgCl_2 \quad \text{eq. 6}$$

Due to its physical properties, e.g. durability, tensile strength, density, titanium was soon found to be of interest in the applications for several high-end products. During the cold war military applications of titanium developed the aviation and nautical departments (Roza 2008), and later on the space race, for the production of key components of spacecrafts (Krebs 2006). Currently, the volume of application of titanium has largely increased, and was estimated that in 2018 c.a. 183 000 tons of titanium foam were produced globally, with China, Japan and Russia being amounting nearly 90 % of the total volume (USGS 2019). According to the same source, over 90 % of titanium is estimated to be consumed as TiO_2, mostly as pigments for paints, paper and plastics, while the remaining 10 % was divided between chemical and alloy applications and use as pure metal.

In the last decades, nanotechnology has been trending both in terms of research and as a result the number of marketed products containing nanomaterials has been increasing (Cancino-Bernardi et al. 2016) Among the many nanomaterials, titanium dioxide nanoparticles, $nTiO_2$, are one of the most widespread nanomaterials in the market, ranging from the most common household to high-end top of the line products (Wahie et al. 2007; Robichaud et al. 2009; Gottschalk et al. 2015). Due to its antibacterial products $nTiO_2$ made their way into cleaning products as well has cosmetics, is also used as a pigment, ranging from food products to paints, plastics and inks (Freyre-Fonseca et al. 2011; Zhai et al. 2015). Depending on the structure it can also show photocatalytic properties, applied on self-cleaning concrete (Shen et al. 2015) and currently studied as a potential waste water treatment method, and superhydrophobicity, that in conjunction with photocatalytic aspect resulted in the self-cleaning glass (Paz et al. 1995).

With the increased usage of TiO_2, a few authors formulated the hypothesis of new potential threat to the ecosystems. Gottschalk et al. (2009; 2011), extensively modeled the inputs of engineered nanoparticles, including $nTiO_2$. First modelled $nTiO_2$ inputs to aquatic environment were very limited, and predictions point to 21 ng L^{-1} in freshwater systems and up to 4 µg L^{-1} when considering effluents from wastewater treatment (WWT) plants (Gottschalk et al. 2009; Gottschalk et al. 2011). More recently, using Danish environment as case study, Gottschalk et al. (2015) modeled the presence of two categories of $nTiO_2$, photostable and photocatalytical, with the first originating from plastics and cosmetics and the latter from paints and construction materials. From the flow analysis results (Table 5), it was evidenced that most $nTiO_2$ released into the aquatic system was the photostable type, as the other was more likely to be recycled or ending up in landfills.

Table 5 – Concentration of photostable and photocatalytical $nTiO_2$ in WWT effluents and sludge and natural compartments

Natural Compartment	**Photostable**	**Photocatalytical**
WWT effluent	3.4 – 92 µg L^{-1}	0.4 – 14 µg L^{-1}
WWT sludge	69 – 1 500 µg g^{-1}	9.3 – 230 µg g^{-1}
Surface freshwater	0.6 – 100 ng L^{-1}	0.05 – 7 ng L^{-1}
Seawater	0.04 – 1 ng L^{-1}	0.004 – 0.099 ng L^{-1}
Freshwater sediments	200 – 28 000 ng g^{-1}	17 – 2 600 ng g^{-1}
Seawater sediments	49 – 1 300 ng g^{-1}	4.3 – 120 ng g^{-1}

Adapted from Gottschalk et al. (2015)

In-situ values measurements are often difficult and thus sometimes considered unreliable (Garner et al. 2017), hence the focus on fate modulation. However other authors have considered to have reliably identified engineered nanomaterials in environmental samples (Kiser et al. 2009; Westerhoff et al. 2011; Tong et al. 2015; Shi et al. 2016). In the case of Kiser et al. (2009) the Arizona WWT plant was noted to release up to 15 µg L^{-1}, two years later Westerhoff et al. (2011) in the same geographical area reported increased upper bound to 25 µg L^{-1}. More recently, in China, Shi et al. (2016) reports values considerably higher in wastewater effluent, ranging from 26.9 to 43.1 µg L^{-1}. Furthermore, the same author denotes a titanium enrichment in receiving water (52 – 86 µg L^{-1}), most likely due to contribution from urban runoff.

Considering the increasing outputs of titanium into the environment, questions arise regarding the dynamic and fate of the anthropogenically titanium and its potential hazardous effects. Several works have evaluated the potential toxic effects (Canesi et al. 2010; Barmo et al. 2013; Huang et al. 2016), while others make risk assessment models (Sharma 2009; Kim 2014; Coll et al. 2016).

1.5.3. Biological role and impact

It's a conventional assessment that titanium has no known biological roles (Arora and Sharma 2016) and, due to its biocompatibility, it has been used in its metallic form for biomedical implants, such as pacemakers or bone fixating plates and screws (Niinomi and Boehlert 2015). In adult humans between 10 to 20 mg of titanium can be found, mostly concentrated in the lungs, liver, spleen and kidney, while in newborns its nearly undetectable (Buettner and Valentine 2012). As such, titanium comes as an acquire element instead of vital one, with intake related to inhalation and dietary sources, related to the nature of white pigmentation (e.g. food coloring and wall painting) (Robichaud et al. 2009; Buettner and Valentine 2012).

In line with these findings, the latest reports by ATSDR (Agency for Toxic Substances and Disease Registry) in 2017 ranked Ti at the 313th while TiO_2 at 685th (ATSDR [X]). Furthermore, the United States Environmental Protection Agency (EPA) has marked titanium dioxide nanoparticles and 5 other nanomaterials as targets of research (EPA [XI]). Meanwhile, several other national and international organization urge the research on this and other nanomaterials, in order to lay the foundation for future environmental guidelines and legislation (Duvall and Wyatt 2011).

Research on $nTiO_2$, is however contradictory and controversial at time, albeit negative impacts on human health having been reported. One of the most controversial studies is in the regard of sunscreen lotions, in which $nTiO_2$ are a main component. *In vitro* studies have shown relation between these nanoparticles and increased brain cell fibrosis, i.e. potential on-set of Alzheimer's disease, as well as several skin related problems (Wu et al. 2008). However, topical application of $nTiO_2$ in rats and pigs shows positive and negative results regarding skin penetration capability, and subsequent absorption into the organism (Wu et al. 2009). The second most common exposure pathway is the lungs, through inhalation of $nTiO_2$ from either degraded building paints or during manufacture (Freyre-Fonseca et al. 2011). In this case results are more concordant in the various *in vitro* and *in vivo* studies, suggesting lung damage could be to the extent of tissue necrosis (Liu et al. 2013a).

Akin to humans, the biological role of titanium as an essential element for marine organisms has been mostly disregarded, however some species are known to have considerably high concentrations of this element. Example of natural sequesterers of titanium are diatoms, with reports up to 1 254 μg g^{-1} (Martin and Knauer 1973), dinoflagellates, up to 33.7 mg g^{-1} (Collier 1953) and sponges, ranging from 100 to 3 500 μg g^{-1} (Bowen and Sutton 1951; Araújo et al. 1999). Other organisms, such as bivalves, were however evidenced to accumulate titanium when exposed, for example, *M. galloprovincialis* was shown to accumulate up to 0.9 mg g^{-1} when exposed to the high concentration of 10 mg L^{-1} of $nTiO_2$ (D'Agata et al. 2014); *R. philippinarum* exposed to 10 μg L^{-1} showed c.a. 0.007 mg g^{-1} (Marisa et al. 2018).

The impact of $nTiO_2$ on aquatic biota was shown to be above the bioaccumulation. As an ultraviolet photocatalyst, in the correct conditions, it can release high amounts of free radical into the water bodies, potentially wiping out bacteria and algae community (Baker et al. 2014; Vale et al. 2016). This is effect is mostly noticeable during the summer, as it is estimated that tons of $nTiO_2$ enter the coastal areas by leaching of sunscreen lotions (Wahie et al. 2007). Effects on aquatic biota is still lacking, mostly focusing on freshwater organisms, and thus far it has been shown that $nTiO_2$ impacts mostly bacteria community, (Vale et al. 2016). To higher tier life forms the effects are

generally sub lethal. On fish damage has mostly localized on gills, with indications of oxidative stress, but was also verified brain damage that is suggested that lead to behavior changes, decreased swimming speed and general activity (Federici et al. 2007; Boyle et al. 2013).

Fewer studies have been reported on marine organisms, however it's a changing trend. A study with injecting $nTiO_2$ in octopus induces minor damage, dose dependent, and also noted the rapid recovery ability, back at baseline under a day (Grimaldi et al. 2013). Trout's have shown gill damage, mostly oxidative stress and inflammation with subsequent breathing issues, and tissue partitioning, focused on intestines and spleen (Baker et al. 2014; Vale et al. 2016) Brain damage was also detected but couldn't be correlated with behavior changes. Macroalgae were noted to be mostly unaffected, attributed to $nTiO_2$ not dissolving, and bivalves' studies inferred that most damage was done through oxidative stress mechanisms (Baker et al. 2014). On mussels, several reports of biochemical alterations with observable oxidative stress have been reported (Canesi et al. 2010; Barmo et al. 2013; Katsumiti et al. 2015; Hu et al. 2017). Benthic organisms were also identified as potentially at risk facing the deposition of $nTiO_2$, nanotoxicity being reported for *T. granosa* (Guan et al. 2018), but organisms such as *A. marina* demonstrated some resiliency to acute exposure (Galloway et al. 2010). Other conjugation effects of $nTiO_2$ presence have also been noted, such as its role as a carrier for phenanthrene accumulation (Tian et al. 2014) or bisphenol-A (Fang et al. 2016). Trophic transference has been proposed for engineered nanomaterials (Laborda et al. 2017), and in the case of $nTiO_2$ it has been confirmed, however direct exposure has generally a greater impact on organisms health (Wang et al. 2016b; Wang et al. 2017; Bhuvaneshwari et al. 2018).

1.6. Research work objectives

The overall aim of this research project is to provide a multidisciplinary insight into the dynamic of Ti(IV) when entering the aquatic ecosystem and its effects on key organisms. The specific objectives are:

Objective 1: To understand the fate of Ti(IV) from anthropogenic sources in coastal environment.

Objective 2: To study the bioaccumulation processes in bivalves exposed to Ti(IV).

Objective 3: To assess the biochemical responses of bivalves exposed to Ti(IV).

Objective 4: To evaluate the biochemical alterations of bivalves in response to combined effects on changes of key environmental variables and on Ti bioavailability.

To achieve these objectives controlled laboratorial experiments were performed on seawater spiked with Ti(IV) and exposure of *Mytilus galloprovincialis* and *Ruditapes phlippinarum*. In addition, the influence of environmental parameters such as pH, temperature and salinity as observed in transitional waters was also studied.

The results presented in the following chapters are derived of the following articles and manuscripts:

Monteiro R., Costa S., Coppola F., Freitas R., Vale C., Pereira E. (2019). Toxicity beyond accumulation of Titanium after exposure of *Mytilus galloprovincialis* to spiked seawater. Environ Pollut. 2019;244:845-854.

Monteiro R., Costa S., Coppola F., Freitas R., Vale C., Pereira E. (2019). Evidences of metabolic alterations and cellular damage in mussels after short pulses of Ti contamination. Sci Total Environ. 2019;650:987-995.

Leite C., Coppola F., Monteiro R., Russo T., Polese G., Lourenço M. A. O., Silva, M. R. F., Ferreira P., Soares A. M. V. M., Freitas R., Pereira E. (2019). Biochemical and histopathological impacts of Rutile and Anatase (TiO_2 forms) in *Mytilus galloprovincialis*. Sci. Total Environ. 134886

Leite C., Coppola F., Monteiro R., Russo T., Polese G., Silva, M. R. F., Lourenço M. A. O., Ferreira P., Soares A. M. V. M., Pereira E., Freitas R. (2020). Toxic impacts of *rutile* titanium dioxide in *Mytilus galloprovincialis* exposed to warming conditions. Chemosphere 252, 126563

Chapter 2

Materials and Methods

2. Materials and methods

2.1. Materials and reagents

All chemical reagents, for both chemical and biochemical procedures, used in the present work were of analytical grade, obtained from certified commercial suppliers without further purification.

Ti^{4+} commercial standard solution (999 ± 5 mg L^{-1}) was acquired from Inorganic Ventures (CGTI1-1), used for both exposures assays and calibration curves with any required dilutions being done in ultra-pure water (18 MΩ cm^{-1}, Millipore Milli-Q system). Titanium dioxide nanoparticles, $nTiO_2$ Degussa P-25, were acquired from Sigma-Aldrich and were characterized and used for exposure experiments. Titanium dioxide powders of specific morphologies (Anatase and Rutile) were acquired from Merck and Alfa Aesar, respectively, and were characterized and used for exposure experiments.

All plastic (polyethylene) and Teflon material related to Ti quantification was properly washed by 24 h immersion cycles in detergent (Extran MA 01), nitric acid (25% v/v), hydrochloric acid (25 % v/v) and then rinsed in ultra-pure water. All ceramic and glassware used in biochemical works were carefully washed in detergent (Derquim 5%) and then thoroughly rinsed in distilled water.

2.2. Titanium quantification method

2.2.1. Acid digestion method validation

The quantification of Ti in solid samples by acid digestion was developed following the guidelines of EPA 3051A (EPA 2007) and a previously developed work by Mieiro et al. (2012).

The method used 0.2 g of sample weighted into a Teflon vessel to which was added the appropriate acid mixture. Samples were left overnight (12 h) at the oven (60 ºC), after which the temperature was raised to 100 ºC for 1 hour. After cooling down, H_2O_2 was added to the reaction vessel to proceed for a last digestion cycle at 80 ºC for 1 h. The samples were collected to polypropylene flasks for a final volume of 100 mL with ultra-pure water and stored at room temperature until analysis.

For the validation, two certified reference materials (CRMs) were used, MESS-3 (marine sediment) and NIST 2976 (mussel tissue), with varying conditions (Table 6). The analysis was done by both inductive coupled plasma mass spectrometry (ICP-MS, Thermo X Series) and inductive coupled plasma optical emission spectrometry (ICP-OES, Jobin Yvon Activa M).

Calibration of both ICP-MS and ICP-OES was ensure by the dilution of Ti commercial standard in acid mixture similar to the samples, with another independent standard being prepared to ensure the validity of the curve. Furthermore, for ICP-OES it was ensure a reading of 3 different wavelengths while for ICP-MS appropriate corrections with the internal standard were made.

Proper calibration was ensured by a minimum of 4 standards, each with coefficients of variation (CV) ranging between 1-15 % (increasing with proximity to LoQ), and a determination coefficient (R^2) of 0.999.

Table 6 – Summary of acid digestion methods tested and studied sample matrices

Method	Acid Digestion			Sample Matrix	
	HNO_3	HF	H_2O_2	Mussel Tissue	Sediment
1	4	2	2	NIST 2976	MESS-3
2	2	1	1		
3	2	0.5	0.5		
4	2	0.5	2		
5	1	0.5	0.5		
6	1	0.5	2		

Note: Acid volumes in mL

2.2.2. Quantification method of Ti in water and tissue samples

Following the validation work, the current accepted quantification of Ti in both water and tissue samples were done by ICP-OES, according to the following procedure.

Collected water samples were promptly acidified with concentrated nitric acid to 1 % (v/v) and directly analyzed within 3 days of collection for total dissolved Ti content (Ti^{4+}).

Tissue samples were first freeze dried and homogenized prior to digestion. Digestion procedure followed validation method nº 1. To a Teflon vessel were added 0.2 g of sample and 4 mL of concentrated nitric acid and 2 mL of hydrofluoric acid. Samples were left overnight (12 h) at the oven (60 ºC), after which the temperature was raised to 100 ºC for 1 hour. Following the cooling down, H_2O_2 was added to the reaction vessels to proceed for a last digestion cycle at 80 ºC for 1 h. The samples were collected to polypropylene flasks for a final volume of 100 mL with ultrapure water and stored at room temperature until analysis.

Calibration curves acquired were always within the aforementioned required quality parameters (Fig. 1), and acid digestion quality control of Ti quantification procedure in tissues was achieved through the use of blank samples (only reagent mixture), a CRM (NIST 2976) and a sample duplicate per digestion cycle. Each of the mentioned parameter were respectively, always below quantification limits (LoQ of 2 µg L^{-1}; 1 µg g^{-1}), 5.83 ± 0.56 µg g^{-1} (within 10 % error margin) and below 20 % deviation (considerable acceptable due to proximity to LoQ).

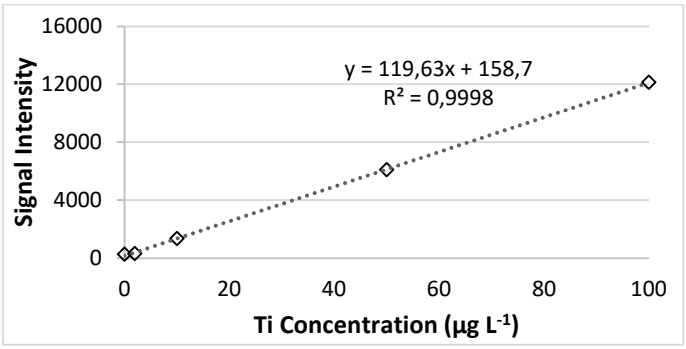

Fig. 1 – Example of Ti calibration curve for ICP-OES (wavelength 336.122 nm)

2.3. Ti^{4+} interactions in marine environment

Biota interaction – pilot test on green macro algae

Sample collection and experimental designed for this experience was based on previous work developed for other metal-based bioaccumulation studies (Henriques et al. 2015; Figueira et al. 2016).

During low tide, green algae *Ulva lactuca* was collected from Ria de Aveiro – Mira channel and brought to the laboratory in plastic bags with local water. Subsequently, using filtered seawater, the algae was cleaned of debris and kept in aquaria for 72 h, filled with filtered seawater at 30 PSU, room temperature (20 ± 1 ºC) and natural light photoperiod condition.

Contaminant exposition was done in 1 L Schott flasks, with c.a. 1.6 g of algae previously (12 h earlier) selected and cut into similar sized disk (c.a. 6 cm), filled with filtered seawater to which was added Ti^{4+} commercial standard to achieve a concentration of 1 mg L^{-1}. For result validity, the experiment was run in triplicate. The experiment was finished after 72 h, with algae being collected and frozen (-20 ºC). Water samples were taken immediately after Ti^{4+} spike and at the end of the experiment.

Stability tests – kinetics of Ti^{4+} in seawater

To assess the stability of Ti^{4+} in seawater, in 15 L aquarium, filled with artificial seawater (obtained by adding commercial salt, Tropic Marin® SEA SALT from Tropic Marine Center, to reverse osmosis water – Aqua-win RO-6080) at controlled salinity, pH and temperature conditions (30 PSU, 8.0 and 17 ºC). Aeration system for water oxygenation and mixing was used to mimic the exposure assays conditions. Contamination spiking was done through the addition of 100 mL of an appropriate concentration Ti^{4+} solution, to obtain three different levels of Ti contamination (5, 50, and 100 µg L^{-1}). The experiment ran for 96 h, each condition done in triplicate, accompanying the exposure assay 2. Water samples were collected at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h.

Stability tests – influences of redox potential

The study of water oxygenation on the stability of Ti^{4+} in solution were done in 1 L flasks, with artificial seawater in the same conditions to the previous kinetic study, with a group of flasks being aerated with air and the other group in an anoxic environment (N_2 bubbling). The experiment ran in triplicate, with the 100 µg L^{-1} condition being chosen to better assess the gradual decrease in Ti concentration. Water samples were taken at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h.

2.4. $nTiO_2$ Degussa P-25 characterization and seawater studies

The $nTiO_2$ Degussa P-25 used were characterized using the transmission electron microscopy (TEM, Hitachi H9000-NA) following the complete analysis set of: a) bright field and dark field imaging; b) electron diffraction pattern; c) EDS chemical characterization.

The samples were prepared following Weiss and Moser (2015) using immersion technique, i.e. immersing the carbon coated copper grid in a dispersion solution of $nTiO_2$ in ultrapure water after being sonicated to achieve maximum particle dispersion, an operational change to the regular drop addition to avoid agglomeration during the drying step (Michen et al. 2015). The grid was then left to air-dry before inserted into the equipment.

Further cross-verification of the structural characterization, morphology and size, of the $nTiO_2$ was done by X-ray powder diffraction (XDR, Philips X'Pert) and dynamic light scattering (DLS, Zetasizer Nano ZS). The former analysis was outsourced to the XDR services provided by LCA while for the latter the $nTiO_2$ was dispersed in ultra-pure water and then analyzed for the average particulate size.

A second set of characterization was done to evaluate the effects of $nTiO_2$ in the marine environment. As such, $nTiO_2$ was dispersed in marine water (filtered saltwater obtained from Ria de Aveiro Lagoon; salinity 30 PSU, pH 8.0) and left for 1 week, after which the dispersion was again sonicated and a carbon grid was made through the immersion technique. Characterization through TEM followed the same steps as previously described.

2.5. TiO_2 powders (anatase and rutile fractions) characterization

The commercially available TiO_2 powders of different morphology were evaluated for structural, microstructural and textural properties, outsourced to both the services provided by University of Aveiro laboratories under the Chemistry Department and the Materials and Ceramic Engineering Department.

Structural and microstructural characterization was done through X-ray powder diffraction (XRD, Philips X'Pert) and SEM (SEM-FEG Hitachi S4100). Textural analysis was obtained by N_2 adsorption-desorption isotherms using a surface area analyzer (Gemini V2.00 – 2380).

2.6. Exposure assays

The investigation of the bioaccumulation and biological effects on the organisms were done through mesocosm experiments in a laboratorial controlled environmental condition. As such, each experiment required several steps for their elaboration, starting from the organism collection, followed by their acclimation to experimental conditions and only then the exposure assay.

Organism selection, collection and acclimation

For the exposure assays two bivalves were used, the mussel *Mytilus galloprovincialis* and the clam *Ruditapes philippinarum*. Standard size chosen for the organisms were 5.7 ± 0.2 cm for mussels and 4.1 ± 0.2 cm for clams, criteria that was kept through all the experiments.

Both organisms were collected at Ria de Aveiro – Mira channel due to being reported as low contaminated area (Freitas et al. 2015b; Velez et al. 2015), and promptly brought to the laboratory for acclimation in 100 L tanks with constant water circulation and filtration.

The individuals went through a minimum period of acclimation of 14 days, while being maintained in artificial seawater (obtained by adding commercial salt, Tropic Marin® SEA SALT from Tropic Marine Center, to reverse osmosis water – Aqua-win RO-6080). During this period, organisms were maintained under controlled temperature (17 ± 1 ˚C), pH (8.0 ± 0.1), salinity (30 ± 1) and photoperiod (12 light:12 dark), resembling the average conditions measured at the sampling site at the collection moment. Mussels were not fed during the first week, after which organisms were fed every other day with Algamac protein plus (approximately 150,000 cells per individual). Water was changed twice in the first week and then was changed on a weekly base.

Dead or un-fit organisms were promptly removed when found and, after the acclimation period was over, the remaining individuals were transferred into their respective aquaria for the exposure assays.

2.6.1. Exposure assay 1 – short-term exposure to Ti^{4+}

To each aquarium, with a final volume of 15 L of artificial seawater, were added 40 organisms (*M. galloprovincialis*) and conditions (temperature, salinity and pH) were maintained as described in the acclimation period (17 ± 1 ˚C; 30 ± 1; 8.0 ± 0.1). Three aquaria were used for each experimental condition, totaling nine aquaria with three different Ti^{4+} concentrations (5, 50, 100 µg L^{-1}), and three more aquaria used for control (no Ti^{4+} contamination) (Fig. 2).

Contamination was done by spiking with 100 mL of Ti^{4+} solutions, with appropriate concentration, prepared in ultrapure water from the commercial Ti standard solution. Salinity and pH checks were made following the additions of the contaminant and no significant alterations were noted.

The experiment ran for 14 day, and during the experimental period water flow and aeration was ensured using one air pump per aquarium. Seawater of each aquarium was changed after 7 days, with the reestablishment of the initial Ti^{4+} concentrations, and the experiment was finished at day 14. Feeding was carried thrice per week (Algamac protein plus, approximately 150,000 cells per individual) and daily checks for unfit or dead (unresponsive to physical stimulus) individuals were made, with their immediate removal upon death.

Water samples for Ti quantification were taken immediately after addition of the contaminant spike (t_0), after 72 h and 7 days (prior to water renewal) with each sample being treated accordingly to previously described methodology. At 96 h and at day 14, 8 individuals per aquarium (24 per condition, 96 in total) were removed and immediately frozen with liquid nitrogen and preserved (-80 ºC) for further processing. For biochemical analysis, 3 organisms per aquarium (9 per condition; 36 per sampling moment; 72 in total) were used. Ti quantification was done using 5 organisms per condition (20 per sampling moment; 40 total).

CTL-R1	**CTL-R2**	**CTL-R3**
$[Ti^{4+}]$ = 0 µg L^{-1}	$[Ti^{4+}]$ = 0 µg L^{-1}	$[Ti^{4+}]$ = 0 µg L^{-1}
C1-R1	**C1-R2**	**C1-R3**
$[Ti^{4+}]$ = 5 µg L^{-1}	$[Ti^{4+}]$ = 5 µg L^{-1}	$[Ti^{4+}]$ = 5 µg L^{-1}
C2-R1	**C2-R2**	**C2-R3**
$[Ti^{4+}]$ = 50 µg L^{-1}	$[Ti^{4+}]$ = 50 µg L^{-1}	$[Ti^{4+}]$ = 50 µg L^{-1}
C3-R1	**C3-R2**	**C3-R3**
$[Ti^{4+}]$ = 100 µg L^{-1}	$[Ti^{4+}]$ = 100 µg L^{-1}	$[Ti^{4+}]$ = 100 µg L^{-1}

Fig. 2 – Exposure assay 1 experimental setup

2.6.2. Exposure assay 2 – mid-term exposure to Ti^{4+} and recovery

To 15 L aquaria, with artificial seawater, 40 organisms (*M. galloprovincialis*) were added and the physicochemical conditions (temperature, salinity and pH) were maintained as described in the previous experiment. Also similar to the previous experiment, three aquaria were used for each experimental condition, for each of the different Ti^{4+} tested concentrations (5, 50, 100 µg L^{-1}), and three more aquaria used for control (0 µg L^{-1} of Ti^{4+}) (Fig. 3).

Contamination was done through spiking with 100 mL of Ti^{4+} solutions, akin to described in the previously assay, with salinity and pH being checked following the additions of the contaminant, also with no significant alterations were noted.

The exposure assay went for a total of 28 days, with each aquarium being aerated through an air pump and water being renewed at a weekly base with reposition of the contamination, through a new spiking. In the following 14 mussels were kept in a depurative state by replacing the Ti spiked seawater by clean artificial seawater. Throughout the experiment feeding was kept at thrice per week (Algamac protein plus, approximately 150,000 cells per individual) and organisms were checked daily for fitness, with unfit or dead individuals being immediately removed upon death.

Ti quantification was done on water samples immediately taken after the contaminant spike (t_0), with further verifications at day 3 and day 7 after the initial spike. Each sample was treated accordingly to previously described methodology. At the end of each phase of the experiment (day 28 and day 28 + 14), 8 individuals per aquarium (24 per condition, 96 in total in each sampling moment) were collected and immediately frozen in liquid nitrogen and preserved at −80 °C. For biochemical oxidative stress tests, 3 organisms per aquarium (9 per condition; 36 per sampling moment; 72 in total) were used, while for bioaccumulation 5 organisms per condition were used (20 per sampling moment; 40 total).

CTL-R1	**CTL-R2**	**CTL-R3**
$[Ti^{4+}]$ = 0 µg L^{-1}	$[Ti^{4+}]$ = 0 µg L^{-1}	$[Ti^{4+}]$ = 0 µg L^{-1}
C1-R1	**C1-R2**	**C1-R3**
$[Ti^{4+}]$ = 5 µg L^{-1}	$[Ti^{4+}]$ = 5 µg L^{-1}	$[Ti^{4+}]$ = 5 µg L^{-1}
C2-R1	**C2-R2**	**C2-R3**
$[Ti^{4+}]$ = 50 µg L^{-1}	$[Ti^{4+}]$ = 50 µg L^{-1}	$[Ti^{4+}]$ = 50 µg L^{-1}
C3-R1	**C3-R2**	**C3-R3**
$[Ti^{4+}]$ = 100 µg L^{-1}	$[Ti^{4+}]$ = 100 µg L^{-1}	$[Ti^{4+}]$ = 100 µg L^{-1}

Fig. 3 – Exposure assay 2 experimental setup

2.6.3. Exposure assay 3 – mid-term exposure to TiO_2 (Anatase vs Rutile)

Five mussels were distributed to each aquarium filled with 3 L of artificial seawater, with physicochemical parameters (temperature, salinity and pH) akin to the ones in the acclimation conditions. Studied contamination conditions were based from the previous studies, substituting the contaminant Ti^{4+} for two commercial TiO_2 powders, of anatase and rutile morphologies, in equimolar concentrations ([Ti_{eq}] expressed in $\mu g\ L^{-1}$). Thus, for each studied concentration (5, 50, 100 $\mu g\ L^{-1}$ of Ti_{eq}) and each of the two TiO_2 powders were used three aquaria, with another three aquaria used for control (0 $\mu g\ L^{-1}$ of Ti_{eq}) (Fig. 4).

Contamination of each aquarium was done through spiking with a previously sonicated 100 mL TiO_2 powder dispersions of appropriate Ti_{eq} concentrations, prepared in ultrapure water through the dilution of a weekly freshly prepared dispersions of 60 and 600 $\mu g\ L^{-1}$ of Ti_{eq} of their respective TiO_2 powders in ultrapure water. Following the spiking the physicochemical properties of the medium were checked, with no variation of the salinity and pH being registered.

The assay followed the 28-day exposure setup, with aquaria being aerated through air pumps (1 per aquarium), and water renewal was done at the start of each new week followed by the restoration of the contamination levels as aforementioned. Feeding (Algamac protein plus, approximately 150,000 cells per individual) was scheduled for three times each week and mussels were health was evaluated daily, and dead organism were promptly removed.

Samples for Ti quantification in water were taken immediately after the spiking (t_0), with further collections after 3 and 7 days. Each sample was conserved through acidification as previously described. At the end of the assay (day 28), of the 5 mussels 4 per aquarium were promptly frozen in liquid nitrogen and stored at – 80 ºC for further processing. For biochemical parameters were used 3 organisms per aquarium, 9 per condition for a total of 63 individuals; and for Ti quantification tissues from 5 organisms per condition were used, totaling 35 mussels.

Control		
CTL-R1	**CTL-R2**	**CTL-R3**
$[Ti_{eq}] = 0$ µg/L	$[Ti_{eq}] = 0$ µg L^{-1}	$[Ti_{eq}] = 0$ µg L^{-1}
Anatase		
C1-R1	**C1-R2**	**C1-R3**
$[Ti_{eq}] = 5$ µg L^{-1}	$[Ti_{eq}] = 5$ µg L^{-1}	$[Ti_{eq}] = 5$ µg L^{-1}
C2-R1	**C2-R2**	**C2-R3**
$[Ti_{eq}] = 50$ µg L^{-1}	$[Ti_{eq}] = 50$ µg L^{-1}	$[Ti_{eq}] = 50$ µg L^{-1}
C3-R1	**C3-R2**	**C3-R3**
$[Ti_{eq}] = 100$ µg L^{-1}	$[Ti_{eq}] = 100$ µg L^{-1}	$[Ti_{eq}] = 100$ µg L^{-1}
Rutile		
C1-R1	**C1-R2**	**C1-R3**
$[Ti_{eq}] = 5$ µg L^{-1}	$[Ti_{eq}] = 5$ µg L^{-1}	$[Ti_{eq}] = 5$ µg L^{-1}
C2-R1	**C2-R2**	**C2-R3**
$[Ti_{eq}] = 50$ µg L^{-1}	$[Ti_{eq}] = 50$ µg L^{-1}	$[Ti_{eq}] = 50$ µg L^{-1}
C3-R1	**C3-R2**	**C3-R3**
$[Ti_{eq}] = 100$ µg L^{-1}	$[Ti_{eq}] = 100$ µg L^{-1}	$[Ti_{eq}] = 100$ µg L^{-1}

Fig. 4 – Exposure assay 3 experimental setup

2.6.4. Exposure assay 4 – effect of temperature on mid-term exposure to r-TiO_2

For this exposure assay two temperature-controlled rooms were used, with one representing the control conditions (stabilized at 18 ± 1 ºC) and the other room simulating the warming effects (22 ± 1 ºC). Mussels were acclimated as previously mentioned and then 5 organisms were distributed into each 3 L aquaria, filled with artificial seawater (30 PSU, 18 ºC, 8.0 or 30 PSU, 22 ºC, 8.0, depending on the room). For data validity each condition was ran in triplicate, and as such, in each of the acclimated rooms there were 12 aquaria, 3 for control conditions (0 µg L^{-1}) and 9 divided between the three studied concentrations (5, 50 and 100 µg L^{-1} of Ti_{eq}) (Fig. 5).

Rutile powder (r-TiO_2) was chosen as the target contaminant and its introduction in the aquaria was done through spiking using 100 mL dispersions sonicated prior to addition. Each contaminant dispersion was prepared by dilution in ultrapure water of freshly prepared dispersion of 60 and 600 µg L^{-1}, also prepared in ultrapure from the r-TiO_2 powder. Following the spiking salinity and pH were verified, with no noticeable significant variation.

The 28-day exposure experiment ran according the normal parameters, with aeration done through 1 air pump in each aquarium and water being renewed weekly, with further reposition of the contaminant. Thrice per week, the organisms were fed (Algamac protein plus, approximately 150,000 cells per individual) and daily checks for unfit or dead individuals were done, with prompt removal of the dead ones.

Weekly, water samples for Ti quantification were taken on three different moments, after spike (t_0) and at day 3 and 7. Samples were stored through acidification as already mentioned. After the four-week exposure period (28 days), the individuals were frozen with liquid nitrogen and stored at -80 ºC for further processing. In this assay 3 organisms per aquarium, 9 per condition for a total of 72 individuals were used for biochemical parameters; and 5 mussels per condition for a total of 40 organisms were used for Ti quantification.

18 ºC		
CTL-R1	**CTL-R2**	**CTL-R3**
$[Ti_{eq}]$ = 0 µg/L	$[Ti_{eq}]$ = 0 µg L^{-1}	$[Ti_{eq}]$ = 0 µg L^{-1}
C1-R1	**C1-R2**	**C1-R3**
$[Ti_{eq}]$ = 5 µg L^{-1}	$[Ti_{eq}]$ = 5 µg L^{-1}	$[Ti_{eq}]$ = 5 µg L^{-1}
C2-R1	**C2-R2**	**C2-R3**
$[Ti_{eq}]$ = 50 µg L^{-1}	$[Ti_{eq}]$ = 50 µg L^{-1}	$[Ti_{eq}]$ = 50 µg L^{-1}
C3-R1	**C3-R2**	**C3-R3**
$[Ti_{eq}]$ = 100 µg L^{-1}	$[Ti_{eq}]$ = 100 µg L^{-1}	$[Ti_{eq}]$ = 100 µg L^{-1}
22 ºC		
C1-R1	**C1-R2**	**C1-R3**
$[Ti_{eq}]$ = 5 µg L^{-1}	$[Ti_{eq}]$ = 5 µg L^{-1}	$[Ti_{eq}]$ = 5 µg L^{-1}
C1-R1	**C1-R2**	**C1-R3**
$[Ti_{eq}]$ = 5 µg L^{-1}	$[Ti_{eq}]$ = 5 µg L^{-1}	$[Ti_{eq}]$ = 5 µg L^{-1}
C2-R1	**C2-R2**	**C2-R3**
$[Ti_{eq}]$ = 50 µg L^{-1}	$[Ti_{eq}]$ = 50 µg L^{-1}	$[Ti_{eq}]$ = 50 µg L^{-1}
C3-R1	**C3-R2**	**C3-R3**
$[Ti_{eq}]$ = 100 µg L^{-1}	$[Ti_{eq}]$ = 100 µg L^{-1}	$[Ti_{eq}]$ = 100 µg L^{-1}

Fig. 5 – Exposure assay 4 experimental setup

2.6.5. Exposure assay 5 – multiple factor assay

Assays occurred in two types of aquaria, 8 L aquarium were used for the study of contaminant effects under salinity and temperature shifts while 17 L aquaria were used for the ocean acidification study. This change for the ocean acidification study was due to experimental design and the need for a different water mixing system (water pump instead of aeration system) for proper homogeneity. To keep an equilibrated organism-water volume ratio, to each of the 8 L aquarium were added 5 mussels (*M. galloprovincialis*) and 5 clams (*R. philippinarum*), while 17 L aquaria had 11 of each organism.

Two contamination conditions were studied, using 100 µg L^{-1} of Ti^{4+} and 100 µg L^{-1} of Ti_{eq} of $nTiO_2$ Degussa P-25. Contamination was done through spiking with 100 mL contaminant solutions, with Ti^{4+} being prepared through dilution of Ti standard in ultrapure water and $nTiO_2$ being disperser in ultrapure water and sonicated prior to addition into the medium. All physicochemical parameters of the medium (salinity, pH and Temperature) were checked following the contamination, with no significant alterations were noted. Samples for Ti quantification were taken immediately after the spike (t_0), to assess initial measured concentration. Each sample was treated accordingly to previously described methodology.

Climate change conditions studied contemplated three salinity ranges (20, 30 and 40 PSU), two different temperatures (17 ºC and 21 ºC) and two pH scenarios (8.0 and 7.6); underlined conditions refer to climate control conditions. Each experimental condition ran in triplicate, with a total of 45 aquaria being used in this experiment; 27 for salinity shift, 9 for seawater acidification and 9 for temperature increase, of which 15 were used for biological control (Fig. 6). Temperature control was achieved through room temperature climatization, and checked daily, while pH manipulation was achieved through automatic CO_2 pumping system (Aquamedic). This system used independent pH probes for each aquarium, all individually calibrated and crosschecked at least twice per week with another pH instrument (Hanna Instruments), with computerized central system programmed to inject CO_2 whenever pH > 7.6.

Feeding and water renewals followed the same procedure in previous experiments, thrice per week (Algamac protein plus, approximately 150,000 cells per individual) and every 7 days, respectively. At the end of each weekly cycle physicochemical parameters of all aquaria were recorded and water samples (50 mL) for total alkalinity analysis were taken from the acidification study aquaria. During the exposure assay organisms were checked for physical fitness, and when found dead were promptly removed.

Total alkalinity analysis followed the Standard Operation Procedure 3 (SOP 3) for determination in seawater (Dickson et al. 2007), using mercuric chloride 0.01% (v/v) for carbon fixation and potentiometric titration method (Gran 1952). Following data treatment used the CO2SYS Calc software (Robbins et al. 2010), using the plotted potentiometric results and the experimental salinity and temperature records, to characterize the seawater carbon content (Moreira et al. 2018a).

The experiment concluded after 28 days, and all organisms were collected and immediately frozen in liquid nitrogen and preserved at −80 °C for further processing. Oxidative stress parameters were checked using 2 organisms per aquarium (6 per condition; 90 per species; 180 in total).

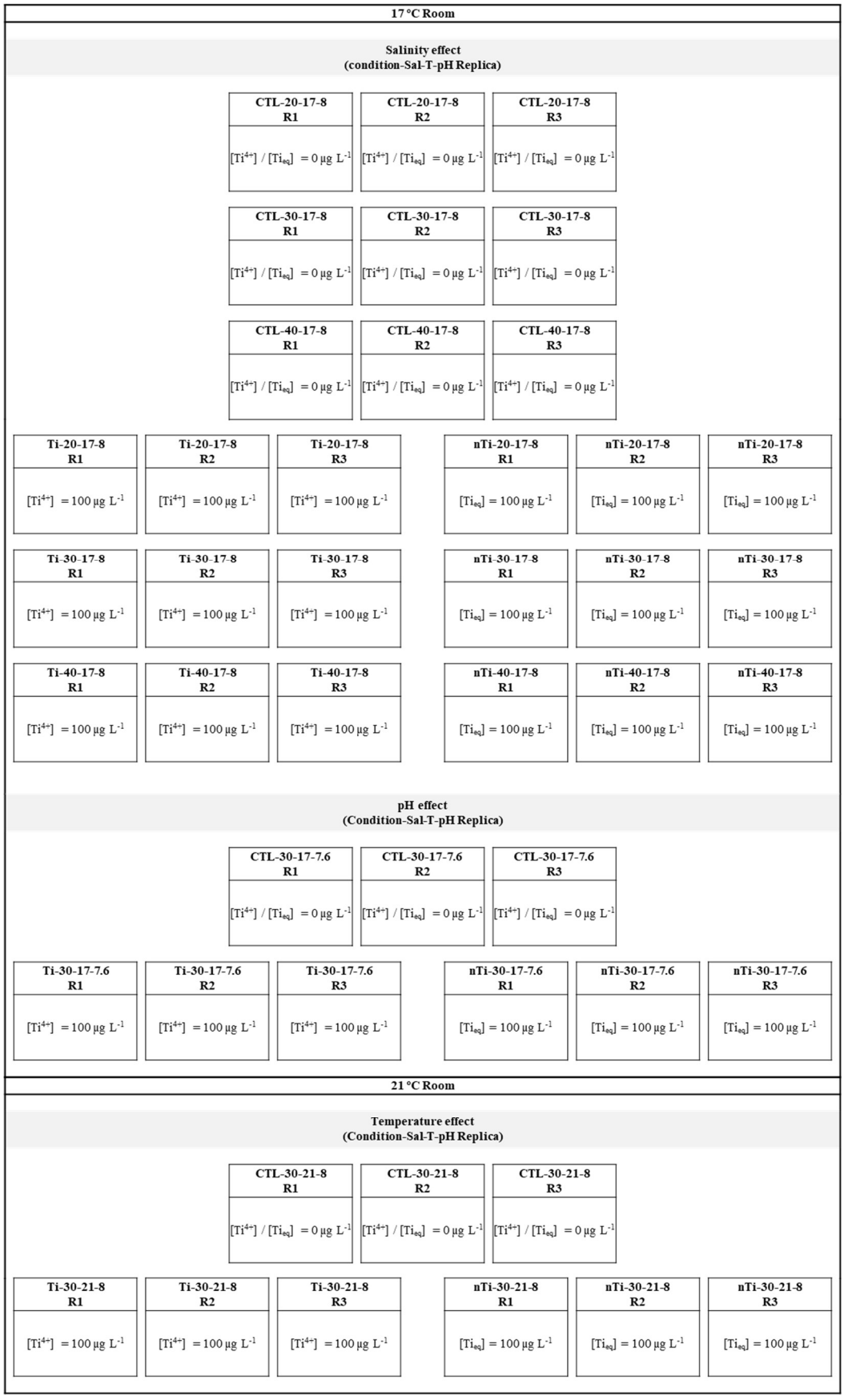

Fig. 6 – Exposure assay 3 experimental setup

2.7. Biochemical and statistical analysis

2.7.1. Biochemical analysis

For the evaluation of the biochemical fitness of the studied organisms a set of biochemical biomarkers were chosen for laboratorial analysis, factoring the energetic content (glycogen, GLY; protein, PROT) and metabolic activity (electron transport system, ETS), the enzymatic (catalase, CAT; superoxidismutase, SOD; glutathione peroxidase; GPx; glutathione reductase, GRed; glutathione S-transferases, GSTs) and non-enzymatic (lipid peroxidation, LPO; protein carbonylation, PC; neurotoxicity – AChE; reduced and oxidized glutathione concentrations, GSH and GSSG, respectively) oxidative stress biomarkers.

Analysis were done spectrophotometrically (microplate reader, BioTek Synergy HT), through the Beer-Lambert law, using either the known molar absorptivity constant (ε) of the target substance or a calibration curve. Quality control was ensured by each sample being analyzed in duplicate (CV < 10 %), blank samples (only reagents) and, when calibrations were involved, the R^2 was ensured to be of at least 0.99.

All analyzed parameters were done followed the respective original author procedures, some with further adaptations, with the most recent updated formats being well described by Almeida et al. (2014), Freitas et al. (2017), Velez et al. (2017) and Andrade et al. (2019b).

Sample preparation and extraction

For the biochemical assays, the whole frozen tissue samples of an organism were mechanically homogenized, kept frozen using liquid nitrogen, and separated into 500 mg aliquots. Each aliquot was extracted with 1 mL of a corresponding buffers and then homogenized by TissueLyser II (Qiagen) for 1 min at 120 Hz. The samples were finally centrifugated at 10 000 g for 20 min (3 000 g for 10 min for ETS) at 4 ºC to obtain the supernatant with the necessary biological fraction for analysis. After extraction samples were always kept frozen (-20 ºC) when stored or in refrigerated conditions (4 ºC) when working.

A set of four extraction buffers were used depending on the parameter for analysis.

ETS extraction was done with a 0.1 mM Tris-HCl buffer (pH 8.5, 15% (w/v) PVP, 153 mM magnesium sulfate and 0.2% (v/v) Triton X-100).

For LPO analysis it was used a trichloroacetic acid buffer (TCA 20% (v/v)).

The extraction buffer for the analysis of the redox status of the cell (GSH and GSSG) was a solution of 0.6% sulfosalicylic acid in potassium phosphate buffer (0.1 M dipotassium phosphate; 0.1 M potassium dihydrogen phosphate; 5 mM EDTA; 0.1% (v/v) Triton X-100; pH 7.5).

Remaining biomarkers were obtained through the potassium phosphate buffer (50 mM potassium dihydrogen phosphate; 50 mM potassium phosphate dibasic; 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) polyvinylpyrrolidone (PVP); 1 mM dithiothreitol (DTT); pH 7.0).

Metabolic capacity and energy related biomarkers

ETS activity was measured following De Coen and Janssen (1997) and King and Packard (1975) methods. The absorbance was measured at 490 nm in 25 sec intervals for 10 min. The amount of formazan formed was calculated using ε = 15 900 $M^{-1}cm^{-1}$ and the results expressed in nmol min^{-1} g^{-1} of fresh weight (FW).

Quantification of GLY content was done according to the sulfuric acid method Dubois et al. (1956), using glucose standards (0-5 mg mL^{-1}). The absorbance was measured at 492 nm and the concentration of GLY was expressed in mg g^{-1} of FW.

PROT content was determined using bovine serum albumin (BSA) as standard (0-40 mg mL^{-1}), following the Biuret method (Robinson and Hogden 1940). The absorbance was measured at 540 nm and the concentration of PROT was expressed in mg g^{-1} of FW.

Oxidative stress enzymatic biomarkers

Activity of SOD was determined by using the method described in Beauchamp and Fridovich (1971) and adaptations performed by Carregosa et al. (2014). The standard curve was determined using SOD standards (0.0001-60 U mL^{-1}). The absorbance was measured at 560 nm. Results were expressed as U g^{-1} of FW, where U (µmol min^{-1}) represents the catalysis rate of enzymatic reduction of nitroblue tetrazolium (NBT).

Activity of CAT was quantified according to Johansson and Borg (1988) and the modifications performed by Carregosa et al. (2014). The standard curve was determined using formaldehyde standards (0-150 µmol L^{-1}) and the absorbance was measured at 540 nm. The enzymatic activity was expressed in U g^{-1} FW, where U (nmol min^{-1}) represents the rate of formaldehyde formation due to enzymatic activity.

Activity of GPx was quantified following Paglia and Valentine (1967). The absorbance was measured at 340 nm for 5 min in 10 sec intervals and the enzymatic activity was determined using ε = 6.22 mM^{-1} cm^{-1} and the results were expressed as U g^{-1} of FW, where U (µmol min^{-1}) represents oxidation rate of NADPH through the enzymatic process.

Activity of GRed was evaluated as described in the method by Carlberg and Mannervik (1985). Absorbance was measured at 340 nm and GRed was determined using ε = 6.22 mM^{-1} cm^{-1}. GRed was expressed in U g^{-1} FW, where U (µmol min^{-1}) denotes the rate of NADPH formation catalyzed by the enzyme.

Activity GSTs was determined according to Habig et al. (1974). The absorbance was measured at 340 nm and the activity of GSTs was determined using the ε = 9.6 mM cm^{-1} for CDNB. Results were expressed in U g^{-1} of FW, where U (µmol min^{-1}) is defined as the rate at which the enzymes catalyzes the formation of dinitrophenyl thioether.

Oxidative stress non-enzymatic markers

Lipid peroxidation (LPO) was measured according to Ohkawa et al. (1979) and modifications referred by Carregosa et al. (2014). The absorbance was measured at 535 nm and LPO levels were determined using $\varepsilon = 156\ mM^{-1}\ cm^{-1}$. LPO levels were expressed in nmol of MDA equivalents formed per g of FW.

Protein carbonylation (PC) was evaluated as described in the method by Mesquita (2014). Absorbance was measured at 450 nm and PC was determined using $\varepsilon = 22\ M^{-1}\ cm^{-1}$. Results were expressed in nmol of protein carbonyls groups formed per g of FW.

Reduced (GSH) and oxidized (GSSG) glutathione concentrations were determined according with Rahman et al. (2006). The absorbance was measured at 412 nm, for both assays, and results were expressed as nmol g^{-1} of FW. Graphical representation can also appear as a ratio of between the oxidized and reduced form, in which case it is dimensionless.

Neurotoxicity

Neurotoxicity was evaluated through the acetylcholinesterase (AChE) activity, determined according the method by Ellman et al. (1961) following the modifications by Mennillo et al. (2017). Absorvance was measured at 412 nm for 5 min, in 30 s intervals, and AChE was determined using $\varepsilon = 13\ 600\ M^{-1}\ cm^{-1}$. Enzymatic activity results were expressed in nmol $min^{-1}\ g^{-1}$ of FW.

2.7.2. Statistical analysis

Exposure assay 1 and 2

Data obtained from biochemical responses and Ti concentrations were submitted to hypothesis testing using permutational multivariate analysis of variance with the PERMANOVA+add-on in PRIMER v6 (Anderson et al. 2008).The pseudo-F p-values in the PERMANOVA main tests were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p < 0.05$) were considered as significantly different.

The null hypotheses tested were: a) for contaminant concentrations (in seawater from the exposure medium and mussels soft tissue) and for each experimental period, no significant differences existed among experimental conditions; b) for contaminant concentrations (in seawater from the exposure experiment and mussels soft tissue) and for each experimental condition, no significant differences existed between experimental periods; c) for each biochemical parameter and for each experimental period, no significant differences existed among experimental conditions; d) for each biochemical parameter and for each experimental condition, no significant differences existed between experimental periods. Graphical representations of significant differences among experimental conditions for different exposure times are represented with lower and upper-case letters, respectively; significant differences between both exposure periods for a given experimental condition are represented with an asterisk.

Principal coordinate analysis (PCO) was obtained by gathering all the obtained experimental data in a single matrix. This matrix was used to calculate the Euclidean distance similarity matrix and was then simplified to the centroid matrix which was finally submitted to ordination analysis by principal coordinates. Presented figures represent the spatial distribution under the determined coordinates with the superimposition of the relevant Pearson correlation vectors (correlation > 0.75).

Exposure assay 3

Results of biochemical responses and Ti concentrations were submitted to a statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA+add-on in PRIMER v6 (Anderson et al. 2008). The pseudo-F p-values in the PERMANOVA main tests were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p < 0.05$) were considered as significantly different.

The null hypotheses tested were: a) for each biochemical response and TiO_2 powders (rutile or anatase) no significant differences existed among exposure concentrations, with significant differences represented in tables with different lower-case letters for rutile and upper-case letter for anatase; b) for each biochemical response no significant differences existed between TiO_2 powders (rutile and anatase), with significant differences represented in tables with an asterisk.

Principal coordinate analysis (PCO) was obtained by gathering all the obtained experimental data in a single matrix. This matrix was used to calculate the Euclidean distance similarity matrix and was then simplified to the centroid matrix which was finally submitted to ordination analysis by

principal coordinates. Presented figures represent the spatial distribution under the determined coordinates with the superimposition of the relevant Pearson correlation vectors (correlation > 0.75).

Exposure assay 4

Results of biochemical responses and Ti concentrations were submitted to a statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA+add-on in PRIMER v6 (Anderson et al. 2008). The pseudo-F p-values in the PERMANOVA main tests were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p < 0.05$) were considered as significantly different.

The null hypotheses tested were: a) for each temperature and biochemical response, no significant differences existed among exposure concentrations, with significant differences represented in tables with different lower-case letters for 18 ºC and upper-case letters for 22 ºC; b) for each concentration and biochemical response, no significant differences existed between temperatures (18 and 22 ºC), with significant differences represented in tables with an asterisk.

Principal coordinate analysis (PCO) was obtained by gathering all the obtained experimental data in a single matrix. This matrix was used to calculate the Euclidean distance similarity matrix and was then simplified to the centroid matrix which was finally submitted to ordination analysis by principal coordinates. Presented figures represent the spatial distribution under the determined coordinates with the superimposition of the relevant Pearson correlation vectors (correlation > 0.75).

Exposure assay 5

Results on biological response (biochemical markers) in mussels soft tissues were submitted to a statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA+add-on in PRIMER v6 (Anderson et al. 2008). The pseudo-F p-values in the PERMANOVA main tests were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p < 0.05$) were considered as significantly different.

The null hypotheses tested were: a) for each contamination exposure condition (CTL, Ti and nTi) no significant differences existed among different environmental tested variations (Sal, pH and T), with significant differences represented in tables with different lowercase letters; b) for each environmental tested variation no significant differences existed between contamination exposure conditions, with significant differences represented in figures with an uppercase letters.

Principal coordinate analysis (PCO) was obtained by gathering all the obtained experimental data in a single matrix. This matrix was used to calculate the Euclidean distance similarity matrix and was then simplified to the centroid matrix which was finally submitted to ordination analysis by principal coordinates. Presented figures represent the spatial distribution under the determined coordinates with the superimposition of the relevant Pearson correlation vectors (correlation > 0.75).

Chapter 3

Results and Discussion

3. Results and discussion

3.1. Ti^{4+} and $nTiO_2$ – quantification, characterization and dynamics in marine environment

3.1.1. Validation of quantification method

Titanium is present in the environment in very low concentrations and present several challenges for its quantification, especially in complex mixtures such as seawater (Poehle et al. 2015). Furthermore, as it has been considered a biologically inert component (Croot 2011) not much research into marine environment has been put until more recent years. As such, there are no standardized tests to provide results on environmentally relevant samples.

The foremost issue to tackle was the necessity to develop an in-house methodology that would enable the accurate measurement of titanium in three major environmental matrix – water, sediment and organism tissues. Inductive couple plasma techniques with both mass and optical detection (ICP-MS and ICP-OES) have been widely used for water and sediment samples have been quite explored, in which LCA (Laboratório Central de Análises) already had internal analytical methods developed and optimized based on pre-existent methods developed by many international, e.g. American Environmental Protection Agency (EPA) and International Organization for Standardization (ISO). Water samples were thus acidified (HNO_3 1%) and analyzed within 3 days of sample collection, ensuring proper preservation and enough acid content to facilitate the atomization process of the already dissolved Ti content (Ti^{4+}). Solid samples, such as organism tissues and sediments, require a prior digestion to solubilize the intended elements (Ti), and thus for the validation method a CRM representing each of the matrices were used – marine sediment by MESS-3 and mussel tissue by NIST 2976.

Microwave assisted digestion method, which is one of the standard methods (EPA 2007), was adapted to fit the methodology in (Mieiro et al. 2012). This was mostly an operational choice considering several factors, such as number of projected samples, time of digestion process, total volume of acid in the vessels, physicochemical properties of Ti (e.g. reactivity and volatility).

Initially the work was supposed to proceed using ICP-MS, due to its broader application and expected lower limits of quantification stemming from the mass detector (Chaves et al. 2010; Khan et al. 2013), however the obtained results contradicted such predicted conclusion.

Regarding the analysis of titanium, ICP-MS has showed several disadvantages, both technical and intrinsic to the element in question. The use of HF to stabilize the titanium in the digestion requires for the exchange of the nebulization system, as HF corrodes the regularly used (silicon/glass material). Furthermore, the analysis of titanium is subject to several interferences from other entities of similar m/z (mass-charge ratio) (Table 7). The isotope Ti-49 may seem a good to focus the analysis however its low abundance tends to difficult its use when samples have very low titanium concentration.

Table 7 – Titanium isotopes and its interferents in ICP-MS analysis

Titanium (m/z)	Major Interferent (m/z)	Isotopic Abundance
Ti-47	$^{31}P^{16}O^{+}$	7%
Ti-48	^{48}Ca	74%
Ti-49	(only minor interferents)	5%.
Ti-50	^{50}V, ^{50}Cr	5%

Adapted from Meija et al. (2016) and May and Wiedmeyer (1998)

Results obtained from this first test on ICP-MS (Table 8) provided a clear insight into the requirements of a proper digestion method. The first revealed issue was that the presence of HNO_3 and HF in enough quantities is crucial for the removal and stabilization of titanium from the sample matrix. Furthermore, it was possible to observe that with the decrease in volume of reagents, the sediment samples were left with leftover residue. Thus, despite the higher acid content which required further sample dilution, only Method 1 provided the best recovery results.

Table 8 – Titanium quantification results of the digestion methods through ICP-MS

Method	LoQ ($\mu g\ g^{-1}$)	[Ti] Tissue ($\mu g\ g^{-1}$)	[Ti] Sediment ($\mu g\ g^{-1}$)	Ti Sediment Recovery Rate
# 1	20	< LoQ	4173	95 %
# 2			3865	88 %
# 3			2166	49 %
# 4			2108	48 %
# 5			2123	48 %
# 6			2048	47 %

Average results with CV < 10% (n = 3)

Although preliminary results were obtained using ICP-MS the quantification limits weren't low enough to provide accurate measurements for the intended research work. As such the exchange from ICP-MS to IPC-OES was made, in which the latter was found to have advantages, such as not having much background noise or interferences due to spectral overlap nor requiring dilution due to broader working parameters. The results obtained from this new approach showed not only a considerably lower detection limit, but also that it was possible to establish the NIST-2976 as a potential internal CRM (Fig. 7).

This latter issue of quality assurance through the use of a proper CRM was thus established using NIST-2976 as an internal standard. As already stated, titanium was never found to be a relevant element to analyze in tissues, and thus at the time it was not found any animal tissue CRMs for this element.

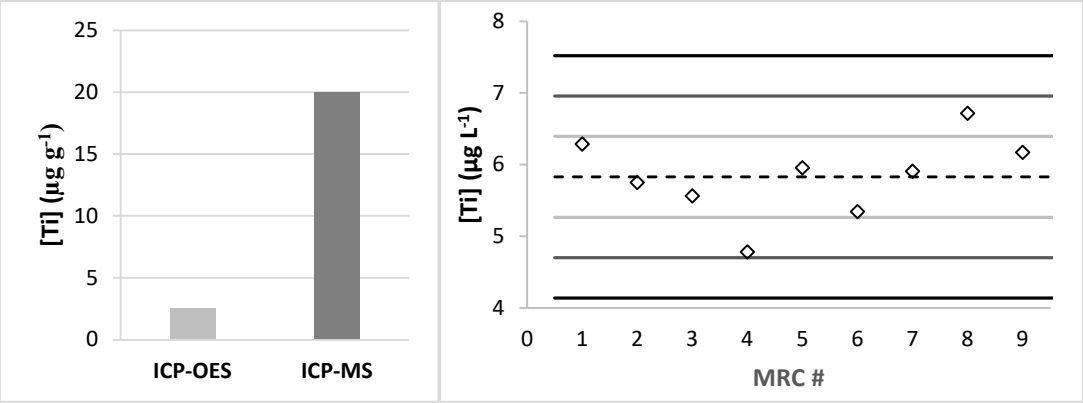

Fig. 7 – A) Limit of quantification for ICP-OES and ICP-MS; B) Control chart for NIST-2976 (Dashed line – average; light grey line – ± sd; dark grey line – ± 2 sd; black line – ± 3 sd)

3.1.2. Ti^{4+} dynamics in marine environment

Variations of Ti at t_0 – nominal concentration vs measured concentration

Titanium is a rather difficult metal to study in the environment, even more so in marine waters, and as such the quantifications done for the experimental setups should take into consideration these difficulties. Furthermore, experimental design introduces some errors when considering the all the preparation steps involved, and henceforth the propagation of error when considering the nominal concentration versus de measured concentrations obtained after contaminant spike.

A first source of error would be from the measurement of the total volume of water, especially in the 15 L aquaria in which there are no proper practical and precise tools available. Previously measured marks were made for practical use and, despite only providing guideline volumes, even at deviation of 0.5 to 1 L the final volume error should be greater than 3 to 6 % which was considered acceptable. Another source of error is in the preparation of the contamination standards, required to minimize the dilution effects of the Ti standard as the concentrations while using mass measurements and considerations of the solvent mixture to acquire an acceptable spiking solution. Further considerations in the preparation of the spikes were taken, such as using trace ultrapure water and trace and a slightly acidification with HNO_3 to keep Ti stable in the spike solution, while not producing any considerable alteration in the salinity or pH of the aquaria.

The preparation of the spikes is thus crucial step, being thus prepared by weight which ensures a much greater control, and as such to properly assess the starting contamination conditions three initial Ti concentrations were considered as follow:

i) Theoretical initial aquarium concentration – $[Ti]_{0t}$

This is the nominal concentration, determined through the measured masses and calculated according to eq. 7, where $m_{S_{Ti}}$ and $\rho_{S_{Ti}}$ are the measure masses (g) and density (g mL^{-1}) of the Ti commercial standard, and V_{spk} and V_{aq} are the spike volume (0.1 L) and the aquarium final volume (15 L).

$$[Ti]_{0t} = {}^{m_{S_{Ti}}}/_{\rho_{S_{Ti}}} \times {}^{V_{spk}}/_{V_{aq}} \qquad \text{eq. 7}$$

ii) Expected initial aquarium concentration – $[Ti]_{0e}$

A variant nominal concentration, determined by the ICP-OES analysis of the spike sample according to eq. 8, where $[Ti]_{spk}$ is the measured concentration (µg L^{-1}) of the contamination spike solution.

$$[Ti]_{0e} = [Ti]_{spk} \times {}^{V_{spk}}/_{V_{aq}} \quad \text{eq. 8}$$

iii) Measured initial aquarium concentration – $[Ti]_0$

Measured concentration at t_0 in the aquarium, resultant from the $[Ti]$ determination by ICP-OES of the water samples acquired right after contaminant spiking (eq. 9).

$$[Ti]_0 = [Ti] \quad \text{eq. 9}$$

This somewhat extensive analysis may seem unnecessary, to the point of not being a common practice, however after checking the obtained results (example provided in Fig. 8) some patterns can be observed.

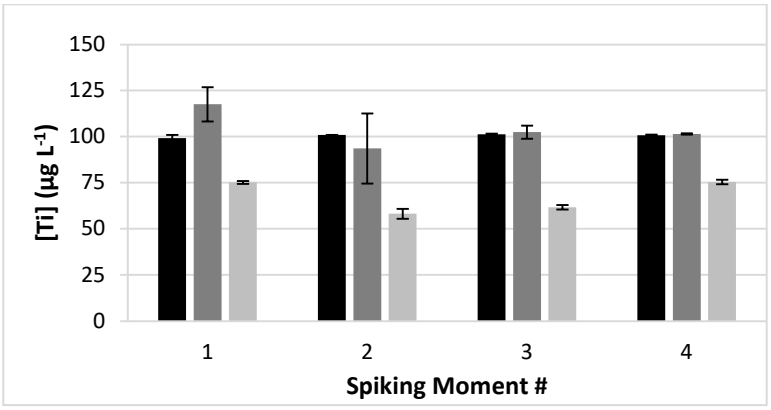

Fig. 8 – Titanium initial concentration in aquarium

(Condition 100 µg L^{-1}; Black – $[Ti]_{0t}$; Dark grey $[Ti]_{0e}$; Light gray $[Ti]_0$)

The first observation is that both theoretical and expected results (black and dark grey bars, respectively) are a higher concentration that the seawater samples (light grey bars) throughout the whole assay. A more in-depth analysis is provided in Table 9, evidencing a more noticeable difference at the condition of lowest concentration (5 µg L^{-1}), with an average reduction of c.a. 55% and even producing 4 values below LoQ – in 2 two separate moments. The other studied conditions (50 and 100 µg L^{-1}) both present a reduction c.a. 34 % of the initial intended concentration.

Table 9 – Data analysis of the different initial Ti concentrations (µg L^{-1})

Condition		t_{0t}	t_{0e}	t_0
5 µg L^{-1}	$\bar{x}$	5.3	5.3	2.5
	CV	7	4	22
50 µg L^{-1}	$\bar{x}$	52	53	35
	CV	6	9	12
100 µg L^{-1}	$\bar{x}$	103	106	71
	CV	6	10	16

Through the analysis of the theoretical and expected initial Ti concentration it should be noted that such difference cannot be faulted into user error (e.g. mistake preparing the spike), or to issues with the commercial standard, since the values are similar. As such, the more reasonable explanation for this substantial decrease of initial Ti concentration should be due to its low solubility in seawater, i.e. quick conversion into oxide species and subsequent precipitation, which may be "catalyzed" with the forced agitation and oxygenation during the aquarium filling and mixing process.

During the whole experiment, the standard preparation and initial Ti concentration on the aquarium could be considered stable (Table 9), with the lowest concentration condition demonstrating a greater variability. This result is however skewered not only due to its different magnitude order, but also because the determinations are near the LoQ of the ICP-OES which already produces values with greater associated uncertainty.

Variation of Ti^{4+} concentration in marine environment

Following the initial spike of Ti^{4+}, and the subsequent spikes after each water renewal, the Ti content in water was followed during the week with close attention to the first 72 h after the spiking. Results noted that, from the 48th hour onwards, independently of the initial concentration, the recorded values were always below LoQ (2 μg L^{-1}), Fig. 9 A-C.

Reported results weren't completely unexpected, since with the work mimicking real environmental scenarios, it should be expected that ultimately the low solubility of this element in seawater would reduce its content to values below the ICP-OES LoQ. This is in agreement with the scarce information regarding titanium dynamics in water, even though most of the references are already quite dated. The base Ti levels in river and sweater are often from over 20 years ago, e.g. (Yokoi and van den Berg 1991; Skrabal 1995), with most of the recent works being focus on technical/method updates. Titanium kinetics was only found to be lightly mentioned once in the literature (Croot 2011), being labelled as "suggested to be very reactive" by another already dated work (Turner et al. 1981).

Taking into consideration the work of Holbrook et al. (2013), despite the differences in study conditions (pool freshwater vs. synthetic seawater), it is evidenced in his work that most of the Ti was sequestered by the filtering system. Furthermore, in that study it is also mentioned that most of Ti was found mostly in the dissolved phase, considered fraction < 1 kD, which correlates well with the supposition of (Skrabal 1995), which states that Ti is lost, by a series of physicochemical processes (e.g. precipitation and aggregation), from the dissolved and/or colloidal phase to the particulate phase when entering the marine system. This information is quite interesting considering the interaction of the contaminant with the organisms being exposed, as this has direct impact on the potential availability and form of the contaminant, and thus affecting the conclusions regarding Ti toxicity.

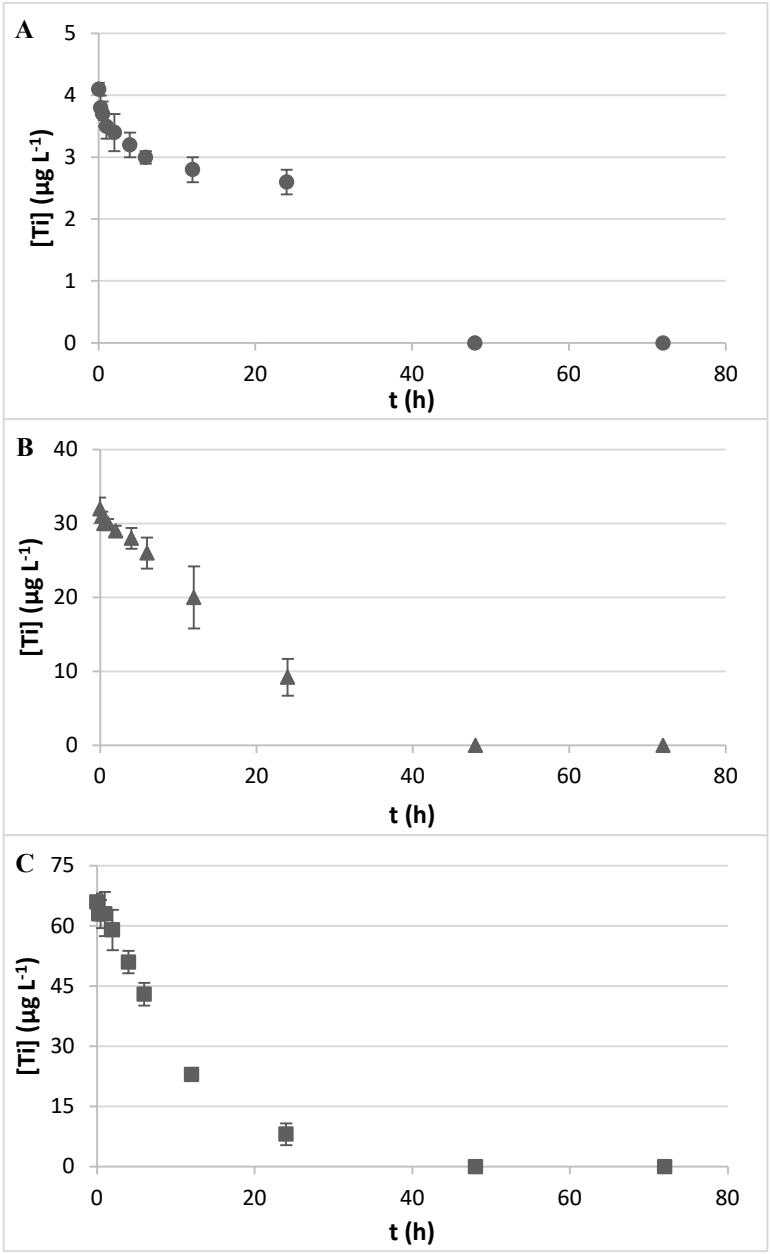

Fig. 9 – Kinetics of titanium content in seawater

(A – 5 µg L^{-1}; B – 50 µg L^{-1}; C – µg L^{-1})

One of the explanations can be drawn by looking into the kinetics of the loss of quantifiable Ti in the aquarium, and through exponential adjustments (eq. 10-12) it has been noted a fitness of the adjustment (R^2) for each of the studied concentrations of 0.88; 0.99 and 0.99, from low to high Ti^{4+} initial concentration.

$$y = 3.6 \times e^{-0.013x} \quad \text{eq. 10}$$

$$y = 33 \times e^{-0.057x} \quad \text{eq. 11}$$

$$y = 65 \times e^{-0.076x} \quad \text{eq. 12}$$

Through these equations it is estimated that average dissolved Ti concentrations on the first day, after contaminant spiking, are 3.1, 19 and 32 μg L^{-1}, while on the second day it is estimated further decreased to 2.2, 4.3 and 4.5 μg L^{-1}; values in increasing order of initial Ti^{4+} concentration. Thus, not only is the initial concentration of Ti^{4+} not reached, but also the residence time of the contaminant is rather limited to a 48-72-hour timeframe. Has such, due to this behavior of the contaminant in the marine environment coupled with the cyclic nature of the exposure assays, the Ti^{4+} contamination could be described as short-exposure bursts of a pulsated nature (Fig. 10).

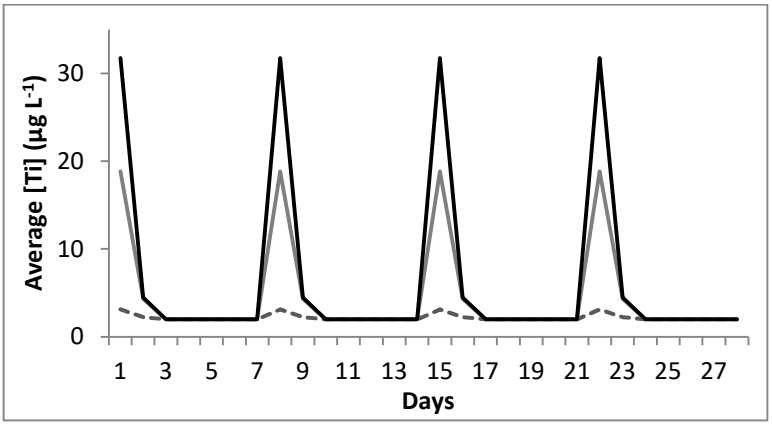

Fig. 10 – Pulsated nature of dissolved Ti contamination

(Estimated initial Ti concentrations: dashed line – 5 μg L^{-1}; gray line – 50 μg L^{-1}; black line – 100 μg L^{-1})

This however doesn't directly translate into a loss of total Ti content in the aquaria. This evaluation is directly related to the dissolved fraction of Ti, which is assumed to be gradually converted into one or more of the insoluble forms, such as TiO_2 or $TiO(OH)_2$ (Turner et al. 1981; Orians et al. 1990). The nature of these particles in the solution are however rather unknown, as it is related to more complex interactions of deposition/suspension in the aquarium as well as effects related to aggregation and even interaction with organic matter, both dissolved and colloidal.

As such when considering the possible impact and exposure pathways of total Ti to marine organisms several questions arise. One of the most important should derive from the Ti availability in marine medium, taking into account both the dissolved and particulate fractions, and which may present a higher danger.

Also considering the required setups of a mesocosm, as they mostly required filtering system to keep the water clean from the biological waste due to the high number of organisms per aquarium, it should be noted the possibility of total Ti loss through filtration. Furthermore, the filtration system is also often used as a form of oxygenation and mixing of the mesocosm, so the alternative also had to take this factor into account.

Influencing factors of experimental design and Ti^{4+} dynamics in marine environment

Setup of a mesocosm experiment is of utmost importance when considering the viability of the results, moreover when the study aims to assess the effects of contamination, which is directly correlated with the physicochemical properties of the medium, the equipment used and the studied conditions have to be well understood and documented.

Thus, prior to the exposure assays some tests have been conducted to assess the dynamics of Ti^{4+} in marine environment, while also using the green algae (*Ulva lactuca*), as a readily available proxy of a marine organisms. Other advantages of this setup were that it not only provides an easier experimental design for a short-time studies, but it has also been reported the algae's capabilities of sorption to various other elements (Figueira et al. 2016; Henriques et al. 2017b).

In this 72-hour pilot experiment, the initial concentration of Ti^{4+} was increased (1 mg L^{-1}) to better follow the kinetics of Ti in the marine environment as well as the partition between the water and algae. The initial measured concentration was considerably lower (c.a. 26%) being reported a value of 740 μg L^{-1}. Results goes in accordance to the previously presented, indicating that as soon as introduced in the system soluble Ti was being lost, most likely through precipitation or sorption.

However, there was a considerable difference at the end of the 72 h of exposure, to which it was now found a retention of soluble Ti c.a. 60 % in the water (Fig. 11). Furthermore, the mass balance through the evaluation of the Ti content in the algae accounted for c.a. 35% of the total Ti introduced in the system, thus only 5 % of Ti could be considered "lost". This loss is within the expected measurement errors and could be explained by deposition of the insoluble fraction or its sorption to the containers.

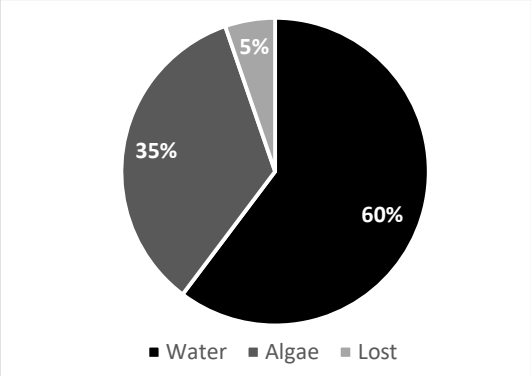

Fig. 11 – Titanium partition at the end of the experiment

Thus, conflicting results regarding the dynamics of soluble Ti in marine environment are evidenced in this experiment, where in previous results after 72 hours all Ti in the soluble fraction was < 2 μg L^{-1} while in this case there is over 400 μg L^{-1}.

To explain these differences it was necessary to explore the differences in experimental design between both studies, in which the previous results are reported on 15 L aquaria with constant aeration (required for water mixing and oxygenation) while this one was done on 1 L Schott flasks with no forced agitation or aeration (algae floating movements and photosynthesis as the only sources of mixing and oxygen). As such, it's through the comparison of these experiments that the importance

of experimental design has been noted and, moreover, that water agitation and oxygenation may be a rather important parameter when defining the exposure assays.

However, in regard to understanding the importance of oxygenation, another test was designed, this time using the initial target concentration of Ti^{4+} of 100 µg L^{-1} – the highest concentration used in the exposure assays.

Results clearly report (Fig. 12) that oxygenation accelerated the decrease of soluble Ti, with an exponential decrease similar to previously reported. In contrast, the reduction of the oxidizing potential of the seawater, through nitrogen bubbling, shows a less steep decrease better adapted to a linear adjustment.

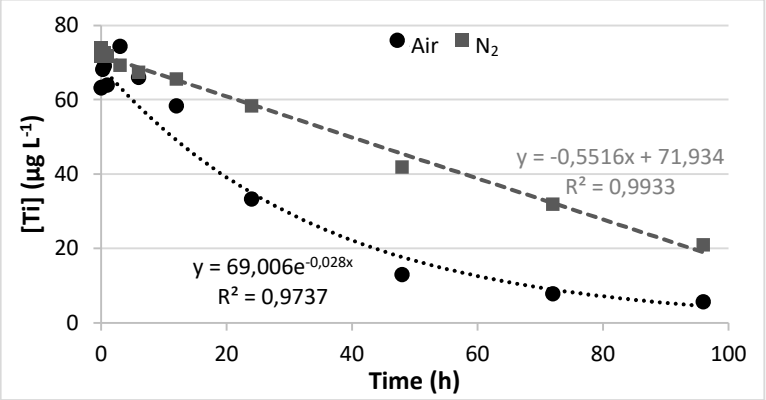

Fig. 12 – Oxygenation effects on Ti stability in seawater

Some slight differences were still noticeable, mainly the presence of quantifiable soluble Ti at 96 h mark, however it is yet again assumed to be related to experimental design differences. As the laboratorial experiments for analysis of these effects are done in much lower volumes (1 L flasks vs 15 L aquaria), which raises issues on the control of parameters such as air flow as well as the mixing of completely different water body-masses.

Air pumping and filtration pumping have thus both been flagged has potential issues when considering the experimental setup, as both systems are commonly used to provide the water flow movements within the aquarium for a proper mixture and oxygenation. Final decision has been reached, opting to use the air pumping system, as this one while mimicking the environmental oxygenation and agitation doesn't remove the total Ti content from the aquarium.

However, in the different exposure assays other variables have been studied, related to the abiotic parameter changes, such has the temperature increase, salinity shifts and event seawater acidification. Each of these parameters have been found to also have slight influences on the concentration of soluble Ti at the spiking moment (Fig. 13).

Shifts from the control conditions (Salinity 30 PSU, pH 8.0, 17 ºC) are noticeable, especially with the cases of salinity increase and pH decrease. It is however of note the variability of the results, which albeit low (CV 6-14 %), won't allow for a complete statically significance difference among all studied conditions. Furthermore, as in previous studies after at the 72 h mark no quantification results report values below LoQ (2 µg L^{-1}), for all conditions.

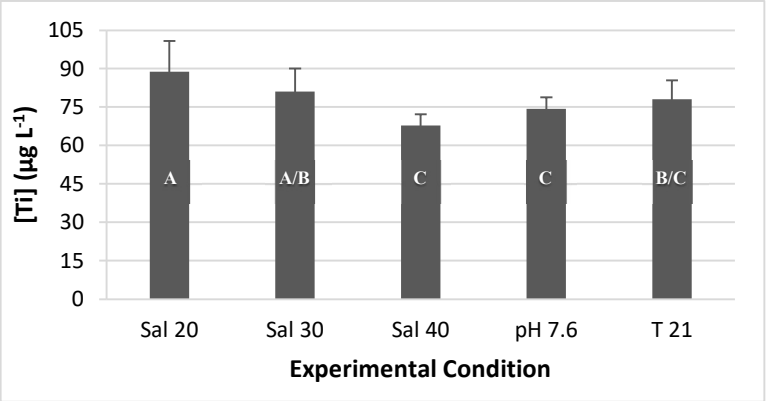

Fig. 13 – Initial concentration after Ti^{4+} spiking (to 100 µg L^{-1}) at different environmental conditions

(Letters represent statistical difference for t-test, p = 0.05)

It could be expected that changes in environmental parameters wouldn't have a major impact on the residence time of Ti^{4+} in marine environment, as in control conditions (30 PSU, pH 8.0, 18 ºC) it already has a very low lifetime in the soluble fraction. Nonetheless, albeit no kinetic studies under climate change conditions were done, the observed initial concentration variation may point to some behavior changes which could ultimately provide answers when considering the toxicity studies (oxidative stress biomarkers).

3.2. Study of TiO_2 powders ($nTiO_2$ Degussa P-25, a-TiO_2, r-TiO_2)

3.2.1. Characterization of $nTiO_2$ Degussa P-25

Bright Field / Dark Field Imaging

Even by the definition of nanomaterial, material with at least one dimension below 100 nm, the Degussa P-25 $nTiO_2$ can be considered quite small, thus requiring a larger magnification to be properly studied. This, in conjunction with the fact they also tend to agglomerate, poses a major problem towards achieving a perfectly focused image, as particles that are on top of each other may be outside of the depth of field.

Such effect can be observed in both TEM images (Fig. 14), where some black/white shadows are clearly of the same particle, however they can't coalesce otherwise the rest of the image would be in a similar state. Furthermore, the darker spots are a clear indication of agglutinated nanoparticles, which greatly reduce the beam transmittance.

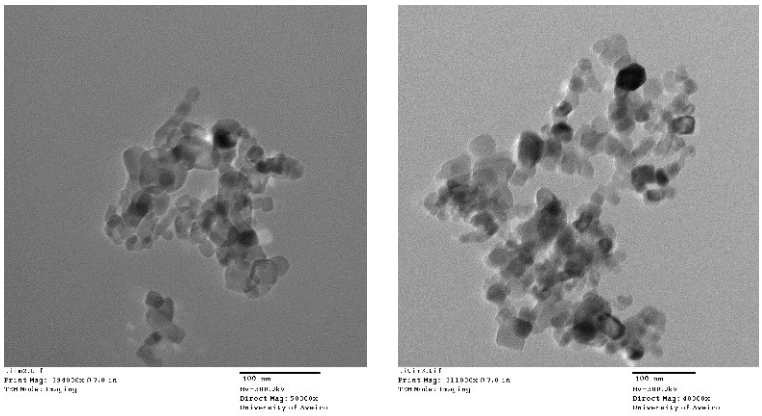

Fig. 14 – Bright field image of two $nTiO_2$ clusters

Regarding size distribution this type of particle agglomeration also often disables the use of Dynamic Light Scattering (DLS) techniques, which instead of evaluating each particle would instead report the size of clusters (reported size 100-500 nm). As such the use of image editing software, ImageJ v1.51d, was required to manually measure the particle size in different clusters. For these two particular clusters the size dispersion was evaluated as being 24 ± 7 nm (n = 75), and the distribution can be observed in Fig. 15.

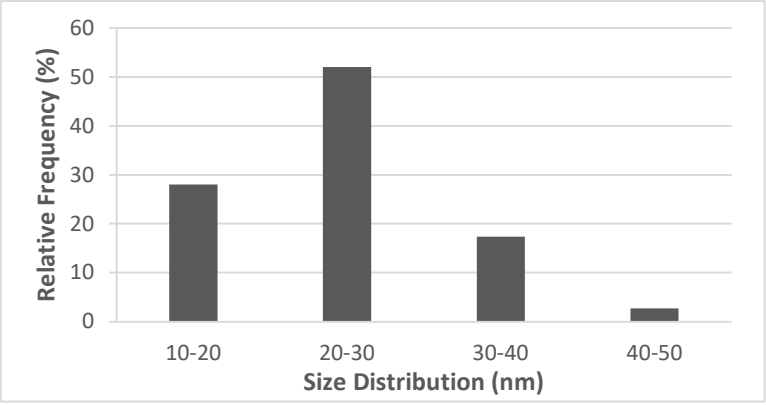

Fig. 15 – Histogram of $nTiO_2$ size dispersion

Crystallinity of the sample was also verified by high-resolution dark field imaging (Fig. 16), in which the lit-up nanoparticles are due to the transmission of the refracted electron beam according to a specific crystallographic plane. As can also be observed they're randomly arranged, explaining the two different images at different beam tilts. Furthermore, it also possible to have a better visualization of the agglutination of the nanoparticles (i.e. different particles on top of each other), as they can almost be selectively lit-up by careful manipulation of the beam tilt angle.

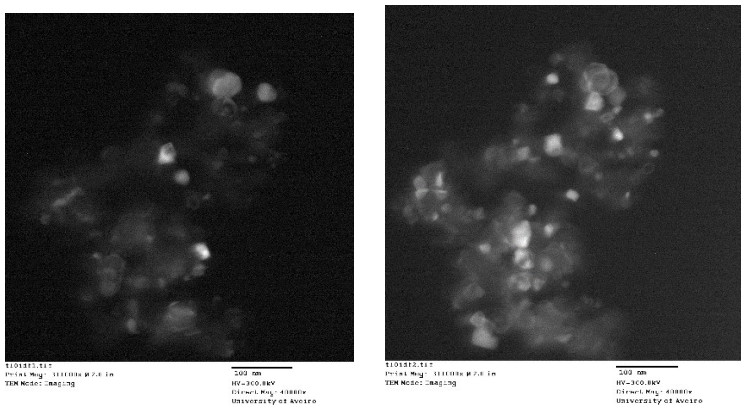

Fig. 16 – High-resolution dark field imaging of a TiO2NP cluster

Electron Diffraction Pattern and X-Ray Diffraction analysis

Since it was impossible to find isolated nanoparticles, the study of the crystallographic structure of the $nTiO_2$ was achieved by targeting a dense nanoparticle cluster and analyze the results obtained from the polycrystal diffraction patterns. The obtained diffraction pattern is thus ring shaped (Fig. 17), each one corresponding to a diffraction plane.

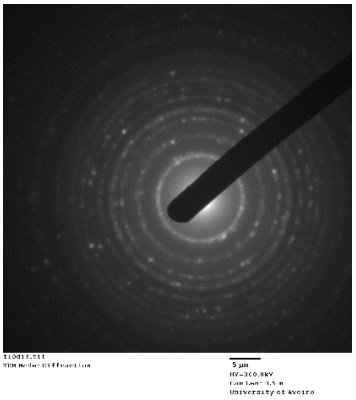

Fig. 17 – Diffraction pattern of $nTiO_2$ (polycrystal)

To determine the crystalline phase of the $nTiO_2$ radius measurements were done (using ImageJ) and correlated with the interplanar distances tabled in the JCPDS files for anatase and rutile. Through comparison it was concluded that the selected diffraction area corresponded to the TiO_2 anatase phase, with each ring obtaining a proper crystallographic plane correspondence (Table 10).

Table 10 – Diffraction pattern rings correspondence to the crystallographic plane

Ring #	Radius (µm)	Crystallographic plane (h k l)
1	6.885	1 0 1
2	10.091	0 0 4
3	12.787	2 0 0
4	14.390	1 0 5
5	16.393	2 0 4
6	17.814	1 1 6
7	19.126	2 1 5
8	20.729	2 2 4
9	22.951	3 0 5

Crystallographic planes from JCPDS 01-071-1166

Using CaRIne Crystallography v3.1 it was possible to obtain the simulated X-ray diffraction pattern (Fig. 18), which coincides with almost all of the rings obtained in the electron diffraction. Only the (2 0 0) and (1 1 6) planes don't have any correspondence in the simulation (3 0 5 is out of scale, $\theta \approx 94^{o}$), however they've previously been identified in the literature (Thamaphat et al. 2008).

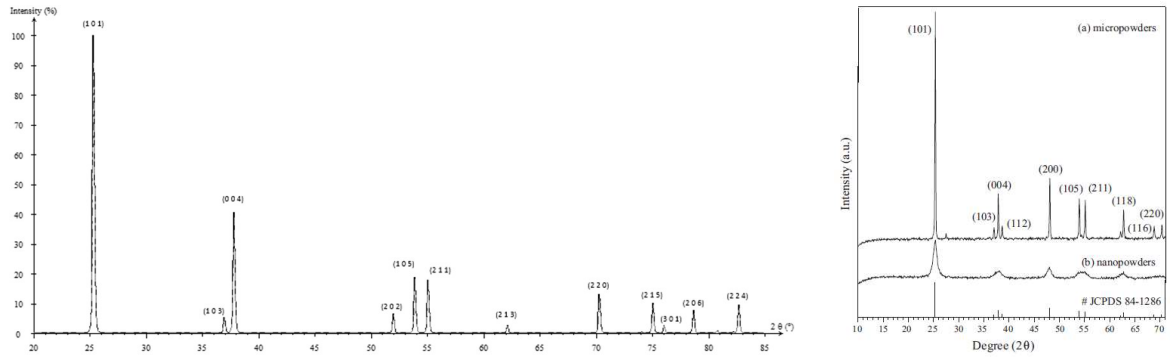

Fig. 18 – X-ray diffraction patterns of TiO_2 anatase phase

(left – CaRIne simulation; right – Thamaphat et al. (2008))

As such regarding crystallography it was finally possible to conclude the spatial distribution of the titanium and oxygen atoms, i.e. the unitary cell, which belongs to the tetragonal group, specifically the ditetragonal dipyramidal (Fig. 19).

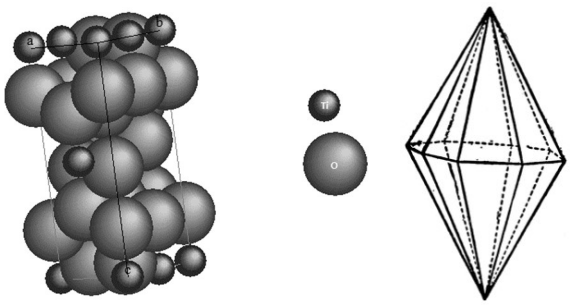

Fig. 19 – Atomic representation and crystal schematic

X-Ray analysis however reveals the existence of two different TiO_2 phases that compose the nanoparticles (Fig. 20), anatase and rutile, in which the latter couldn't be found using electron diffraction pattern due to its lesser proportion.

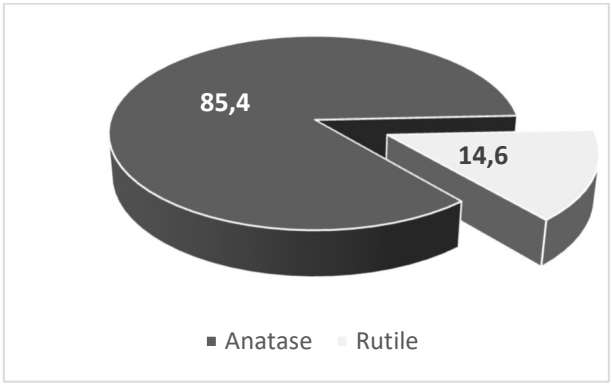

Fig. 20 – Anatase/Rutile ratio in Degussa P-25

Energy-Dispersive X-Ray Spectroscopy (EDS)

EDS analysis only objective was to confirm the purity of the $nTiO_2$. Through the analysis of the EDS spectra (Fig. 21) it was confirmed the high degree of purity, since besides the titanium and oxygen peaks there is only the copper peak that is related to the sample grid.

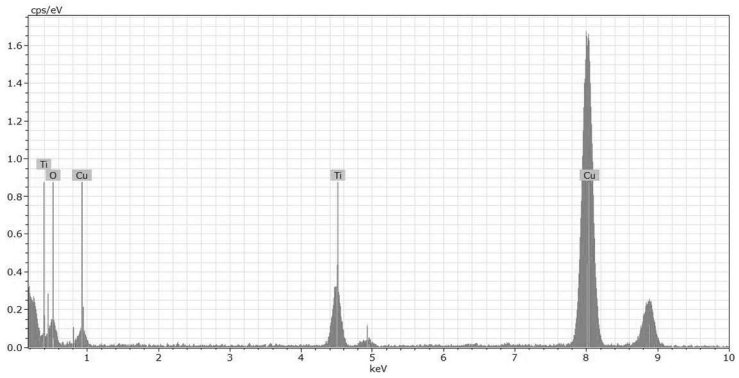

Fig. 21 – EDS spectra of $nTiO_2$

From the characterization it has been noted that $nTiO_2$ was within the purity and crystallinity specifications of manufactory (99.9 %; 85 / 15% - anatase / rutile), however size dispersion was greater than reported. The nanoparticles were noted to easily form agglomerates. Furthermore, Ohno et al.(2001) has revealed the occurrence of formation of selective phase agglomerates, with anatase particles averaging 25 nm and rutile 85 nm. Such difference wasn't verified in present work as no rutile agglomerates were found, which isn't unexpected due to the sheer difference in the fractions of each phases revealed by the XRD analysis.

3.2.2. Study of $nTiO_2$ Degussa P-25 in seawater

Seawater is a highly complex matrix, with high content in both organic and inorganic components, which difficult the application current analytical techniques. It is however due to this problem that the application of TEM techniques may provide new insight onto possible transformations of $nTiO_2$ in this environment.

Thus, in this experiment a dispersion of Degussa P-25 $nTiO_2$ was prepared in filtered seawater, while the sampling grid was prepared by immersion technique a week later in order to allow sufficient time for potential physicochemical transformations to occur.

Similar to the previous case the investigation started with bright field imaging, however some problems were promptly detected. The first one was that even at lower magnifications it could be observed several big dark spots and some smaller better-defined scale of gray structures, which still were much bigger than the previously characterized nanoparticles (Fig. 22).

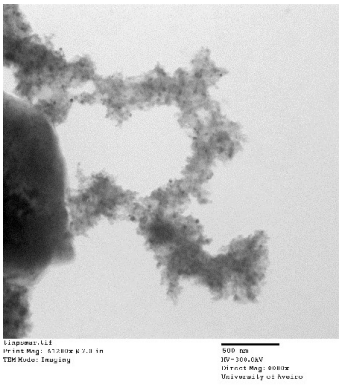

Fig. 22 – Bright field imaging $nTiO_2$ suspension in seawater at low magnification (8000x)

From the obtained results it was suspected that the bigger black stains may be related to the presence of previously dissolved organic matter, which was collected by the grid during the sampling, while the other better-defined structures may be different ionic components of the seawater (e.g. salt). This is further corroborated by the difficulties during imaging process, as the sample would continuously "slide" due to electrostatic effects (mostly charge accumulation by the organic matter).

Therefore, the next step was to use dark field imaging of the gray areas in order to find zones with crystalline structures, from which the search for the "hidden" $nTiO_2$ could be started.

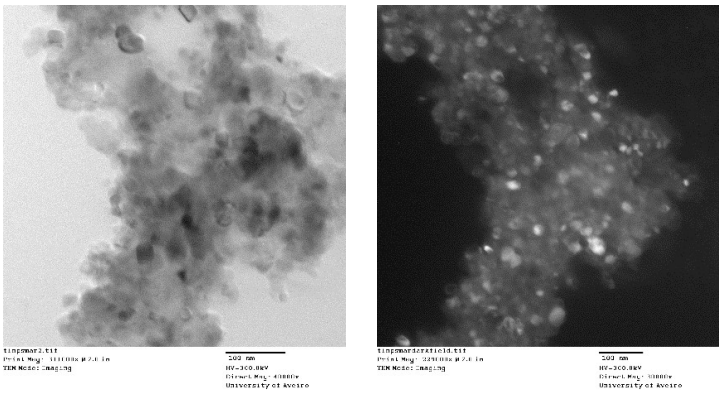

Fig. 23 – Bright field and correspondent dark field imaging of $nTiO_2$ suspension in seawater

Nonetheless the high salt content rendered the efforts almost useless (Fig. 23), as not only would the nanoparticles lit-up but also the several crystalline salt structures, which are also present and in a much higher percentage.

As such the last effort to detect the $nTiO_2$ was left to the EDS analysis, since there was no possibility to otherwise uncover the nanoparticles or even check if they were in fact in the sample. Thus, by obtaining the EDS spectra (Fig. 24), it was possible to confirm the presence of titanium, as well as several components of the seawater matrix (NaCl, $MgCl_2$, SO_4^{2-}).

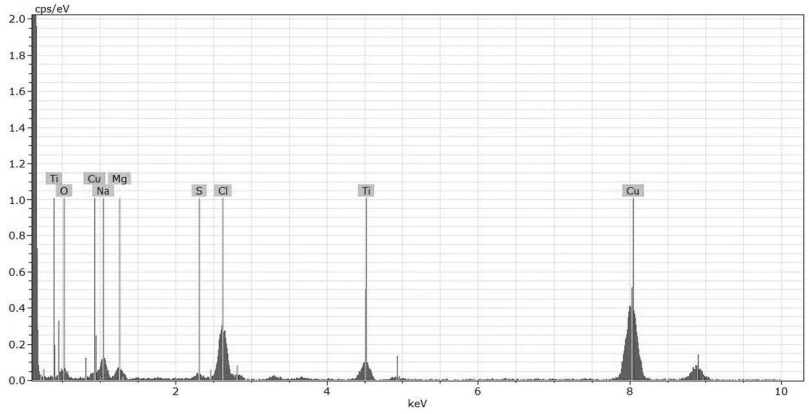

Fig. 24 – EDS of $nTiO_2$ suspension in seawater

However, EDS doesn't provide information regarding the structural characteristics of the titanium, although it is known that very likely is still in TiO_2 state (it mostly sediments in saltwater). Regarding if it is still a nanoparticle or of it bonded/sorbed to another component of the seawater matrix, it remains unknown.

An attempt at using electron diffraction showed the easily identifiable cubic system spot pattern of NaCl halite crystalline structure (Fig. 25), which is was also represented using CaRIne. The observed rings, albeit not very bright, match the spots position thus there is some tenuous contribution of other nearby crystals towards the obtained diffraction pattern.

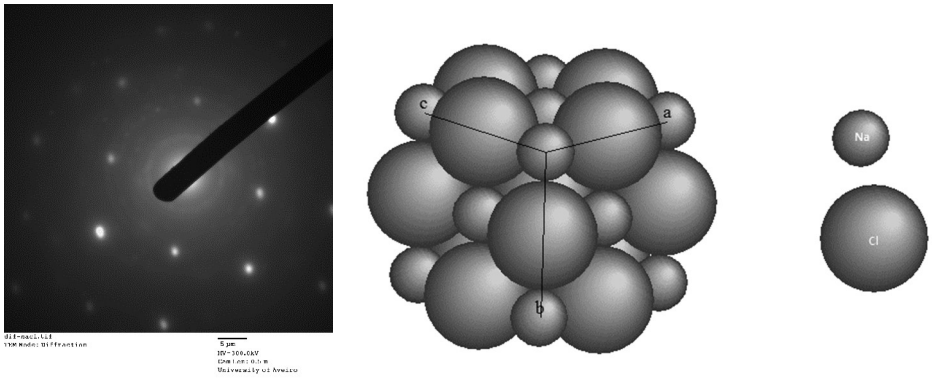

Fig. 25 – Diffraction pattern of NaCl and corresponding atomic spatial arrangement

Several attempts at finding other diffractions patterns were made, however the results were continuously brought to the NaCl cubic arrangement (which is expected due to its high content), or the presence of diffuse rings from which it is impossible to calculate a pattern (Fig. 26)

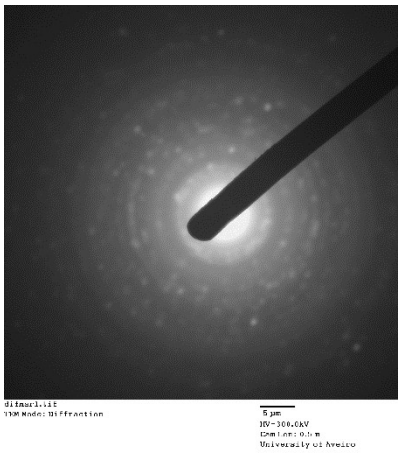

Fig. 26 – Diffraction pattern as diffuse rings of $nTiO_2$ suspension in seawater

3.2.3. Characterization of TiO_2 powders (a-TiO_2 and r-TiO_2)

Commercially available TiO_2 powders of different morphologies, anatase and rutile, for use in the exposure assays were characterized as no manufacture certificate was provided.

Through XRD (Fig. 27), each of the obtained TiO_2 powders was confirmed to be of a single morphology, anatase ($I4_1/amd$ tetragonal space group) and rutile ($P4_2/mnm$ tetragonal space group).

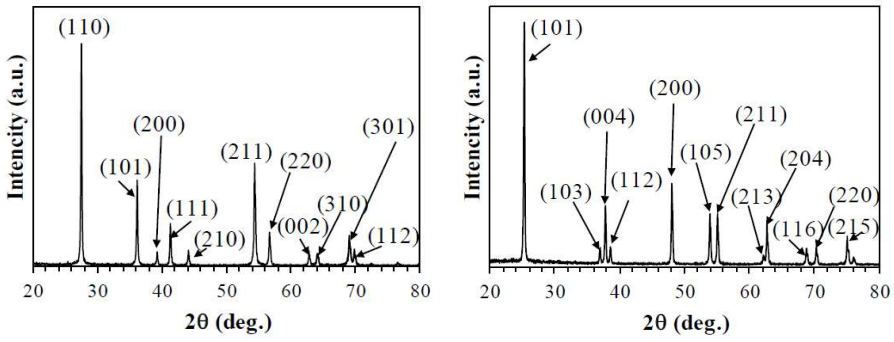

Fig. 27 – XRD pattern of anatase (left) and rutile (right) powders

Size distribution histograms (Fig. 28), revealed differences in terms of their size, with rutile particles being considerably larger than the anatase ones. From the obtained results, it has been noticed that a-TiO_2 powder can be characterized as a nanopowder, since at least one of the dimensions is < 100 nm, despite the average size ranging to values greater than 100 nm. Rutile particles, however, were noticed for their greater size and thus better relating to a definition of a micropowder (average size on the μm scale). Nonetheless it should be noted that rutile particles easily formed clusters, and no isolated were obtained for proper measurements.

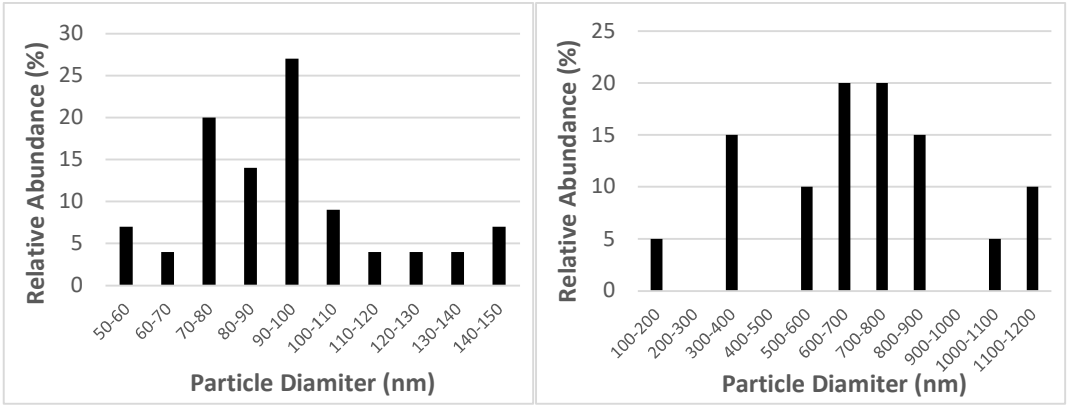

Fig. 28 – Size distribution histograms of anatase (left) and rutile (right) powders

The full characteristics, morphological and textural, of both powders are presented in Table 11, better evidencing the disparity in size and surface area between both particles, parameters which are inversible proportional to each other.

Table 11 – TiO_2 powders full characterization parameters

Sample	**Crystal system (cell parameters)**	**ρ [a] ($g.cm^{-3}$)**	**d_{parBET} [b] (nm)**	**d_{parXRD} [c] (nm)**	**d_{parSEM} [d] (nm)**	**S_{BET} ($m^2 \cdot g^{-1}$)**
a-TiO_2	Tetragonal (anatase) (a=b=3.7924 c=9.5304)	3.38	186	50	94	8
r-TiO_2	Tetragonal (rutile) (a=b=4.5922 c=2.9578)	4.27	428	-	694 [e]	3

a) Calculated using the lattice parameters

b) Calculated through the equation: $d_{parBET} = \frac{6}{S_{BET}\,\rho}$ considering the spherical shape of the particles where S_{BET} corresponds to specific surface area and ρ is the density

c) Crystallite size obtained through the Scherrer equation: $d_{parXRD} = \frac{k_{sf}\lambda_{Cu}}{FWHM\cos(\theta)}$ where k_{sf} corresponds at dimension less shape factor, λ_{Cu} is the wavelength of the X-ray (Cu Kα radiation, λ = 1.5406 Å), $FWHM$ is full width of peak at half maximum in radians and θ is the Bragg angle

d) Calculated based in diameter of 45 particles, using the Image J

e) Value results from particulate aggregates.

3.3. Bioaccumulation and oxidative stress studies

3.3.1. Short-term Ti^{4+} exposure assay

Objective

This study aimed to evaluate the toxicity under three different Ti^{4+} contamination scenarios using the mussel *M. galloprovincialis*, a widely used biomonitor, in conditions similar to the ones present in the Ria de Aveiro Lagoon.

Thus, variations on the Ti^{4+} content in water and the organism tissues in conjunction to with the oxidative stress and metabolic biochemical parameters were evaluated after 96 h and 14 days of exposure.

Results

Ti content in both water and tissue during the experiment are reported in Table 12. The concentration of dissolved titanium in the aquaria decreased sharply with time, reporting values below LoQ (< 2 μg L^{-1}) after 72 h post spiking moment.

Mussels tissues evidenced similar values between the biological control (no contamination) to the two lower exposure conditions (5 and 50 μg L^{-1}), while the organisms in the most contaminated aquaria were noted to have a slight increase in Ti content.

Table 12 – Titanium concentration in water and mussel tissues of exposure assay 1

Condition	Ti^{4+} in Water (μg L^{-1})			Ti in Mussels (μg g^{-1})		
	t_0	72 h	7 days	96 h		14 days
CTL (0 μg L^{-1})	< 2	< 2	< 2	1.6 ± 0.3[a]		< 0.9
C1 (5 μg L^{-1})	2.7 ± 0.5	< 2	< 2	1.5 ± 0.3[a]		< 0.9
C2 (50 μg L^{-1})	38 ± 2	< 2	< 2	1.4 ± 0.3[a]		< 0.9
C3 (100 μg L^{-1})	80 ± 7	< 2	< 2	2.5 ± 0.3[b]	*	3.1 ± 0.5

Letters indicate difference between conditions and asterisk differences between exposure periods (p = 0.05)

Biochemical parameter results are represented in Fig. 29, including all the oxidative stress and metabolic parameters of the mussels exposed to the scenarios of Ti^{4+} contamination.

Regarding the metabolic capacity, no statistically relevant changes were found for the lower exposure condition at any of the sampling moments. However, for mussels at 100 μg L^{-1} Ti aquarium, it has been noted a decrease in ETS activity at 96 h followed by a pattern reversal, after 14 days, with its activity increase in relation to the biological control (non-contaminated mussels). When comparing the same conditions at different time, it has been noted two contrasting situations of statistical difference in ETS activity – a decrease at the 50 μg L^{-1} and an increase at the 100 μg L^{-1}.

Both energy reserves, total protein and glycogen, showed a similar trend of increasing content with increasing contaminant concentration, with PROT showing higher concentrations than the control in all conditions at both times while GLY only registered a significant increase at concentration of 50 μg L^{-1} and above. Between sampling times and within the same condition it's

noteworthy the greater disparity in GLY content, with statistically relevant difference at the two higher concentrations, with PROT showing no relevant variation.

Enzymatic oxidative stress conditions evaluated consisted of SOD, CAT, GPx and GSTs.

SOD activity reported no significant variation between different times for the same condition, however it was noted an increase activity with increasing concentration, for a given time when compared to control conditions, for 50 and 100 μg L^{-1} at 96 h and 100 μg L^{-1} after 14 days. Activity of CAT in mussels revealed a slight increase of activity at 96 h for both 5 and 50 μg L^{-1} conditions and a major increase at the highest contaminated concentration, white at 14 days of exposure only the 100 μg L^{-1} showed significant activity increase. No relevant differences were found between times for each of the studied conditions. An increased GPx activity was noted in all contaminated conditions for both exposure times, with incremental increase with increasing contaminant concentrations at the 96 h mark. After 14 days it was noted a decrease in the enzymes' activity, albeit still higher than the control condition, but without any difference among the different concentrations, which led to the significant variation between times for each of the studied contaminated conditions. The GSTs were noted for having no statistical differences among conditions for each of the studied moments. Differences in relation to their respective control conditions were noted at 96 h, with increased activity at both 50 and 100 μg L^{-1}, and at 14 days, only for the 100 μg L^{-1} condition.

Non-enzymatic oxidative stress parameters were studied through LPO, GSH and GSSG.

Cellular damage was evidenced through LPO content increase with increased Ti concentration, especially at the two higher contaminated conditions both at 96 h and 14 days. No variation has been noted within the same condition for both sampling moments. No statistically significant variations were noted in GSH analysis, both between conditions and between exposure times. Graphical analysis infers a content decrease with increasing Ti concentration and exposure time, however result dispersion won't allow for mathematical certainty. GSSG reported a rather significant difference in content between 96 h and 14 days for each of the studied conditions, with a sharp reduction of its content at the latter. At 96 h there it was also noted an increased GSSG content with increased dissolved Ti in the water, while after 14 days these differences were only reported at the 50 and 100 μg L^{-1}.

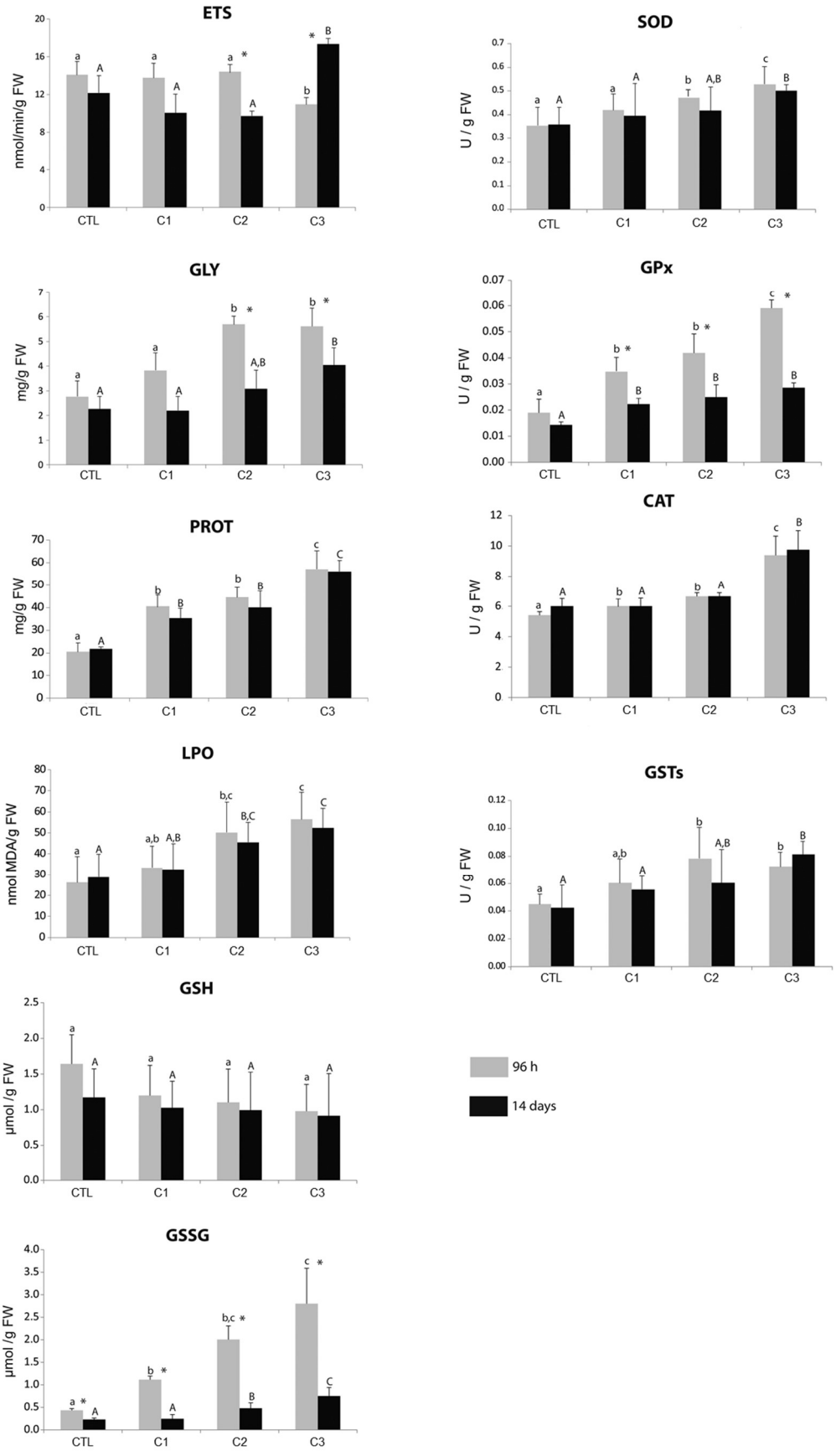

Fig. 29 – Biochemical fitness parameters of mussels due Ti^{4+} exposure (96 h and 14 days)

Discussion

Anthropogenic pollution of the marine environment is one of the greatest dangers of the current times, and it is necessary to understand how organism react to contamination in short-time exposure scenarios. Especially in estuarine systems, due to both cyclic and potentially abrupt changes, organism tend to be vulnerable to anthropogenic pressures such as effluent discharge or contaminant runoff , which can be further exacerbated through highly variable system dynamics (Ritter et al. 2002; McLusky et al. 2004). This study experiment thus takes into account the current knowledge gap regarding the effects of Ti(IV) as a potential environmental contaminant, which is now more relevant due to its increased used and release into the environment.

From the previous Ti^{4+} dynamics studies, it had been an ever-present worry regarding the design of the experimental for the exposure assay, mostly due to the permanence time of soluble Ti in the marine environment. When many of the exposure assays are done in 28-day time intervals (Freitas et al. 2015b; Coppola et al. 2016), the already noted pulsated nature of Ti led to the shortening of the exposure assay to 14 days, with a collection at 96 h reflecting the moment at which no soluble Ti was expected to be present in the aquaria.

As such, during this experiment it can be concluded that the mussels were exposed to an exponentially decreasing soluble Ti concentrations after each spiking moments, for a total of two contamination pulses. This can be greatly contrasted with other contaminants which are known to have longer permanence times in marine environment, such as Cd, Hg and Pb (Henriques et al. 2017b), and thus Ti availability and its biological effects may be greatly fluctuate.

However, this variability may not only be attributed to the potential loss of availability of soluble Ti through precipitation into its oxides. Mussels have been found to successfully depurate metal contaminants (Wang and Fisher 1997; Yap et al. 2003; Yap et al. 2004) and thus, while not expected such low values right after expected total loss of soluble Ti in aquaria at 96 h, a decrease in Ti content may not be completely strange when considering nearly half of each week had no appreciable contamination. The sampling process however hadn't taken this into consideration; thus, it was unable to measure the intake/depuration ratio of Ti by measuring its levels in tissues at much shorter intervals.

As such, it has been noted that only at the highest concentration the organism couldn't compensate the intake with their natural depurative capabilities, and thus providing results slightly above the baseline. This has been confirmed in both exposure times, with reported vales increasing to nearly twice the baseline levels at 96 h, however it should be noted that values are rather close to LoQ (0.9 µg/g), and thus are at the limits of the currently available techniques.

Results however take a turn when looking at the biochemical parameters, with observable variations in the organisms' biochemistry contrasting the nearly unremarkable bioaccumulation factors previously registered. This was denoting that, for the three exposure scenarios, the organism had responded accordingly with remarkable changes to their metabolic and energetic status, as well as evidencing damage and defense strategies to prevent injuries due to contamination.

At 96 h it was observed an upkeep or lowered metabolic rate, i.e. maintenance or reduction of ETS activity, in conjunction with the increase in energy reserves (GLY and PROT) which were signs of metabolic changes in the mussels due to Ti exposure. Canesi et al. (2010) in their work with

M. galloprovincialis, albeit with $nTiO_2$ and other nanomaterials, also noted an increase in lipid content after 1 day of exposure. The mussels' metabolic activity in the present exposure assay, with the exception of the highest concentration condition, remained fairly constant however, but this didn't directly translate into biochemical inaction.

The different antioxidant enzymes (SOD, CAT and GPx) were activated, at varying degrees depending on the enzyme, with GPx being the one with more pronounced fluctuations in its response to the oxidative stress. This response however wasn't enough to avoid cellular damage, as noticed by the increased LPO in all exposure conditions, with more pronounced effects at the 100 $\mu g\ L^{-1}$ condition. These results further indicate a prevalence of biochemical effects past the Ti accumulation period, i.e. past 96 h mark, which is inferred that after contamination mussels suffer from a ROS overproduction status allied to a limited capability to eliminate such xenobiotics.

At the end of the experiment (14 days), oxidative stress biomarkers evidence further changes in the mussels' health and activity.

Mussels have been reported to lower their metabolic rate in cases of oxidative stress, being one of the major defense mechanisms the lowering of the filtration capacity to prevent damage (Gosling 2003; Ortmann and Grieshaber 2003) thus also reducing the intake of harmful substances and prevent damage. This mechanism is not only supported by the observed data, as there is a trend of decreasing ETS at higher contaminant exposure, but also explaining how the Ti content in the tissues further diminished to values below LoQ (0.9 $\mu g\ g^{-1}$). Glycogen, as the most readily available energy source, was notably more depleted at both 50 and 100 $\mu g\ L^{-1}$ conditions, which can be attributed to its used for the defense mechanisms which were further triggered on the second contamination pulse.

In regard to the most contaminated condition, the conjugation of the increased metabolic rate and decrease in energy reserves must be explained by the need to activate their antioxidant defenses. With the lowering of the metabolic rate (e.g. filtration capacity and thus less feeding) energy was being depleted for recovery following the first contamination pulse. Then the second contamination pulse pushed the mussels beyond their recovery capacity, and thus was required the resume of their activity, even though that potentially led to further Ti accumulation. Furthermore, the antioxidant and biotransformation mechanisms might not have been further activated as the mussels had no means to respond to the added stress.

From both moments of exposure, it was thus noticed a cumulative effect of each contamination pulse, in which the biochemical activity still has no time to return to basal conditions despite the nearly half week of soluble Ti free environment. This is however an evidence of the weakness of working with Ti as a contaminant, primarily in the degree that it was impossible to derive the speciation and dispersion when considering it conversion into the colloidal oxides. Thus, it remains unknown its fate in the aquarium – either suspension and potentially entering the mussels through filtration feeding or deposition and elimination from the system. So far it has been assumed that biochemical effects reported were due to the trace levels of Ti found in the tissues, which its presence are harmful and ultimately the mussels actively acted to depurate it.

Nonetheless the metabolic and energetic effects resultant from this experiment are concordant with those found in the literature to $nTiO_2$ exposure to the same mussel (Canesi et al. 2010; Barmo et al. 2013). Through both authors works it was also confirmed the increased ROS burst caused by

Ti with increased concentration, as well as the proportional response in relation to CAT antioxidant activity. Regoli and Giuliani (2014) have stated that antioxidant defense system is a complex mechanism, composed by a wide array of reactions, which is observed in the current results as all 3 antioxidant enzymes (SOD, CAT and GPx) appear to be working in tandem to eliminate ROS and prevent harmful effects. GPx was perhaps the one enzyme to show the greatest fluctuations during the exposure assay, with its peak activity at 96 h. However due to the lack of works relating this enzyme to Ti(IV) it was difficult to assess its variation in relation to the contaminant nature.

The efforts of the antioxidant enzymes weren't completely fruitful, and thus the observable increase in biotransformantion enzymes (GSTs), dependent only on the exposure condition, which act as mediators for the GSH/GSSG scavenging mechanism (Regoli and Giuliani 2014). This system which maintains the redox status of the cell is thus capable of capturing ROS, and thus slowly being converted from the reduced (GSH) to the oxidized (GSSG). Such effects were also observed in the obtained results, with the increase of GSSG at both times but more pronounced at 96 h, further supporting some inability of the enzymatic responses and the gradual loss of overall antioxidant defenses.

Having mentioned both the GSH/GSSG and GPx it should be noted their interconnection, as GPx antioxidant action also works through GSH/GSSG cycle, degrading H_2O_2 into H_2O and O_2. Thus, the increased activity of GPx is usually followed by reduction of GSH and increase of GSSG which is the occurrence in the present work, as well as Henriques et al. (2019) represented by the decrease of GSH/GSSG ratio by the *M galloprovincialis* exposure to gadolinium. As such GPx key role may lie in the fast early on acting effects to work in tandem with the GSH/GSSG system. This allows for energy and defenses being focused on the early moments, after which CAT and SOD or the leftover GSH content should work on the xenobiotics' removal.

Ultimately, ROS content weren't timely removed and has GSH was being depleted it onset cellular damage (LPO increase), which has also been reported in the literature of $nTiO_2$ exposure for aquatic species such as *Oncorhynchus mykiss* (Federici et al. 2007) and *Danio rerio* (Xiong et al. 2011). Exposure times in these works were within the same timeframe of the present work, 14 days for the former and 96 h for the latter, which sets a precedence of Ti bases materials harmful effects.

In conclusion, it appears that Ti contamination has a capacity to show toxicity beyond accumulation, which are likely a characteristic to contaminants with low permanence time and quickly excreted by the organism. The presented effects albeit not fatal, it denounces potentially short-time impairing effects and its cumulative impact may pose a threat to the ecosystem.

3.3.2. 28-day exposure of Ti^{4+} and 14-day recovery assay

Objective

This experiment aimed to assess the toxicity under three different Ti^{4+} contamination scenarios and the organisms' recovery potential. The mussel *M. galloprovincialis* was used as a biomonitor and average environmental conditions of the present Ria de Aveiro Lagoon were taken as baseline.

Thus, variations on the Ti^{4+} content in water and the organism tissues in conjunction to with the oxidative stress and metabolic biochemical parameters were evaluated after 28 days of exposure and following further 14 days of recovery.

Results

Table 13 report the concentrations of Ti in aquarium water, average for the 4 spiking moments, and mussel tissues, at each sampling moment.

Behavior of soluble Ti in seawater displayed the already mentioned sharp decrease with time, reporting values ranging from 50% to 29 % lower than expected within moments of spiking, from the less contaminated condition to the highest respectively. Following spiking, it was similarly noticed that after 72 h no soluble Ti had been found at quantifiable levels on the aquaria.

Regarding the Ti levels found on tissues it was evidenced a baseline level ranging from 2.1 – 2.3 μg g^{-1} for the control conditions. However measured Ti in the whole soft tissue of mussels after exposed to Ti^{4+} (28 days) was found to have no significant variation in relation to this baseline, showing only a slight increase on the average Ti levels. After exchanging to clean seawater (28 + 14 days) Ti content in soft tissue was evaluated c.a. 2.3 μg g^{-1} for all exposure conditions.

Table 13 – Titanium concentration in water and mussel tissues of exposure assay 2

Condition	Ti^{4+} in Water (μg L^{-1})			Ti in Mussels (μg g^{-1})	
	t_0	72 h	7 days	28 day	28 + 14 days
CTL (0 μg L^{-1})	< 2	< 2	< 2	2.1 ± 0.6^{a}	2.3 ± 0.6^{A}
C1 (5 μg L^{-1})	2.5 ± 0.5	< 2	< 2	2.8 ± 0.6^{a}	2.3 ± 0.6^{A}
C2 (50 μg L^{-1})	35 ± 4.0	< 2	< 2	2.3 ± 0.6^{a}	2.0 ± 0.6^{A}
C3 (100 μg L^{-1})	71 ± 11	< 2	< 2	2.9 ± 0.4^{a}	2.5 ± 0.4^{A}

Letters indicate difference between conditions, lower case for 28 days and upper case for 28 + 14 days (p = 0.05)

Fig. 30 presents the biochemical parameters results, which include metabolic and energetic variations as well as enzymatic and non-enzymatic oxidative stress biomarkers.

Exposure to Ti (28 days) was noted to have altered the metabolic capacity of the mussels, indicated by the decrease of ETS activity with increasing contaminant concentration, with significant variation between each of the conditions. Following depuration period (28 + 14 days) it was however noticed a recovery in ETS activity, with full recovery only for C1 and with C2 and C3 showing lower but similar values. For all conditions, ETS activity was significantly higher at 28 + 14 days period.

Energetic reserves (GLY) after Ti exposure were significantly higher in all conditions in relation to control conditions, pattern which was also maintained following the 14-day recovery

period. This variation was however more pronounced for C3 at 28 days period and for both C2 and C3 at the end of the experiment (28 + 14 days). Variations within the same conditions at both sampling moments were only found on the two highest exposure conditions (50 and 100 µg L^{-1}), in which the GLY content was higher at 28 + 14 days for the former and lower for the latter, respectively.

SOD was noted to reduce its activity with increasing Ti concentration, as shown by the significant decrease for all exposure conditions when compared to control mussel values after the 28-day exposure period. Returning them to clean conditions (28 + 14 days) maintained a similar pattern, with C2 and C3 still showing values significantly lower than baseline levels for non-contaminated organisms. No statistically different values of SOD activity were noted between post-contamination and post-recovery were found for any of the studied conditions. Antioxidant activity of GPx was noted for its significant increase with increasing Ti level of exposure (28 days), in which each scenario had significantly higher values in regards to the control condition. A sharp decrease in enzymatic activity was also noted when comparing the post-contaminated (28 days) with post-recovery (28 + 14 days), with reported values lower but similar in all exposure conditions (5 – 100 µg L^{-1}), albeit higher than the baseline when compared to control mussels (28 + 14 days). For the post-contamination period (28 days) CAT activity followed GPx trend, in which the values were not only above control conditions but it also increased with the concentration gradient (C1 < C2 < C3). The activity of this enzyme after de recovery period (28 + 14 days) continued to increase similarly to the previous trend, with this increment of activity being significantly higher in all contaminated scenarios when compared to the values reported at 28 days. When compared to control mussels, GSTs activity was noticeably higher after 28 days, with increased values in all conditions, while at 28 + 14 days this increase happened only at C2 and C3. Furthermore, this increase was of a greater magnitude post-contamination, with significant higher values at 28 days in all exposure conditions at 28 days.

LPO values indicate rather significant impact on the cellular damage, evidenced by the increase values in all conditions following the 28-day exposure period when compared to control condition. This increase was similar for the lower contaminated conditions (C1 and C2) with significantly higher impact when exposed to 100 µg L^{-1}. The 14-day recovery period showed the LPO content returning to control (non-contaminated mussels) levels, with the exception of C3 which was still slightly higher. Oxidation status of the cell, evaluated through GSH content, denoted a sharp decrease with increasing contaminant concentrations after Ti exposure (28 days), with significant decrease between each of the studied scenarios (CTL > C1 > C2 > C3). After recovery in clean seawater (28 + 14 days) it was noticed an average increase in GSH content for both lower exposure conditions (C1 and C2), while C3 evidenced values similar to post-contaminated moment. However, this increase was only statistically significantly at 50 µg L^{-1} condition, with values reported at C1 28 + 14 presenting a slightly higher variability and thus no mathematical resolution between moments was achieved.

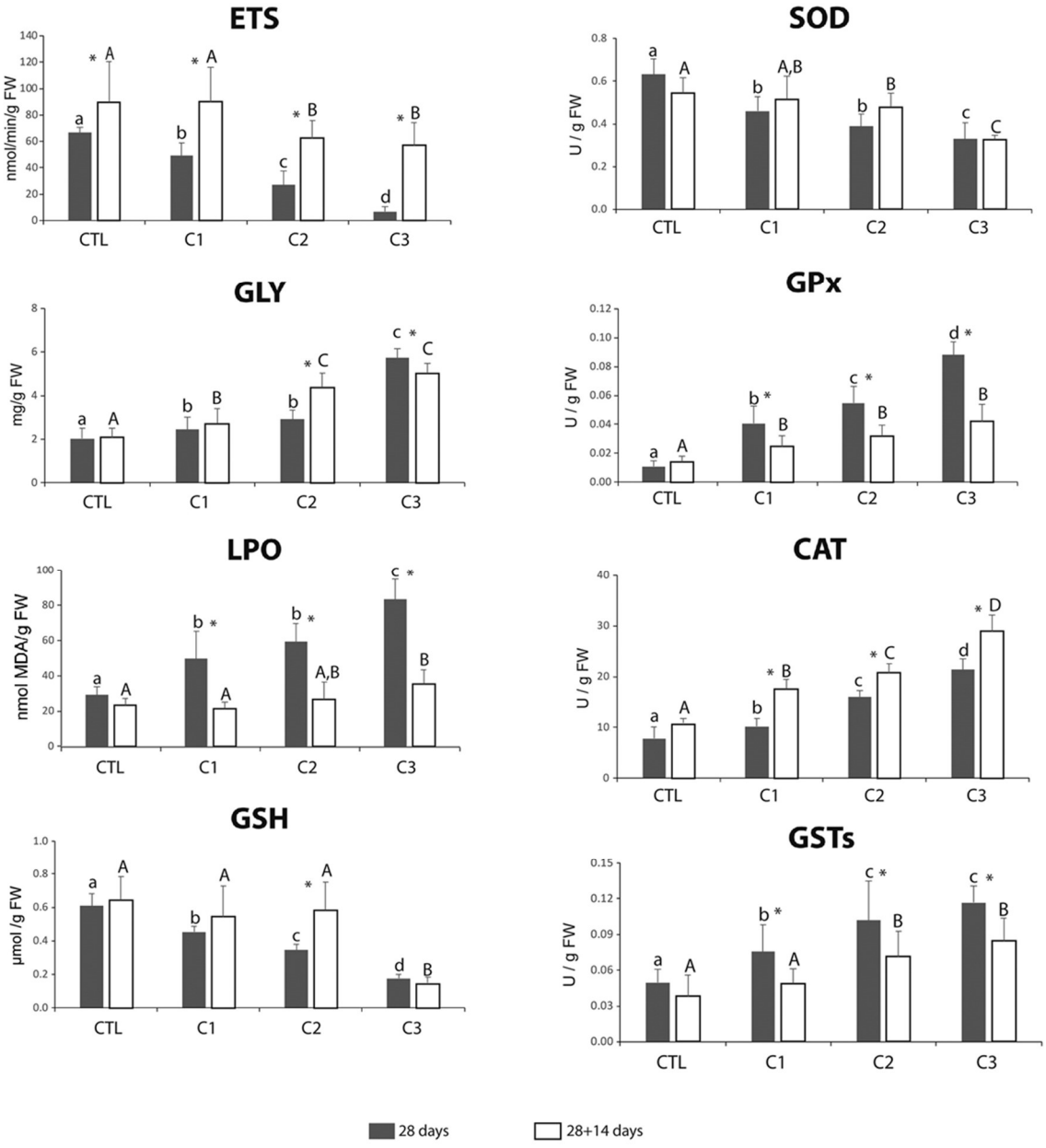

Fig. 30 – Biochemical fitness parameters of mussels due Ti^{4+} exposure (28 days and 28 + 14 days)

A PCoA (Principal Component Ordination Analysis, simplified as PCO), arranged biomarkers in a two-dimension arrangement, with PCO1 (horizontal dimension) explaining 78.3 % of total variance and PCO2 (vertical dimension) factoring 16.1 % of the total variability (Fig. 31).

Through the horizontal dimension it was possible to separate the control conditions at both pre and post recovery (28 and 28 + 14 days – CTLa and CTLb), the lower contaminated condition at 28 and 28 + 14 days (C1a and C1b) and the mid-contaminated scenario post recovery (C2b), in the negative quadrants, from the remaining conditions (C2a, C3a and C3b – high Ti contamination scenario pre and post recovery and mid-contaminated scenario pre recovery) in the positive quadrants. PCO1 was determined to be better explained by SOD and ETS activity as well as GSH content, with high positive correlation ($r > 0.8$), and are related to the control conditions at both periods (CTLa and CTLb) and the mussels' recovery at the two lowest concentrations (C1b and C2b).

The vertical dimension, while factoring a minor of the total variance, allowed for the resolution of pre and post recovery periods (28 days vs 28 + 14 days), on which the positive side is dominated by the post contaminated conditions (28 days, CTLa, C1a, C2a and C3a) and the negative side by the post recovery conditions (28 + 14 days, CTLb, C1b, C2b and C3b). LPO and CAT appear to be the main drivers of such separation, and are related mainly with mid-exposure scenario pre recovery (C2a) and high contamination scenario post recovery (C3b).

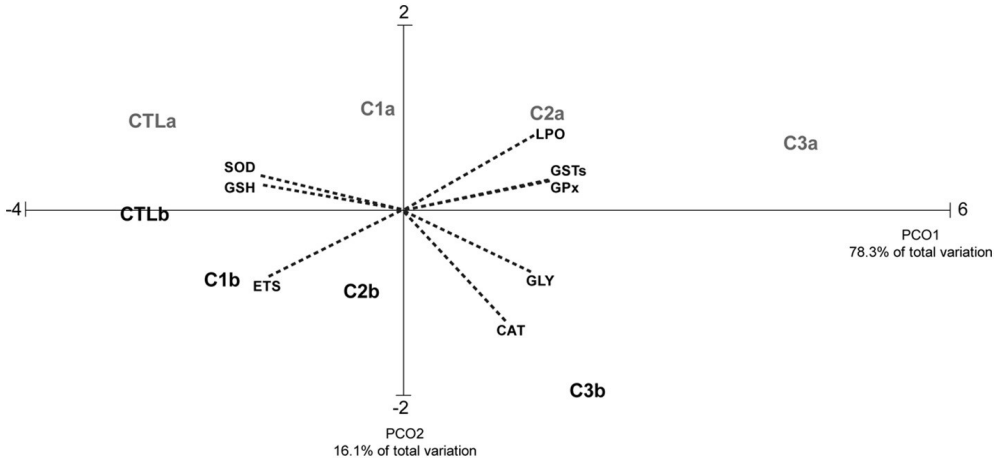

Fig. 31 – Principal component ordination analysis of biomarkers

(a – 28 days; b – 28 + 14 days; CTL – control mussels; C1-C3 – 5-100 μg L^{-1})

Discussion

In this study the experimental design followed a more conventional approach to the impacts of contamination, using a 28 day exposure assay like the myriad of works found in the literature for marine organisms (Du et al. 2008; Wang and Wang 2014; Lei et al. 2015; Han et al. 2018). Toxicity related to oxidative stress studies as thus been widely accepted for mid-term periods (28 days), as should provide enough timeframe for organisms to react to the different stressors, ranging from pollutants to abiotic parameters changes (e.g. temperature and salinity), and fully activate their defense mechanisms. Present work goes a step forwards, not only evidencing the after-effects of the pollution (soluble Ti as a stressor), but also assessing the individuals' capabilities to recover after a two-week period in a non-contaminated environment.

Results from water analysis post Ti^{4+} spiking report the already mentioned fast decrease in concentration, which is considered completely gone (< LoQ – 2 μg L^{-1}) in 72 h. With the weekly water renewal schedule, and subsequent reestablishment of Ti^{4+}, it was thus considered that mussels experienced 4 pulses of contamination. As such, not only were the measured initial concentration over 30 % lower than the intended nominal parameters, which could decrease the potential toxic effects, but at each water renewal cycle the individuals should have a cleaner latter half of the week (c.a. 96 h), and thus a potentially more advantageous environment to recover.

Results of Ti content in tissues are supportive of the aforementioned claim, as it was only found a residual amount on tissues non-discernable from the baseline values (non-contaminated control mussels) for any of the exposure scenarios (5, 50 and 100 μg L^{-1}). In conjunction with the values registered in water samples, it has been hypothesized that there is a competition between the uptake and excretion of Ti by the mussels due to their depuration capabilities (Wang and Fisher 1997; Yap et al. 2003; Yap et al. 2004). As such, at the moment of Ti spiking intake should be the predominant, effect which should last 2 to 3 days at most, following which the mussels would than start excreting Ti and return to baseline values. Furthermore, following the 14-day recovery in clean seawater, residual Ti values in tissues were statistically equal to post-contamination ones (in studied conditions, CTL and C1-3). This result further solidifies the efficient depuration capability, of which after 4 contamination pulses, even at the higher exposure concentrations mussels were able to return to their baseline values. Thus, all Ti reported values should be considered residual non-toxic values, as the biochemical parameters will further discuss.

It should however be noticed that result variability ranged from 14% to 29 %, which analytically could be considered excessive, taking into account the operational standard of 10 % for acceptance. Nonetheless, values measured for residual Ti in tissues are very close to LoQ, which greatly influences their certainty due to their deviation to the center mass of the calibration curve (Miller and Miller 2018). As such, determinations of already highly manipulated samples required the acceptance of a broader operational cut-off criteria, while the certified reference material kept in check the validity of the digestion.

Regarding the biochemical biomarkers, and starting with the metabolic and energetic parameter, it was noticed a severe metabolic decrease (lower ETS) with associated increase in energy reserves (higher GLY) at the end of the 28-day exposure when comparing the contaminated conditions to the control mussels. This is a regular behavioral mussel defense response to adverse stimulus, in which the decrease of activity is due to their closure of the valves (Gosling 2003; Anestis

et al. 2007). This form of defense relies on the reduced filtration volume, that attempts to reduce the intake of any foreign and harmful substances, and as a consequence of reduced cellular activity the energy reserves are consumed at a slower rate, thus the inversely proportional relation.

However, once mussels return to clean conditions (28 + 14 days), it was noted a substantial increase of metabolic rate, with the increase of ETS activity in all exposure conditions. This recovery was only complete for C1, achieving values similar to control conditions, indicating Ti might have a prolonged after effect at higher concentrations. Glycogen also showed an increased content at the end of the recovery period, in all conditions except C3, which indicates a two-fold response from the mussels. At the lower contaminated conditions (C1 and C2), the increased metabolic rate may not have been directly followed by the expenditure of GLY, used by the antioxidant defenses, and thus lagging behind. Increased activity also assumes a resume of regular feeding behavior, which also signifies an increased energy intake. In the case of mussels at C3, the after-effects of exposure may thus be severe enough to dysregulate the formerly mentioned mechanisms, culminating on the reduction of energy reserves. Still relative to the energy reserves, it should also be noticed that other sources of energy could have been consumed under stressful conditions, such as the case of lipids as it has been reported for different bivalves (Darriba et al. 2005; Pardeshi 2015).

Effects of Ti exposure at the 28-day mark were not only noticeable at the metabolic level but also through the cellular damage indicator (LPO), which increased considerably following the increasing Ti gradient. This has been attributed to the xenobiotics formed within the cell (ROS), which actively acted on the lipid of the cell membrane, degrading it. Source of ROS had however to be due to extraneous substances, in this case a contaminant (Ti), as the natural ROS production due to mitochondrial activity has been excluded due to its reduced activity. Damage was nonetheless recoverable in most cases at 28 + 14 days, as seen in the decrease to near baseline values in C1 and C2, with C3 still showing repercussions from the contamination.

The extent of cellular damage can be observed by the cell redox status through the GSH content. Reduced glutathione is present in the intracellular fluid and is the main responsible for its reductive status, and in its solo activity may act as a ROS scavenger, depleting itself to lessen the burden and damage of other cellular organelles (Arora et al. 2012). As such, when considering the extent of reduction of GSH content post contamination period it can be concluded that defenses were gradually worn down by the successive Ti pulses. Ultimately, the cellular defenses were unable to protect the cell and led to the onset of LPO. This damage was recoverable, as GSH content returned to control values at 28 + 14 days in the lower contaminated conditions (C1 and C2). C3 however shows no recovery whatsoever, which is also congruent with prior results (ETS and LPO) of prolonged aftereffects.

The rapid degree at which GSH content was consumed following Ti exposure, infers more than a passive defensive mechanism. GSH has been known to act in tandem with GPx to actively counteract the presence of ROS (Zitka et al. 2012), which is in accordance to presented results. As GSH content decreased with increased Ti exposure, GPx activity followed the inverse pattern by increasing with the increased contamination. This is evident for all the exposure scenarios, as the each of GSH content and GPx activity at 28 days has been well defined according to Ti exposure (GSH – C1 > C2 > C3; GPx – C1 < C2 < C3). Such effect has been widely reported on bivalves, in the case of exposure to $nTiO_2$ (Huang et al. 2018b; Marisa et al. 2018) as well as other types of

contaminants such as metals (Hg) (Coppola et al. 2017) and drugs (cetirizine) (Teixeira et al. 2017). Following the recovery period (28 + 14 days), GPx activity reduced considerably, albeit above control values, thus indicating it was still acting to eliminate leftover ROS but with a lower priority in regard to other defenses.

The biotransformantion enzymes GSTs, function similarly to GPx, in which it acts through conjugation reaction with GSH to degrade endogenous and exogenous xenobiotics (e.g. ROS) (Dasari et al. 2017). As such, GSTs pattern was noticed to be nearly equal to GPx, increasing with increasing Ti concentration after exposure (28 days), followed by a considerable decline post-recovery (28 + 14 days). This exposure effects were similar to observed in other bivalves in cases of different stressors (contamination and temperature, respectively) (Nunes et al. 2017; Coppola et al. 2018b), with the work by Belabed et al. (2013) evidencing the same GSTs pattern when *Donax trunculus* was exposed to Cd and then left to recover.

Another antioxidant enzyme, SOD, provided an interesting result at the end of the 28-day exposure period with its decreased activity as the contamination gradient increased. SOD acts primarily on the superoxide radical ($O_2^{\bullet-}$) converting it into hydrogen peroxide (H_2O_2), which could then be either acted upon GPx and/or CAT (Regoli and Giuliani 2014). As such, acting as the first antioxidant enzyme, SOD is depleted early on and the extent of depletion is more severe with increased concentration. Huang et al. (2018b) observed this effect when *Mytilus coruscus* was exposed to $nTiO_2$, noting an increased SOD activity in 3 days followed by an attenuation in 3 days and reduction in 7 days. Other tests on *Limnoperna fortune* (Girardello et al. 2016) revealed a sharp decrease in SOD content in the hemocytes after 2 h with slight recovery in 4 h, indicative of the fast acting by SOD followed by the activation of antioxidant defenses to further supply the enzyme. Furthermore, the increased activities of both GPx and CAT enzymes at the end of the Ti exposure supply enough basis to indicate the active role of SOD, as well as its ultimately depletion and eventual substitution by these second-in-line defense mechanisms. At the end of the experiment (28 + 14 days), SOD activity presented a slight recovery at both C1 and C2 conditions, indicative of the gradual recovery of the organisms. However, at the highest exposure scenario, SOD remains at the pre-contamination levels indicative of its steady use to achieve basal conditions, which is in accordance to results provided by both LPO and GSH which emphasize the need for further recovery.

The resultant H_2O_2 from SOD activity continues its chain on the oxidative defense mechanism and thus its removal is relegated to GPx and CAT for its break down into H_2O and O_2 (Regoli and Giuliani 2014). GPx, as already mentioned, was found to be the first line of defense, acting expressively (by up to 400 % increased activity in relation to control condition) at the end of the exposure period; but then, at 28 + 14 days, either reducing its activity due depletion or being relegated to a secondary role. CAT however took a steadier and more prolonged role, showing increased activity at the end of the contamination period with further increase at the end of the recovery period. The increased CAT activity was also more pronounced with increasing exposure concentration, further confirming the presence of ROS at the end of the recovery period and the mussels' inability to completely recuperate, especially in the case of C3.

In regards to antioxidant activation and inhibition, results are often divisive when considering the information in the literature, and cases such as presented in the current experiment. Activation of the antioxidant enzymes has been well document when bivalves face stressful conditions (Ahmad et al. 2011; Freitas et al. 2014; Gomes et al. 2014; Benali et al. 2017; Marchi et al. 2018a) however situations of one mechanism superseding other and even enzymatic inhibition have also been reported (Borg and Schaich 1984; Verlecar et al. 2008; Almeida et al. 2015; Freitas et al. 2015a; Freitas et al. 2015b; Luna-Acosta et al. 2015). Under such complex effects it has been proposed that under continuous oxidative stress conditions the excessive ROS production may impar enzymatic activity (Hodgson and Fridovich 1975), which could translate into a reduction of its activity.

The observed SOD activity decrease due to contamination may thus not only be explained under the depletion effect, but also due to antioxidant inhibition as reported by (Pandey et al. 2003; Manduzio et al. 2004; Min and Kang 2008; Falfushynska et al. 2009; Falfushynska et al. 2010). This decrease in activity can lead to the overabundance of the superoxide radical which in turn will further negatively impact the antioxidant defenses (Falfushynska et al. 2009; Canesi et al. 2010). With a slower action by SOD the activity of CAT would also be prolonged, and as SOD activity increases so would CAT to remove the resultant H_2O_2, thus providing an alternative explanation to the obtained results.

Li et al. (2010), in their work with *Oncorhynchus mykiss*, reported that following exposure to different contaminant it was noticeable an increase in antioxidant enzymes (CAT and SOD) after 7 days. However, after 21 and 42 days of exposure both enzymes activities had decreased significantly, to which was proposed that SOD may have overproduced H_2O_2 and thus CAT eventually failed to timely catalyze this ROS specie. With CAT failure the remaining antioxidant enzymes followed suit in a cascading inhibition effect. Such effects of SOD fluctuation weren't observed in present experiment, as timeframe for assessing potential SOD increase was much earlier than first sampling moment (28 days), at which time SOD inhibition should be prevalent. The extreme enzymatic inhibitory effects weren't also noted, as CAT appears to have a continuous and gradually more expressive effect especially after the recovery period.

As final remarks, it should be noted that following the four contamination pulses provided no Ti increment on mussel tissues, attributed to both abiotic factors (low permanence time of Ti^{4+} in water) and biotic factor (decrease metabolic rate and successful excretion/depuration). Nonetheless, despite the lack of bioaccumulation, it was observed effects of oxidative stress with considerable loss of reductive cell capability (lower GSH) even though antioxidant defenses had been activated (CAT, GPx and GSTs). This led to cellular damage (increased LPO) at 28 days, which was partially reverted, following recovery (28 + 14 days), with similar trend to the cell redox status. Antioxidant defense mechanisms were interpreted however questions regarding inhibition/depletion still remain and need to be further explored.

3.3.3. Short-term and Mid-term exposure comparison

Interactions between Ti(IV), be it in its soluble or oxide form in the soluble fraction, and the marine environment have been noted to be poorly understood. This lack of understanding and new rushed works trying to assess toxicity may be missing important steps from which groundwork should be first laid down.

In regards to soluble Ti^{4+} dynamics in seawater, it has been noted that it quickly removed from the water, probably through precipitation as an oxide and following sedimentation effects (Sillanpää et al. 2011; Liu et al. 2013b; Monteiro et al. 2019a; Monteiro et al. 2019b). Furthermore *M. galloprovincialis* has been inferred to quickly metabolize and excrete the accumulated Ti, hence the trace levels found on tissues similar of below baseline set by the control individuals. This has been observed in both experiments, Table 14, from which even at the highest exposure condition, Ti content cannot be considered anything more than residual. Results obtained are limited due to technical restrictions, however studied conditions are within reported environmental and predicted modeled conditions (Westerhoff et al. 2011; Gottschalk et al. 2015; Xia et al. 2017), henceforth should be of significance for future reference.

Table 14 – Titanium concentration in water and mussel tissues of exposure assays 1 and 2

Condition	**Ti in Mussels (µg g^{-1})**			
	96 h	14 days	28 day	28 + 14 days
CTL (0 µg L^{-1})	1.6 ± 0.3	< 0.9	2.1 ± 0.6	2.3 ± 0.6
C1 (5 µg L^{-1})	1.5 ± 0.3	< 0.9	2.8 ± 0.6	2.3 ± 0.6
C2 (50 µg L^{-1})	1.4 ± 0.3	< 0.9	2.3 ± 0.6	2.0 ± 0.6
C3 (100 µg L^{-1})	2.5 ± 0.3	3.1 ± 0.5	2.9 ± 0.4	2.5 ± 0.4

Oxidative stress conditions present some problems when comparing different works. Has observed in previous results and discussions comparisons had to be made evaluating pattern and trends instead of absolute values. This was due an inherent trait of the biomarkers' assays, which are not only highly dependent on factors such as the species, type of tissue, in-vivo vs in-vitro, but also due to operational factors ranging from the operator, the reagents and even the laboratory environmental conditions (e.g. room temperature which affect enzyme kinetics). Thus, it was not unusual for the absolute value of the response of the same parameters be considerably variable even for the same species (Coppola et al. 2017; Andrade et al. 2019a; Monteiro et al. 2019a; Pinto et al. 2019). In order to minimize external variability sources, each parameter was thus analyzed in a single batch, i.e. for a given parameter the samples were analyzed using the same reagents, within the same session by the same operator.

The first exposure assay conclusion with inference of cumulative impact of successive Ti^{4+} contamination pulses are validated by the second exposure assay. Results from metabolic activity (ETS) which showed an ambivalent response to short-time exposure were revealed that at the end of a full 28-day exposure assay the organisms had a clear reduction in activity.

Antioxidant enzymatic defenses comparison provided new insights onto the probable off-set order. At earlier exposure times (96 h and 14 days) SOD appears to be slightly increasing after which (28 days) has been noted to show a decreasing trend. This may signify that early SOD action

converted a considerable amount of superoxide radicals into the hydrogen peroxide, and thus it either starts to be inhibited by the new ROS species or no energy is being funneled into its activity (a sort of enzymatic depletion) as other enzymes such as GPx, CAT and GSTs start to be more important for the cell current status. It is in fact noticed an early on-set (96 h) of the GPx activity denouncing the need for H_2O_2 removal, with similar trend (great GPx activity in more contaminated scenarios) also observed in organisms at the end of 28 days. Activity breaks on this enzyme have been noticed twice, attributed to a combined effect from the energy shortage and lower metabolic activity and the need to activate other enzymatic pathways (GSTs and CAT). Both of these latter two enzymes act at seemingly different time interval, with GSTs working first followed by CAT. This can however be explained by the GSH/GSGG system, in which at earlier moments the greater GSH cellular content managed to supply both GPx and GSTs. Meanwhile, as it depletes and is converted into GSSG, the antioxidant defenses reliant on this component start to lose their expression and are thus superseded by CAT which showed its importance only at later moments.

These complex interaction between the enzymatic and non-enzymatic mechanisms are in accordance to the cellular damage (LPO) found in the cell. At earlier moments with the high GSH content and GPx and GSTs activity cellular wall damage was avoided at the lowest Ti concentration. However, with the gradual depletion of GSH and decreased activity of aforementioned enzymes, higher LPO levels were found in all exposure scenarios. This is observable on Fig. 32, which represents the a tendential increase of LPO in relation to the respective control. The trend shows a slight delay on the activation of the oxidative defenses with offset of lipidic peroxidation (96 h). After the second contamination pulse (14 days) the antioxidant defenses were still able to neutralize further damage, however, this was the tolerance limit for the organism defenses, as after the following two Ti^{4+} pulses increasing damage has evidenced, significantly so for the highest concentration.

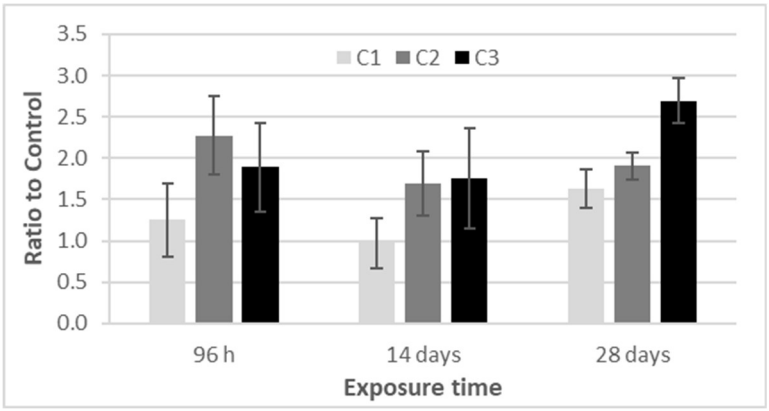

Fig. 32 – LPO ratio to control organisms

In conjugation, both experiments reveal the capability of *M. galloprovincialis* to cope with potential cases of Ti^{4+} pollution, by successfully developing defense mechanisms against exogenous xenobiotics. However, prolonged exposure may lead to an antioxidant stressful status which is highly dependent on the contaminant concentration. Moreover, although damage was found to be non-lethal and recoverable, the biochemical impact was noted to have left prolonged after-effects that will take a considerable amount of time to return to baseline levels.

3.3.4. Mid-term exposure to TiO_2 – Anatase vs Rutile

Objective

This exposure assay intended to appraise the induced toxicity of two different TiO_2, in three different levels of contamination, towards the mussel *M. galloprovincialis*, a widely used proxy species in marine environment, in conditions similar to the ones present in the Ria de Aveiro Lagoon.

Thus, variations on the Ti content in water and the organism tissues in conjunction to with the biochemical biomarkers were evaluated after 28 days of exposure.

Results

Results of Ti quantification in water and mussel tissues samples are presented in Table 15. Obtained results from water samples for the control condition were always below LoQ (2 µg L^{-1}) while samples from the contaminated aquaria weren't possible to be accurately determined due to technical limitations. In the case of the tissue samples, it was recorded residual levels of Ti in all tissue conditions with similar values between the control groups and the contaminated conditions (2.1 – 2.5 µg g^{-1}). The exceptions were C3 of both TiO_2 polymorphs and rutile-C2, which presented slightly higher concentrations (increment up to twice the baseline values).

Table 15 – Titanium concentration in water and clam tissues of exposure assay 4

Condition		**Ti in Water (µg L^{-1})**			**Ti in Mussels (µg g^{-1})**
		t_0	72 h	7 days	28 day
CTL		< 2			2.1 ± 0.3 $^{a, A}$
a-TiO_2	C1	N.A.			2.4 ± 1.0 ^{a}
	C2				2.5 ± 0.4 ^{a}
	C3				4.5 ± 0.3 ^{b}
r-TiO_2	C1	N.A.			2.3 ± 0.8 $^{A, B}$
	C2				2.8 ± 0.2 ^{B}
	C3				5.3 ± 0.7 ^{C}

N.A. – Not Available

Letters indicate difference between conditions, lower case anatase upper case for rutile (p = 0.05)

Biochemical parameters results, which include metabolic and energetic variations as well as enzymatic and non-enzymatic oxidative stress biomarkers, can be observed in Table 16.

Mussels exposed to both TiO_2 powders for 28 days were noted to have very slight variations in both metabolic and energetic parameters. ETS activity remained at baseline levels in all exposure conditions in the case of rutile, while for anatase only C2 evidenced a clear increase in activity with the remaining values not being statistically different. Between powder morphology, in each tested concentration, there was however no significant difference. In what regards to energy reserves, GLY content was noted to clearly increase for all studied conditions and both contaminants in relation to control values, albeit no statistical differences were found between each of the scenarios or contaminants. PROT content showed a significant decreased content at C1 (both polymorphs) and

C3 (anatase), while no significant variation was found between a-TiO_2 and r-TiO_2 for any of the TiO_2 exposure levels.

Antioxidant responses were evaluated through SOD, CAT, GPx, GRed and GSTs activities.

SOD activity, when compared to control values, was significantly lower in the case rutile-C2 while it was increased at rutile-C1 and anatase-C2. At the lowest concentration exposure scenario (C1) rutile showed a significantly higher SOD activity, contrarily, at the mid-concentration exposure scenario (C2) it was the anatase form that evidenced a superior SOD activity. At the highest concentration of exposure both values were similar and near baseline values. CAT was found to be nearly non-responsive, with nearly all reported activities being near baseline levels. The sole exception occurred for anatase-C2, which showed significantly higher activity when compared to both the baseline and rutile values. Results from GPx showed a generally increased activity of this enzyme in all studied conditions, which was also similar for both forms of TiO_2. The only exception was anatase-C1, which kept its baseline value, reporting also a significantly lower value when compared to rutile activity. The activity of GRed was noted to be heavily impacted at the lower levels of exposure, with a significant increase for both studied contaminants, while the remaining scenarios maintained a near baseline results. Only at C2 it was found a statistically different results between rutile and anatase, however both results were within control levels. GSTs were found to be significantly higher for rutile-C3 while significantly lower for anatase-C2 and C3, when compared to control levels. Remaining values were noticed to be similar to control condition, and thus only at C2 and C3 it was found significant different patterns between a-TiO_2 and r-TiO_2

Cellular damage however produced more diverse results better evidencing the impact of TiO_2 exposure. LPO levels were found to have no relation to the exposure concentrations but with the TiO_2 morphology. Rutile powder reported baseline values of LPO in all exposure conditions, while anatase exposure was noted to have increased LPO values but similar in all tested concentrations. Carbonylation of the proteins (PC levels) were statistically higher in the case of rutile-C2 and lower for rutile-C1 and anatase-C3 when compared to baseline levels. Comparing both morphologies at each of the studied exposure scenarios it was noticed that all PC levels were significantly different, with anatase being higher at C1 while lower at C2 and C3.

Neurotoxicity, evaluated through acetylcholinesterase activity, was noted to be heavily dose dependent, achieving maximum values in both contaminants at C2, while still being significantly higher than control values at C3 (both powders) and C1 (anatase). As such, only at the lowest concentration the neurotoxic effects were noted to be statistically different between anatase and rutile phases.

Table 16 – Biochemical parameters in mussels in the experiment of exposure to a-TiO_2 and r-TiO_2 at 5, 50 and 100 µg L^{-1} of Ti_{eq}

Condition		GLY mg g^{-1} FW		PROT mg g^{-1} FW		ETS nmol min^{-1} g^{-1} FW		SOD U g^{-1} FW		CAT U g^{-1} FW		GPx U g^{-1} FW		GSTs U g^{-1} FW		GRed nmol g^{-1} FW		LPO mmol MDA g^{-1} FW		PC nmol g^{-1} FW		AChE nmol min^{-1} g^{-1} FW	
CTL	-	11.6 ± 3.3	(a, A)	42.6 ± 7.8	(a, A)	11.4 ± 2.7	(a, A)	0.93 ± 0.54	(a, A)	3.63 ± 0.31	(a, A)	0.14 ± 0.02	(a, A)	0.075 ± 0.027	(a, A)	0.037 ± 0.022	(a, A)	28.3 ± 9.5	(a, A)	1.45 ± 0.19	(a, A)	0.010 ± 0.007	(a, A)
a-TiO_2	C1	18.2 ± 5.1	(B)	28.5 ± 10.0	(B)	14.5 ± 12.8	(A, B)	0.72 ± 0.27	(A) *	3,43 ± 0.49	(A)	0.12 ± 0.04	(A) *	0.060 ± 0.01	(A)	0.078 ± 0.020	(B)	41.1 ± 10.9	(B) *	1.46 ± 0,16	(A) *	0.020 ± 0,003	(A)
	C2	16.8 ± 4.0	(B)	39.3 ± 5.2	(A)	18.0 ± 5.4	(B)	1.74 ± 1.39	(B) *	4.58 ± 0.47	(B) *	0.17 ± 0.03	(B)	0.048 ± 0.006	(B) *	0.050 ± 0.005	(A) *	55.1 ± 27.8	(B) *	1.41 ± 0.32	(A) *	0.031 ± 0.008	(B) *
	C3	19.7 ± 3.8	(B)	30.0 ± 7.8	(B)	13.3 ± 7.6	(A ,B)	0.96 ± 0.65	(A, B)	3.75 ± 0.49	(A)	0.19 ± 0.02	(B)	0.045 ± 0.017	(B) *	0.040 ± 0.021	(a) *	56.0 ± 31.6	(B) *	1.15 ± 0.13	(B) *	0.020 ± 0.007	(B) *
r-TiO_2	C1	15.6 ± 2.1	(b)	32.6 ± 11.5	(b)	13.3 ± 7.1	(a)	1.72 ± 1.04	(b) *	3.62 ± 0.57	(a)	0.17 ± 0.03	(b) *	0.057 ± 0.014	(a)	0.061 ± 0.022	(b)	19.3 ± 6.1	(b) *	1.22 ± 0.13	(b) *	0.009 ± 0.004	(a)
	C2	15.9 ± 4.2	(b)	36.4 ± 5.5	(a, b)	14.4 ± 7.5	(a)	0.33 ± 0.11	(c) *	4.00 ± 0.62	(a)	0.15 ± 0.01	(b) *	0.069 ± 0.008	(a) *	0.035 ± 0.009	(a) *	32.8 ± 4.2	(a) *	1.87 ± 0.24	(c) *	0.034 ± 0.006	(a) *
	C3	20.9 ± 7.6	(b)	38.6 ± 16.3	(a, b)	11.8 ± 4.6	(a)	0.58 ± 0.36	(a, c)	3.49 ± 1.02	(a)	0.16 ± 0.04	(a, b)	0.103 ± 0.027	(b) *	0.045 ± 0.013	(a,b)	22.9 ± 9.8	(a, b) *	1.55 ± 0.3.	(a) *	0.022 ± 0.011	(b) *

Lower-case letters indicate difference between control and r-TiO_2 along the concentration gradient (p = 0.05)

Upper-case letters indicate difference between control and a-TiO_2 along the concentration gradient (p = 0.05)

Asterisk (*) indicates difference between r-TiO_2 and a-TiO_2 within the same concentration of exposure (p = 0.05)

Results from the biochemical parameters were plotted in a 2D arrangement through PCO analysis (Fig. 33), with PCO1 (horizontal axis) factoring 32.8 % and PCO2 (vertical axis) accounting for 29.8 % of total variation. PCO1 showed high correlation to PC, GSTs and SOD (0.77, 0.89 and 0.75, respectively) while PCO2 was closely related to CAT and AChE (0.75 and 0.96, respectively).

It's observed a clear spatial separation among the vertical axis (PCO2), with the mid and high exposure concentrations (C2 and C3), regardless of TiO_2 powder form (anatase/rutile), near the null values or in the positive side. Mussels (CTL) under control condition and both of TiO_2 lowest concentration of exposure (C1) were presented in the negative side of PCO2. PCO1 separated according to morphology, with mid to high concentration (C2 and C3) of exposure being related to the negative and positive sides for anatase and rutile forms, respectively. Control condition mussels (CTL) and the lowest exposure concentration (C1) were also separated accordingly, with CTL at the positive side, anatase at null values and rutile in the negative side.

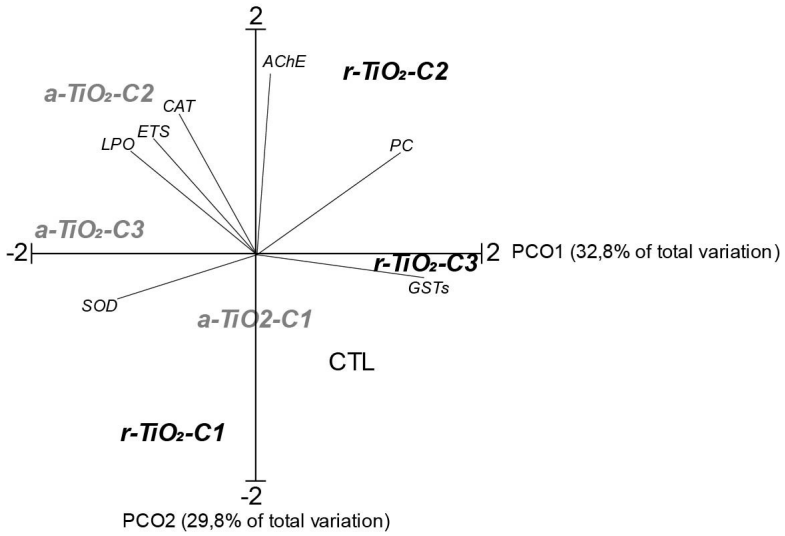

Fig. 33 – Principal component ordination analysis of biomarkers for the anatase vs rutile experiment

(Grey – a-TiO_2 – anatase; Bold Black – r-TiO_2 – rutile; Narrow Black – CTL – control; C1 – 5 µg L^{-1}; C2 – 50 µg L^{-1}; C3 – 100 µg L^{-1})

Discussion

Present exposure assay builds on the previous obtained results of Ti^{4+} exposure, pushing forward into the study of TiO_2 powders, as these may be some of the most readily available current sources of Ti contamination of the marine environment, or at the very least act like a close proxy. Thus, maintaining the commonly used approach of the 4-week exposure trials, mussels were used as a marine key-organisms to study the potential toxic effects induced by the exposure to TiO_2 powders.

As in the previously reported results, Ti accumulation in tissues was mostly at the residual level, albeit some increased content was found at greater exposure conditions. Especially in the marine environment, where nanoparticles tend to more easily aggregate (Baker et al. 2014), the increased concentration may facilitate such cluster formation (Ciacci et al. 2012) which can ultimately enable the increased intake by organisms (Ward and Kach 2009). However, as already discussed, the inherently efficient depuration capabilities of mussels (Wang and Fisher 1997; Yap et al. 2003; Yap et al. 2004) may promptly reduce the observable bioaccumulation effects. As such, as also previously claimed, a decreasing amount Ti content in water conjugated with the depurative abilities of mussels was the final remark in the case of the previous Ti^{4+} case studies. However, no Ti content in water has been provided in the present study which limits such claims.

In fact, several attempts at quantification of TiO_2 in seawater were made. Starting with direct analysis, akin to Ti^{4+} procedure, it was promptly discarded as it was noticeable a white powder in each of the sample containers on the higher concentration samples at t_0, which is characteristic of TiO_2 deposition. In the literature there are reported some methods aimed for direct analysis of TiO_2 in ICP-related techniques (Shaw et al. 2013; Bocca et al. 2018; Gondikas et al. 2018). However, they either require unavailable specialized equipment or are highly unpractical for the currently developed work, i.e. constant sonication moments prior to analysis and contamination of the ICP equipment due to surfactants and other additives.

Different attempts at acid digestion were made (not mentioned in chapter 2), but ultimately failed due to different factors. Dilution of the sample during the digestion process was one of the main problems, which would increase the operational LoQ of the ICP-OES from 2 μg L^{-1} to 5 μg L^{-1}. As such, any attempts at the quantification of the lowest concentration samples (C1, 5 μg L^{-1}) would invariably produce the report of below LoQ. For the remaining conditions (50 and 100 μg L^{-1}) chemical and stability issues were the main problems, mostly due to the chemical reactions between TiO_2 and Ca^{2+} present in the sample and HF which is the main agent for Ti solubilization. After digestion, samples are apparently clear but quickly start to present a slight turbidity in the form of a white colloidal substance. Chemically this could either be due to the deposition of either TiO_2 or CaF_2 (Patnaik 2002), which are both insoluble white powders in water, one resultant from an incomplete digestion, the other from the reaction between HF and Ca^{2+} in the seawater. As such, the "not available" descriptive in Table 15, isn't due to the failure to quantify Ti, i.e. values below LoQ, but that the obtained results had such variability within their own replicates that were considered meaningless and not representative of the true initial concentration of Ti in the aquaria.

Nonetheless, it should be noted that no white precipitate was visually observed on 72 h and 7-day samples, which could represent that most of TiO_2 in the aquaria should have been deposited. This finding was also in accordance with prior claims from other authors (Jiang et al. 2009; Canesi et al. 2010; Zhu et al. 2011) in regards to the behavior of $nTiO_2$ in marine environment, having

concluded that the high ionic strength of seawater shrinks the energy barrier between particles and thus more easily enabling the agglomeration and deposition process.

A final mention regarding the effects of seawater in the TiO_2 particles, it should be noted that it was impossible to do any characterization on physicochemical changes after the exposure period. As already mentioned in previous (3.2.2) characterization is nearly impossible in seawater due to interferents, moreover with the low concentrations of the exposure assay it would be nearly impossible to find enough TiO_2 particulates to provide any meaningful data. As such, any discussion regarding biological effects had to solely take in account the initial morphological characteristics (presented on 3.2.3), which should be stressed the major differences in size and surface area between a-TiO_2 (particulates on the micrometric scale) and r-TiO_2 (aggregates on the micrometric scale).

Structural properties, such as size and morphology, have been noted to heavily influence the contaminant (TiO_2) toxicity (Katsumiti et al. 2015) and this work is one of the first instances in which two TiO_2 polymorphs have been reported to provided different metabolic responses. Rutile apparently provided no ETS variation while anatase was consistently above baseline limits, especially at mid-level exposure scenario. Little metabolic impact has been noted to occur under low contamination conditions (Coppola et al. 2017; Andrade et al. 2019a), thus for rutile exposure the concentration may not be enough to impact the mussels'. The increased metabolism in the case of anatase exposure, more pronounced in the mid exposure concentration, may be related to an activation of the antioxidant and biotransformantion defenses (SOD, CAT, GPx and GSts). The activation of these mechanisms to prevent potential damage demand higher energy consumption, henceforth the increased mitochondrial activity. Such effects have been demonstrated by Monteiro et al. (2019b), in which the exposure to Ti^{4+} incurred an increased ETS in order to activate GPx, CAT and GSTs. According to these results, morphology may be critical feature of TiO_2 impact on metabolism, but concentration also plays a significant role in the mechanistic defenses of mussels.

Concurrently, energy parameters provided contrasting results, with glycogen reserves being considerably higher in every exposure scenario, while protein content was consistently lower than baseline, regardless of TiO_2 polymorphs. In the case of GLY content, the most readily available energy source, it indicates that mussels under exposure scenarios may be avoiding using their energy reserves, similarly to the case of Coppola et al. (2018a) in which *M. galloprovincialis* was exposed to arsenic. Correlating these results with the metabolic activities, which was maintained for rutile and increased for anatase, it would be indicative that energy demands weren't enough to require GLY consumption. In either case, it could be inferred that the tested concentrations weren't high enough to provide significant impacts and/or other energy sources were being used. In the case of the latter option, the usage of protein as energy source has also been reported in the literature (Darriba et al. 2005; Pardeshi 2015). As such, if protein was being used for energy and enzyme content isn't being increased, PROT level decrease may be indicative of low toxicity under tested concentrations. Monteiro et al. (2019b), results may be presented has the contrasting effect, in which both GLY and PROT increase with Ti^{4+} exposure, related to the significant increase in enzymatic activity.

Furthermore, it has been previously noted that the metabolic and energetic aspects are deeply related to the enzymatic activities, and as such, since the variation of the former are limited, the impact on the latter should also be diminutive. And it was exactly this effect that was observed in this work, as the variations of CAT are nearly non-existent and even GPx presented only slight

increases (< 33 %) in contrast to the very expressive effects with Ti^{4+} exposure (Monteiro et al. 2019a; Monteiro et al. 2019b). Canesi et al. (2010) reported similar CAT and GST non-responsiveness when exposure concentration to $nTiO_2$ was below 200 μg L^{-1} after 24 h, while GPx low fluctuation is in accordance to the finding of Barmo et al. (2013) in case of low concentration 96 h exposure, thus present study scenarios may not be sufficient aggressive to provoke biochemical responses. Furthermore, as TiO_2 is a short-lived contaminant, similar to the previous studies of Ti^{4+}, the organisms may promptly adapt to the experimental conditions and further stabilize their defensive measures as previously observed (Monteiro et al. 2019a). GRed, appears to have been slightly influenced by dosage, with increased expression only at C1 for both forms, and SOD presents only increased activity for rutile at C1 and anatase at C2. SOD in particular has been noted to be inhibited by TiO_2, therefore not unexpected presenting such low values (Falfushynska et al. 2009; Falfushynska et al. 2010). Nonetheless, these results were once again an indication of the influence of dosage and morphology of TiO_2 on the biochemical responses. However, correlating the lack of significant antioxidant responses to the lower PROT content and maintenance of ETS activity it could be inferred that impact on organisms wasn't enough to fully activate enzymatic defenses.

Cellular damage results are the parameters that more easily evidence the disparities between both TiO_2 powders. Lipid peroxidation (LPO), appears to be the primary indicator of cellular damage, registering the most difference between both polymorphs with anatase presenting the more toxic effects. Presented results of LPO were in agreement with metabolic and antioxidant defense, in which the lack of effects by rutile powder is in line with the lack of damage markers, henceforth the low toxicity at the studied concentrations. Contrarily for anatase, the slight activation of antioxidant defense may have counteracted some, but not all induced damage as observed by the increase in LPO. It should however be noted that anatase powder was determined to be of a smaller size, and as such higher negative impact in the cell are supported by the literature (Katsumiti et al. 2015; Jimeno-Romero et al. 2016).

Acetylcholinesterase (AChE) is an enzyme that play a vital function on nerve system (Fulton and Key 2001), and its variation has been widely used as a biomarker towards stressors such as contaminants (Matozzo et al. 2005; Gomes et al. 2013; Gonzalez-Rey et al. 2014). In this particular case, the increased activity has been attributed to an attempt to reduce the neurotransmitter excess in the synaptic clefts (Rosa et al. 2016), and such results have also been found in other works (Pan et al. 2012; Xia et al. 2017). This increase has however been denoted as an overcompensation mechanisms (Badiou et al. 2008) or may even be related to cell apoptosis (Zhang et al. 2002).

In conclusion, both forms of TiO_2 were responsible for bioaccumulation and alterations of the biochemical responses. Increased concentration led to increased bioaccumulation, while dosage effects were observed in some biochemical responses. However, the critical differences in toxicity were noticeable to be of morphological nature. Rutile particles were shown to provide relatively small biochemical alterations and nearly no damage was induced to the mussels. On the other hand, mussels exposed to anatase particles showed significantly increased damage markers, mostly attributed to the smaller size of the particles that may have induced higher toxicity due to higher surface area and reactivity. Nonetheless, such effects were sub-lethal (mortality < 1 %), and it is expected that the induced damage to be recoverable, has the extent of the extent of biochemical alterations were lower that the results obtained at previous Ti^{4+} studies.

3.3.5. Effect of temperature on mid-term exposure to r-TiO_2

Objective

This work, published under Leite et. al (submitted), attempted to evaluate the toxicity of r-TiO_2 in three contamination scenarios and considering the effects of warming. The mussel *M. galloprovincialis* was used as a key-organism of the Ria de Aveiro Lagoon, taking into account the current and predicted environmental characteristics of this system.

Thus, variations on the Ti content in water and the organism tissues in conjunction to with the biological parameters (histopathological and biochemical biomarkers) were evaluated after 28 days of exposure.

Results

Ti quantification results are provided in Table 17 and, as in the case of the previous study, results in water samples are reported not available due to inconsistency of values provided by the ICP-OES. As already explained, difficulties related to chemical incompatibilities and the limitations of working with ICP quantification techniques won't allow for proper quantification of Ti in a complex medium such as seawater. On tissues, however, results are also similar to previous studies, with all conditions presenting residual amounts of Ti in tissues, with C1 and C2 not being statistically different from the CTL, with a slight increase at the highest exposure conditions (C3). At elevated temperature it was observed a similar trend, with individual at C3 reporting higher bioaccumulation than control (CTL) mussels. However, at the highest exposure concentration (C3) mussels at control temperature (18 ºC) presented higher Ti tissue content than mussels in warming conditions (22 ºC).

Table 17 – Titanium concentration in water and clam tissues of exposure assay 4

Condition		**Ti in Water ($\mu g\ L^{-1}$)**			**Ti in Mussels ($\mu g\ g^{-1}$)**
		t0	72 h	7 days	28 day
18 ºC	CTL	< 2			2.1 ± 0.3^{a}
	C1	N.A.			2.4 ± 1.0^{a}
	C2				2.5 ± 0.4^{a}
	C3				$4.5 \pm 0.3^{b\,*}$
22 ºC	CTL	< 2			1.8 ± 0.7^{A}
	C1	N.A.			$2.3 \pm 0.6^{A,}$
	C2				2.2 ± 0.6^{A}
	C3				$3.3 \pm 0.4^{B\,*}$

N.A. – Not Available

Letters indicates difference between conditions, lower case 18 ºC upper case for 22 ºC ($p = 0.05$)

Asterisk indicates statistical difference between temperatures for the same exposure concentration ($p = 0.05$)

In Table 18 are presented the full results of the biomarkers related to the oxidative stress performance, metabolic and energy status in the mussels.

In terms of metabolism, at normal temperature (18 ºC) exposure to r-TiO_2 didn't significantly change independently of the concentration, however in warming scenarios (22 ºC) a significant increase, in relation to the respective control group, was found at both lower and medium exposure concentrations. At C1 this increase was rather substantial, being the only case in which results obtained at 22 ºC was significantly higher than the 18 ºC counterpart.

The energetic balance of the cell had also some fluctuations due to contamination. At 18 ºC all exposure conditions reported higher GLY content in relation to the control group, albeit similar between each other, while at 22 ºC it was only significantly higher at C3. For each of the exposure conditions the only significant different was only found at CTL, which reported a GLY content at 22 ºC. Total protein content (PROT) in mussels exposed to TiO_2, regardless of temperature or concentration, was consistently lower than in mussels in their respective control (CTL) groups. At 18 ºC this difference was more pronounced at C1 while at 22 ºC there was a systematic decrease in PROT with increasing contaminant concentration. No statistical differences were noticed between exposure concentrations for any of the temperatures.

Antioxidant responses were evaluated through SOD, CAT, GPx and GSTs activities.

Antioxidant activity of SOD at control temperature (18 ºC) showed a significant increase at C1 and a decrease at C2 and C3 when compared to control (CTL) organisms. At 22 ºC the trend was completely the opposite, with increasing SOD activity with increasing contaminant concentration. Temperature was noted to have a suppressive effect at lower concentrations, and differences between similar concentrations at different temperatures were always significant. Catalase activity at 18 ºC was kept at baseline levels in all contamination concentrations, while at 22 ºC its activity tendentially decrease with increased concentration but it was only significantly lower at C2. Differences between similar exposure concentrations at different temperatures were noted at C1 and C3, both values higher at 22 ºC. GPx showed a near non responsiveness, with no fluctuations at 22 ºC and only significant increases at C1 and C2 at 18 ºC, when compared to the respective control organisms. Only at C2 it was found differences between enzymatic activities at different temperatures. GSTs response at 18 ºC was only significantly higher than CTL for the highest exposure concentration (C3). In warming conditions (22 ºC) all values were within the CTL group response. Within the same exposure concentration, it was noticed a decrease in GSTs activity with increased temperature in all conditions, except for C1.

Lipid peroxidation reported similar or lowered values in all exposure scenarios, regardless of exposure concentration or temperature, when compared to their respective control (CTL) organisms. In warming condition (22 ºC), however, LPO was significantly higher in all tested conditions when compared to results at control temperature (18 ºC).

Neurotoxicity was noticed to have a dependency on both temperature and dosage. At lower exposure temperature (18 ºC) dosage effect was observed at C2 and C3, both showing increased AChE levels. However, at 22 ºC, temperature effect towered over the dosage effect with consistently higher but similar values among all contamination scenarios, exception to 18 ºC C2 which was the highest reported values of AChE.

Table 18 – Biochemical parameters in mussels in the experiment of exposure to r-TiO_2 (5, 50 and 100 µg L^{-1} of Ti_{eq}) at 18 ºC and 22 ºC

Condition			GLY mg g^{-1} FW		PROT mg g^{-1} FW		ETS nmol min^{-1} g^{-1} FW		SOD U g^{-1} FW		CAT U g^{-1} FW		GPx U g^{-1} FW		GSTs U g^{-1} FW		LPO mmol MDA g^{-1} FW		AChE nmol min^{-1} g^{-1} FW	
18 ºC	CTL	-	11.6 ± 3.3	(a) *	42.6 ± 7.8	(a)	11.4 ± 2.7	(a)	0.93 ± 0.54	(a) *	3.63 ± 0.31	(a)	0.14 ± 0.02	(a)	0.075 ± 0.027	(a, b) *	28.3 ± 9.5	(a) *	0.01 ± 0.007	(a) *
	r-TiO_2	C1	15.6 ± 2.1	(b)	32.6 ± 11.5	(b)	13.3 ± 7.1	(a) *	1.72 ± 1.04	(b) *	3.62 ± 0.57	(a) *	0.17 ± 0.03	(b)	0.057 ± 0.014	(a)	19.3 ± 6.1	(b) *	0.009 ± 0.004	(a) *
		C2	15.9 ± 4.2	(b)	36.4 ± 5.5	(a, b)	14.4 ± 7.5	(a)	0.33 ± 0.11	(c) *	4.00 ± 0.62	(a)	0.15 ± 0.01	(b) *	0.069 ± 0.008	(b) *	32.8 ± 4.2	(a) *	0.034 ± 0.006	(b) *
		C3	20.9 ± 7.6	(b)	38.6 ± 16.3	(a, b)	11.8 ± 4.6	(a)	0.58 ± 0.36	(a, c) *	3.49 ± 1.02	(a) *	0.16 ± 0.04	(a, b)	0.103 ± 0.027	(c) *	22.9 ± 9.8	(a, b) *	0.022 ± 0.011	(c)
22 ºC	CTL	-	16.5 ± 3	(A) *	46.3 ± 11.2	(A)	13.1 ± 5.6	(A)	0.17 ± 0.15	(A) *	6.09 ± 3.63	(A, B)	0.16 ± 0.04	(A)	0.042 ± 0.01	(A, B) *	121 ± 46	(A) *	0.022 ± 0.008	(A) *
	r-TiO_2	C1	18.8 ± 5.6	(A, B)	36.8 ± 6.7	(B)	26.6 ± 2.1	(B) *	0.55 ± 0.28	(B) *	5.55 ± 1.34	(B) *	0.19 ± 0.02	(A)	0.048 ± 0.006	(A)	60.4 ± 28.9	(B) *	0.025 ± 0.006	(A) *
		C2	16.1 ± 2.4	(A)	34.3 ± 4.1	(B, C)	18.7 ± 3.0	(C)	1.06 ± 0.73	(B, C) *	3.92 ± 0.59	(A)	0.17 ± 0.02	(A) *	0.033 ± 0.01	(B) *	79.2 ± 68.2	(A, B) *	0.027 ± 0.004	(A) *
		C3	23.8 ± 8.8	(B)	29.3 ± 8.4	(C)	10.4 ± 5.2	(A)	2.10 ± 1.46	(C) *	4.55 ± 0.92	(A, B) *	0.19 ± 0.04	(A)	0.042 ± 0.01	(A, B) *	70.5 ± 56.7	(A, B) *	0.023 ± 0.007	(A)

Lower-case letters indicate difference between control and r-TiO_2 at 18 ºC along the concentration gradient (p = 0.05)

Upper-case letters indicate difference between control and r-TiO_2 at 22 ºC along the concentration gradient (p = 0.05)

Asterisk (*) indicates difference between 18 ºC and 22 ºC within the same exposure condition (p = 0.05)

A 2D arrangement of biochemical responses was arranged through PCO analysis (Fig. 34). The first principal coordinate (PCO1) represents 35.9 % of the variability, highly correlated to CAT, GSTs and LPO (0.80, 0.80 and 0.83, respectively). The second axis (PCO2) represents 23.2 % of variability, correlated with PROT and SOD (0.81 and 0.98, respectively). PCO1 clearly separates the mussels according to the temperature of exposure, with mussels at 18 ºC in the negative side and at 22 ºC in the positive side. PCO2 further helps distinguishing the concentration gradient effects. Mussels at 22 ºC showed a separation directly related the concentration of exposure, starting at the most negative point of PCO2 with control organisms (CTL-T 22), then appearing by order each subsequent concentration, with the highest of PCO2 registered for mussels exposed to the highest concentration (C3). The mussels at control temperature (T 18), didn't show such orderly separation. At the positive side the lowest concentration (C1) was clearly separated from the remaining exposure scenarios, with a further coalescence of the control and the highest exposure condition (CTL and C3).

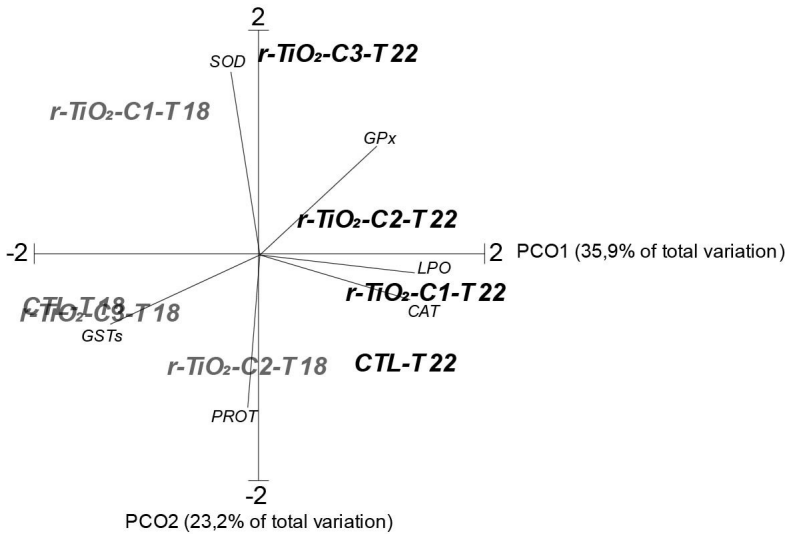

Fig. 34 – Principal component ordination analysis of biomarkers in the r-Tio2 exposure experiment at 18 ºC and 22 ºC (Black – 22 ºC; Gray – 18 ºC; CTL – control mussels; C1-C2-C3 – 5-50-100 µg L^{-1})

Discussion

TiO_2 forms have been noted as potentially harmful substances to the aquatic environment and thus have been put on the watchlist of the scientific community. From the prior study it has been noted major differences between the most commonly available powders of a singular phase, a-TiO_2 and r-TiO_2, while their different toxicity has been mostly due to their inherent characteristics (notably size). Furthermore, despite the damage found on mussels being rather low with little to no mortality and mostly minor shifts in biochemical performance, this later study aims to obtain further insights on toxicity changes related to climate change.

In terms of climate change, temperature shifts are some of the foremost studies related to marine species (MaMahon and Ussary 1995; Anestis et al. 2007; Anestis et al. 2010). Furthermore, not only have these studies indicated temperature as one of the stressors for biochemical alterations, with a rather varied defense mechanisms being employed by different species and conditions, but that this abiotic change can also have a more widespread effect. Especially when considering the contaminants, temperature may have a crucial role, either by affecting its availability or even through behavioral changes of the organisms, and thus the focus of these interactive effects on current research (Pfeifer et al. 2005; Verlecar et al. 2007; Izagirre et al. 2014; Kamel et al. 2014).

As such, present study picked up on the studied TiO_2 form which has shown the lowest potentially threating effect on the marine environment, rutile, and changed the environmental factors, considering the predicted increase of 4 ºC by IPCC 2014, to test for potentially interactive effects which could enhance its toxicity.

Similar to the previous study, the results regarding of the behavior of rutile in water were impossible to obtain, as both quantification and characterization of particles once entering the aquaria couldn't be done by any of the available techniques. Aeration of the aquaria may act as mixing agent, keeping some particles suspended in the aquaria, however, due to the medium conditions, saltwater, particles will more easily tend to cluster and deposit (Ciacci et al. 2012; Baker et al. 2014) and thus the available fraction to be ingested by the organisms may be lowered (Ward and Kach 2009). Mussels were also noted to easily depurate contaminants (Wang and Fisher 1997; Yap et al. 2003; Yap et al. 2004) and thus from a combination of these effects it can be then explained the rather residual Ti concentration in tissues. Only at the highest contaminated scenario (C3) it was noted an increase of Ti content, which is congruent with most of the previous studies with Ti^{4+}/TiO_2 exposure (3.3.1., 3.3.2., 3.3.4.), but it should be noticed a lower bioaccumulation at C3-22 ºC when compared to C3-18 ºC, which is most likely attributed to behavioral changes of the organisms due to temperature increase.

From the obtained PCO (Fig. 44), it was clearly noticed that biochemical performance helped in the separation of the studied conditions into two major groups, related to the control temperature (18 ºC) and the warming conditions (22 ºC). Furthermore, contaminant concentration was also found to be a well separated within their own conditions (temperature), which further denotes the importance of the dosage effect in terms of the organisms' response. As such, it's imperative to associate the Ti bioaccumulation of the mussels to the biochemical activity of the different measured parameters, even though the residual amount of Ti was only significantly higher at C3 (both temperatures). It has already been proposed, and accepted (Monteiro et al. 2019a; Monteiro et al. 2019b), that despite only being measured the residual amounts of Ti in tissues, mussels should pass

through a cycle of accumulation and depuration which will ultimately affect their biochemical performance, at different levels depending on the initial Ti concentration of exposure. Moreover, temperature effects on the bioaccumulation of contaminants, such as observed in this work, have also been reported with mixed results. Some reports, for the same mussel species at comparable temperatures, noted increasing accumulation with increasing temperature (Attig et al. 2014; Coppola et al. 2018a), in the case of exposure to Ni and As, while it was also reported no impact at on cadmium bioaccumulation (Nardi et al. 2017).

The contaminant uptake and the metabolism as often been interlinked when studying cases of oxidative stress, as stressors may induce alterations on the respiratory and feeding behavior of the organisms (Gosling 2003; Anestis et al. 2007). In this study, it has been noted that at control temperature (18 ºC) no metabolic change was noticeable despite the incremental addition of rutile while mussels in warming scenarios (22 ºC) increased their metabolic rates in lower contaminated scenarios (C1 and C2). From the control temperature results it should be noticed the low toxic effect of rutile, as either it could be considered non-toxic or that the organisms effectively defended without much impact on its normal metabolic activity. However, in warming conditions, it could be derived that either the toxicity of the material or the sensitivity of the organism is increased, under rather limiting conditions. As no metabolic changes were found on control organisms, between both temperatures, it could be inferred that increased toxicity under increased temperature was the more likely effect. Thus, at lower concentrations the organisms were able to activate their metabolism to activate their defensive mechanisms, ability which was suppressed as the rutile concentrations was increased. Metabolic suppression due to contaminant concentration is a well-documented effect in mussels, already reported for Ti^{4+} exposure in prior works (Monteiro et al. 2019b) as well as other metals (Henriques et al. 2019; Pinto et al. 2019).

The energetic balance of mussels in this work showed that the organisms were able to maintain or even increase their main energy source (GLY), even at the higher contamination conditions. This was noticeable at both temperatures, at which even at CTL-22 ºC the GLY content significantly increased, denoting the importance of temperature as a stressor. However it is at the highest concentrations in warming condition (C3-22 ºC) which shows the greatest GLY content, probably a result of lowering the metabolic activity to prevent potential damage (Gosling 2003; Anestis et al. 2007). Nonetheless, PROT content showed the exact reverse behavior, with lower concentrations at contaminated conditions with the lowest PROT content being reported at C3-22 ºC. These results, combined with the increased GLY content, may denote a primary use of proteins as energy source and as such the total protein content was lowered at increasingly stressful conditions. However, it should be noted that enzymes, as they are proteins, are also accounted in this parameter which could also indicate that enzymes are being used to counteract potentially oxidative stress situations, while such stress should not be enough to deplete the currently produced defenses and induce the formation of new enzymes. This status of near maintenance of energetic status as been reported in the literature, however it is often associated with a decrease in metabolic activity to keep such energy-activity balance. In the case of Ti^{4+} exposure such effects have already been previously discussed (Monteiro et al. 2019a; Monteiro et al. 2019b), with the reported increase in GLY content, while other studies with bivalves noticed similar effects, increase GLY content in *Mytilus galloprovincialis* due to As exposure (Coppola et al. 2018a) or *Macoma balthica* due to Cd exposure (Duquesne et al. 2004), decrease PROT content due to carbon nanotubes exposure (Marchi et al. 2018a; Marchi et al. 2019).

Activation of oxidative stress defensive mechanisms is the natural reaction of organisms to response to stress situations, such as warming and contaminants, which produces ROS (Regoli and Giuliani 2014), and since it was noticed variations at both metabolic and energetic status it was expected to also observe variations of the enzymatic activities which compose such complex mechanism. However primary response enzymes, such as CAT, SOD and GPx, were noticeable non responsive at control temperature (18 ºC) which further supports that all tested concentrations of rutile have little to no toxic effect. It should however be noticed that both at C2 and C3 SOD response was suppressed, effect which was also previously evidenced and already in prior studies with Ti(IV) contaminants (Girardello et al. 2016; Huang et al. 2018b; Monteiro et al. 2019a). This non-responsiveness of the antioxidant enzymes could however be related to the response of an increased detoxification capabilities through the activation of GSTs, which is observed at C3, and henceforth further supporting its importance at lower temperatures. This combination of biochemical parameters, non-responsiveness of CAT and SOD and increase in GSTs at control temperatures, has been previously reported by Coppola et al. (2018a) in their study with mussels exposed to As and Ale et al. (2019) in the case of mussels exposed to silver nanoparticles. In warming conditions antioxidant defense mechanisms change rather significantly, with CAT being inhibited along the concentration gradient, SOD presenting the exact opposite effect while GPx maintain its non-responsiveness. Furthermore GSTs activity was also noticeably different at 22 ºC, reporting values near baseline on all conditions, which is uncharacteristic taking into account the information from the literature which generally reports increased activity with increased concentration (Matozzo et al. 2013; Boukadida et al. 2017). As such, in this case SOD may has been the principal defense mechanisms of protection, clearly being responsible for the degradation of ROS generated by the contaminant, while such increase in activity is in-line with other works such as Matozzo et al. (2013) Pirone et al. (2019).

At the biochemical level no significant cellular damage as lipidic peroxidation (LPO) was found. At control temperature LPO levels are near baseline in all exposure concentrations which denotes that the oxidative stress defenses help up, in this case most likely through the activation of GSTs. However, in warming conditions it was noted that LPO values spiked, and thus temperature may have been the major stressor towards the organisms during this experiment. Furthermore, in high temperature conditions the presence of r-TiO_2 tendentially decrease the LPO in mussels, which is contradictory to most reported effects (Freitas et al. 2017; Coppola et al. 2018a). SOD activation due to the presence of contaminant after its inhibition due to temperature, as shown by Parry and Pipe (2004), may be the only explanation for the observed results.

Neurotoxicity, through acetylcholinesterase activation, as been previously reported in previous work. At 18 ºC the increase in AChE which was also observed in works such as Xia et al. (2017) and Pan et al. (2012) has been attributed to an attempt to reduce neurotransmitter excess in synaptic clefts (Rosa et al. 2016). Other explanation, despite not related to mussel cells, have noted the increase in AChE levels in cases of cell apoptosis (Zhang et al. 2002). In increased temperature scenarios it was noted an increase in AChE baseline activity, which may have then superimposed to the dosage effects, and thus not being any variation between contamination scenarios.

In conclusion, rutile powder, the least toxic of the single phase commercially available powders, was noted to have nearly no toxicity even in warming conditions at present tested conditions. Especially in warming conditions, the dual effect of reducing metabolic activity when exposed to TiO_2, allied to the increased antioxidant activity (highlight for SOD), was clearly sufficient to avoid increased damage. Overall, damage was rather limited and as in the previous cases it should be recoverable if organisms return to the adequate environment.

Temperature rise however noticeably increased the damage markers baseline and the presence of extraneous substances increased the mussels' metabolism, indicative the mussels facing stressful situations which could be detrimental if prolonged. According to Menge et al. (2008) modulations, warmer temperatures may induce increased reproduction and growth, however Petes et al. (2007) pointed that thermotolerance was a critical factor on biodiversity of coastal areas. Furthermore, it has already been reported that in some areas mussels have been reaching their upper thermal tolerance limits (Michaelidis et al. 2014). Thus, despite in present study reporting a sub-lethal toxic effect under warming conditions, warming scenarios are still of concern.

3.3.6. Multi-parameter exposure assay

Objective

This experiment aimed to assess the toxic potential Ti(IV) (Ti^{4+} and $nTiO_2$) under environmental change conditions observed in transitional systems. The mussel *M. galloprovincialis* and clam *R. philippinarum* were used as a bioindicators and average environmental conditions (temperature and pH levels) of the Ria de Aveiro Lagoon (bivalves sampling area) were taken as baseline condition.

Prior notice

Mussels (*M. galloprovincialis*) biochemical assays reported values, on the control group, with variability exceeding the quality parameters (CV > 50 %) and as such they've been excluded.

Ti quantification in clams' (*R. philippinarum*) tissues, at date of submission, was still pending due to equipment (ICO-OES) malfunction and maintenance. Results will be acquired later to complete the work.

Results and Discussion

Quantification of Ti in water samples

In this experiment aquaria were contaminated with two different Ti(IV) species, the Ti^{4+} standard, used in the earlier works (3.3.1. and 3.3.2.), as well as the $nTiO_2$, one of the most widely used TiO_2 nano-powders composed by both anatase and rutile forms (as determined in 3.2.). The initial concentrations chosen for this experiment was the 100 $\mu g\ L^{-1}$, which translates the most severe contamination exposure studied. These values were also near the range of predicted $nTiO_2$ input levels in the aquatic medium (Gottschalk et al. 2015), as well as reported levels found in water effluents from wastewater treatment plants and receiving waters for $nTiO_2$ (Westerhoff et al. 2011; Shi et al. 2016).

In Table 19 it's presented the concentrations of Ti in soluble fraction following Ti(IV) spiking in the aquaria. Similarly to the reported in the previous works, when spiking with Ti^{4+} the results report significant decreases to the intended nominal concentration (Monteiro et al. 2019a; Monteiro et al. 2019b). In the case of $nTiO_2$ it was impossible to obtain proper determination, as analogous to the works with anatase and rutile powders (3.3.4 and 3.3.5), the available methodology wouldn't allow for proper determination through digestion and further analysis by ICP-OES.

In relation to the control group (Sal 30 PSU, pH 8.0, T 17 ºC), it was noticeable decrease in the concentrations of Ti in the dissolved fraction immediately following the spiking of the aquaria. Furthermore, such decrease was significant when considering the increasing salinity or decreasing pH, which denotes an influence of the physicochemical characteristics of the medium on Ti(IV) dynamics. The increasing salinity was noticed to gradually decrease the Ti concentration in water, which is supported by literature information (French et al. 2009; Keller et al. 2010; Lv et al. 2016) for $nTiO_2$. Due to titanium inherent low solubility in seawater (Orians et al. 1990), the narrow variation of working parameters of temperature (18 ºC to 21 ºC) and pH (8.0 to 7.6) weren't expected to significantly affect these measurements. Such inference was confirmed in the case of temperature

increase, in which no significant difference was found to control environmental condition. Furthermore, Lv et al. (2016) reported only a maximum of 16 % increase in sedimentation following a 25 ºC temperature increase. In the case of pH, acidification may lead to increased agglomeration as it brings closer to its isoelectric point range (Gumy et al. 2006; Miller et al. 2010), however such slight decrease may have no significant impact on TiO_2 stability (Dunphy Guzman et al. 2006). The decrease in concentration following spiking were thus more likely due to a forceful oxygenation when filling and homogenizing the aquaria of greater volume (15 L), which unintentionally increased the rate of TiO_2 formation as previously observed in (3.1.2.).

Table 19 – Titanium concentrations in water samples of exposure assay 5

Condition	**Ti^{4+} in Water ($\mu g\ L^{-1}$)**			**$nTiO_2$ in Water ($\mu g\ L^{-1}$)**		
	t0	72 h	7 days	t0	72 h	7 days
CTL (all conditions)	< 2	< 2	< 2	-	-	-
Sal 20	89 ± 12 [a]	< 2	< 2			
Sal 30-pH 8.0-T 18	81 ± 9 [a, b]	< 2	< 2		N.A.	
Sal 40	68 ± 4 [c]	< 2	< 2			
pH 7.6	74 ± 5 [c]	< 2	< 2		N.A.	
T 21	78 ± 7 [b, c]	< 2	< 2			

N.A. – Not Available; Letters represent statistical difference (p = 0.05)

Sal 30-pH 8.0-T 18 is the control environmental conditions

Following 72 hours, no Ti was found (< 2 $\mu g\ L^{-1}$) in the soluble faction, in the case of Ti^{4+} spiking, for any of the studied conditions. This once again denoted its short-permanence time of in solution as it is quickly converted into the insoluble oxide and it aggregates and sediments. In the case of $nTiO_2$, as it is introduced in the aquaria only aggregation and sedimentation should be taking effect and thus its residence time may be considerably shorter. In the work of Lv et al. (2016), it was shown a considerable decrease of $nTiO_2$ in seawater within 7 h, while Morelli et al. (2018) noted a permanence time up to 48 hours (90 % removal which was the technical measurement limit). However, in both cases concentrations of $nTiO_2$ were of a different magnitude (10 $mg\ L^{-1}$) which has been noted to heavily impact its aggregation dynamics (Keller et al. 2010). Furthermore, measurements were non-quantitative, using DLS to indirectly follow the particle aggregates in relation to t_0 measurement, thus introducing a certain measurement bias.

Effect of salinity

Aquatic organisms present in the transitional systems, such as clams and mussels, experience a cyclic change on the conditions, due to tides, and even periodic alterations, rainy seasons (winter) (Elliott and Quintino 2007; Dauvin and Ruellet 2009), and thus have adapted to possess a greater resiliency to these variations (Elliott and Quintino 2019). However, salinity shifts have been evidenced as an important stressor for marine biota (McLeod and Wing 2008; Carregosa et al. 2014), and in many cases it can be the source of the malformations or even increased mortality (Coughlan et al. 2009; Munari et al. 2011; Verdelhos et al. 2015).

Contamination of the marine systems under salinity shifts poses a difficult challenge to researchers, as there are considerations on the overall effects brought by the changes on the physicochemical parameters of the medium. As a primary effect, it should be considered the responses of the organisms to changes in the medium such as the increase or decrease of salinity (Roy et al. 2007; Hamer et al. 2008; Coughlan et al. 2009). Bivalves' health tends to vary in response to salinity shifts, however biochemical studies have further identified situations of increased vulnerability when other stressors, such as contaminants, are involved (Carregosa et al. 2014; Moreira et al. 2016; Marchi et al. 2018b). Furthermore, metals and nanoparticles also interact with the medium differently depending on the ionic strength, which tends to increase the precipitation and agglomeration rate (French et al. 2009; Wang et al. 2014) and thus making them more bioavailable through the filtration (Ward and Kach 2009). As such, the former interactions between the changes in the marine systems and its repercussions on the biological response of the organisms as well the chemical alterations of the contaminants, results on peculiar iterative effects when studying the biochemical performance under both stressors. For example, on *M. galloprovincialis*, *R. philippinarum* and *C. angulata*, these iterative effects have been under the scope of the scientific community for a wide array of contaminants (e.g. arsenic, carbon nanotubes, drugs) (Correia et al. 2016; Moreira et al. 2016; Marchi et al. 2018b; Freitas et al. 2019). However, when considering Ti(IV) as a potential contaminant, no studies were found evaluating its toxicity in salinity shift scenarios.

In the present work, the biochemical performance of *R. philippinarum* was tested under three different salinities (20,30 and 40 PSU) for two Ti(IV) contaminants (Ti^{4+} and $nTiO_2$), with complete results being presented on Table 20. The PCO analysis (Fig. 35) reveals the relationship between the evaluated biochemical results under the studied factors (type of contaminant and salinity). Its observable that the graphical representation explains 83.5 % of the total variation, and the analyzed biochemical parameters presented as vectors ($r > 0.75$), denoted their importance in the separation of the different conditions.

PCO1 explained 74.4 % of the total variation, separating salinity conditions along the horizontal axis, with clams exposed to the highest salinity (40 PSU) at the positive side and clams exposed to the lowest salinity (20 PSU) grouped at the negative side, while clams at control salinity (30 PSU) were presented in the middle of the axis. ETS, GSH/GSSG ratio, GSTs and SOD presented high correlation with clams exposed to the lowest salinity ($r = 0.91$, 0.88, 0.95 and 0.94, respectively) while PROT and LPO were highly correlated with clams exposed to the highest salinity level ($r = 0.88$ and 0.76, respectively).

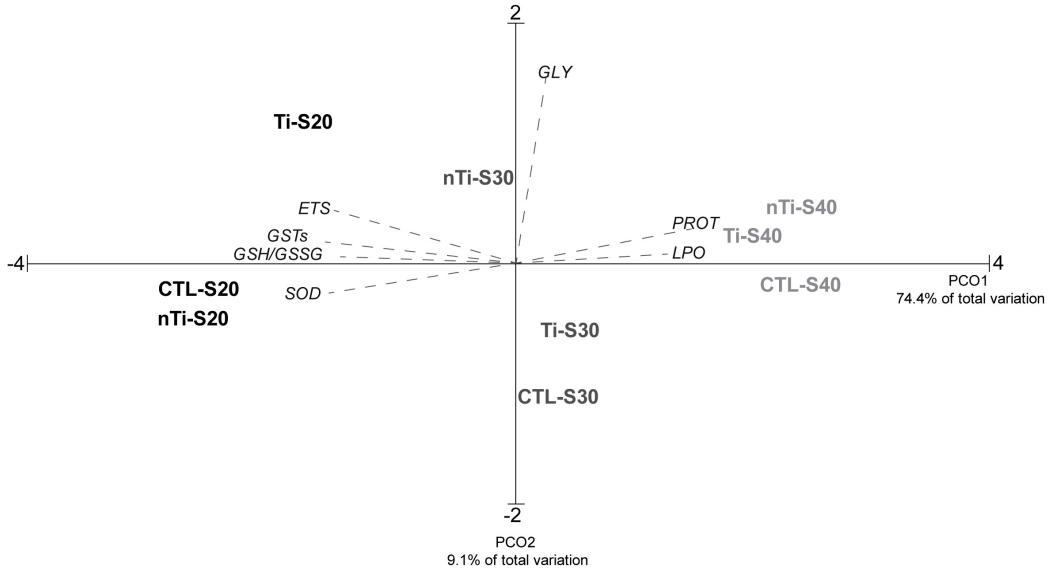

Fig. 35 – Principal component ordination analysis of biomarkers for the salinity shift experiment

(Control organisms (CTL), Ti^{4+} exposed organisms (Ti) and $nTiO_2$ exposed organisms (nTi) at salinity 20 PSU (Black), 30 PSU (Dark Grey) and 40 PSU (Light Grey))

High metabolic capacity observed in clams exposed to the lowest salinity can be attributed to the higher energy demands for the activation of the defense mechanisms (Choi et al. 2001), as seen also by the higher correlation to both SOD and GSTs enzymes. High metabolic activity has been noted to increase ROS production as byproduct of mitochondrial activity which could in turn increase LPO levels (Gibbin et al. 2017). However, clams in the low salinity environment shown no significant damage (LPO), which was attributed to the activation of antioxidant defenses (higher activity of both SOD and GSTs), and thus maintaining intracellular reductive status (high GSH/GSSG ratio). Similar situations in which SOD increased at lower salinities have been reported, for *C. sinensis*, *M. modiolus*, *V. corrugata*, *R. decussatus* and *R. philippinarum* (Li et al. 2012; Carregosa et al. 2014; Zhan et al. 2018). Furthermore, Carregosa et al. (2014) also observed that *R decussatus* and *R. philippinarum* increased GSTs enzymatic activity when salinity was lowered from 28 to 21 PSU and 28 to 14 PSU, for each species respectively.

At high salinity conditions (40 PSU), independently on the contaminant presence or type, high LPO levels were observed highlighting the negative impacts of salinity towards clams' cells membranes. Similar effects have been observed in different bivalves, such as *R. philippinarum* and *R. decussatus* (28 to 35 PSU) (Velez et al. 2016a) and *M galloprovincialis* (25 to 35 PSU) (Freitas et al. 2019). It was also observed by van der Gaag (2016) that salinity increase was a major stress driver for mussels, in which at 40 PSU *D. polymorpha* and *M. leucophaeata* suffered 100 % mortality within 14 days. High PROT content that characterizes clams under the highest salinity condition may reveal tendencies in the utilization of energy reserves. In the work of Velez et al. (2016b), *R. philippinarum* was noticed to used different energy sources depending on salinity levels, favoring protein at lower salinity and lipids at higher salinities. Furthermore, the lower metabolic activity (ETS) may lead to a decrease in energy consumption and therefore an increase of potential energy reserves such as PROT, also observed by Velez et al. (2016b). This effect has been formerly noted by Kim et al. (2001) through the reduction of oxygen consumption, in which it was proposed that outside optimum salinity conditions mussels tended to remained closed for longer periods.

The vertical axis, PCO2, had a much lower representativeness explaining solely 9.1 % of total variation, with high correlation towards GLY content towards the positive side. Despite its lack of expression, it still allowed for some resolution related to contaminant exposure, in all but the highest salinity conditions.

At lower salinity (20 PSU), it was clearly demarked a higher GLY content in clams exposed to Ti^{4+}, yet, at control salinity (30 PSU) the highest energy reserves were in turn related to $nTiO_2$ exposed clams. Monteiro et al. (2019a) had already identified increased GLY content under Ti^{4+} exposure, which has been attributed to a defensive mechanisms to avoid the intake of foreign substances. Under lowered salinity, the decrease in ionic strength may have led to an increased availability of Ti^{4+}, has evidenced by the water measurements following contaminant spike, thus revealing this demarcating feature. However, at 30 PSU the increased ionic strength may have a greater influence of $nTiO_2$ aggregation, increasing the aggregates size (Keller et al. 2010) as well as its bioavailability (Ward and Kach 2009), and thus the self-defense tactics may be more biased now towards the nanoparticles.

Everything considered, salinity was shown to be the main driver for the biochemical response alterations, with either contaminant playing only a minor role in the reported responses. Furthermore, the reported results point to a nearly inconsequent effect of Ti(IV) towards organisms in highly dynamic systems, such as estuaries. As the contaminant concentrations used in this work were within the levels reported as being discharged into the environment, it stands that, currently, $nTiO_2$ may not play a major role as a contaminant in transitional waters.

Table 20 – Biochemical parameters in clams in the experiment of exposure to Ti^{4+} and $nTiO_2$ at different salinities

Condition		GLY mg g^{-1} FW	PROT mg g^{-1} FW	LPO mmol MDA g^{-1} FW	ETS nmol min^{-1} g^{-1} FW	SOD U g^{-1} FW	GSTs U g^{-1} FW	GSH/GSSG -
CTL	Sal 20	2.42 ± 0.53 (a; A)	6.06 ± 0.70 (a; A)	6.09 ± 0.68 (a; A)	67.09 ± 12.97 (a; A)	1.92 ± 0.60 (a; A)	0.35 ± 0.04 (a; A)	5.43 ± 0.79 (a; A)
	Sal 30	1.94 ± 0.81 (a; A)	6.65 ± 1.29 (a; A)	8.42 ± 0.84 (b; A)	22.99 ± 8.27 (b; A,B)	1.07 ± 0.35 (b; A)	0.23 ± 0.04 (b; A)	3.55 ± 0.84 (b; A)
	Sal 40	2.31 ± 0.79 (a; A)	12.43 ± 2.13 (b; A)	9.68 ± 1.98 (b; A)	17.79 ± 6.51 (b; A)	0.09 ± 0.05 (c; A)	0.22 ± 0.03 (b; A)	3.08 ± 0.95 (b; A)
Ti	Sal 20	4.04 ± 1.05 (a; B)	7.56 ± 1.53 (a; A)	8.38 ± 0.70 (a; A)	65.13 ± 15.45 (a; A)	1.56 ± 0.20 (a; A)	0.37 ± 0.03 (a; A)	5.17 ± 0.63 (a; A)
	Sal 30	2.64 ± 1.19 (a; A, B)	8.31 ± 1.03 (a; B)	8.26 ± 0.51 (a; A)	14.60 ± 5.27 (b; A)	0.99 ± 0.24 (b; A)	0.25 ± 0.04 (b; A)	4.36 ± 0.69 (a, b; A, B)
	Sal 40	3.31 ± 0.87 (a; A, B)	11.25 ± 1.79 (b; A)	7.71 ± 1.20 (a; A)	14.97 ± 2.00 (b; A)	0.12 ± 0.04 (c; A)	0.17 ± 0.03 (c; B)	3.69 ± 0.64 (b; A)
nTi	Sal 20	2.04 ± 0.52 (a; A)	6.02 ± 0.94 (a; A)	7.04 ± 0.79 (a; A)	53.10 ± 11.22 (a; A)	1.51 ± 0.34 (a; A)	0.37 ± 0.03 (a; A)	7.66 ± 0.39 (a; B)
	Sal 30	4.11 ± 2.01 (a; B)	6.51 ± 1.17 (a; A)	7.20 ± 0.64 (a; B)	32.48 ± 11.75 (b; B)	0.54 ± 0.15 (b; B)	0.27 ± 0.05 (b; A)	5.25 ± 1.00 (b; B)
	Sal 40	3.67 ± 1.11 (a; B)	11.11 ± 0.95 (b; A)	9.18 ± 2.29 (a; A)	15.41 ± 4.83 (c; A)	0.18 ± 0.05 (c; B)	0.16 ± 0.03 (c; B)	3.15 ± 0.84 (c; A)

Lower-case letters indicate difference between salinity within the same exposure conditions (p = 0.05)

Upper-case letters indicate differences between exposure conditions for the same salinity (p = 0.05)

Effect of pH

Lowering the pH of seawater has a widespread impact on the physicochemical characteristics of seawater, especially when considering the chemical equilibriums of the carbonate system (Marsh 2008). In the present assay it was studied a decrease of 0.4 units of pH in relation to control conditions (8.0). From the analysis of Table 21, it was identified that under the increased CO_2 pressure the carbonate system was deeply altered, mainly when considering the differences of the available carbonate (CO_3^{2-}). Moreover, this reduction of the dissolved carbonate, mostly converted in hydrogencarbonate (HCO_3^-), proves to be a significant issue as it was accompanied by a reduction of the saturation. In the case of both calcium carbonate polymorphs (calcite – Ca and aragonite – Ar) the saturation rates decreased by over 50 %, with only calcite maintaining a value over 1. According to Marsh (2008), aragonite will tendentially dissolve and the precipitation of calcite will be impaired, and thus organism which have shells or exoskeletons may be potentially harmed (Ries et al. 2009; Hendriks et al. 2010; Barker and Ridgewll 2012). The clam *R. philippinarum* has been noted to have shells mainly made of aragonite polymorph (Kim et al. 2015) and thus its vulnerability towards this new lower pH environment may induce further stress to its survival

Table 21 – Carbonate system parameters of the aquaria seawater

Condition	T ºC	pH	TA µmol kg^{-1}	pCO_2 µatm	$[HCO_3^-]$ µmol kg^{-1}	$[CO_3^{2-}]$ µmol kg^{-1}	Ω_{Ca} -	Ω_{Ar} -
CTL	16.5	7.6	2405 ± 138	1959 ± 304	2295 ± 141	45.7 ± 4.1	1.1 ± 0.1	0.7 ± 0.1
Ti	16.6	7.6	2369 ± 145	1746 ± 275	2246 ± 138	52.3 ± 5.3	1.3 ± 0.1	0.8 ± 0.1
$nTiO_2$	16.4	7.6	2511 ± 195	1734 ± 271	2385 ± 201	52.3 ± 5.2	1.3 ±0.1	0.8 ± 0.1
S 30 – pH 8.0*	16.5	8.0	2428 ± 74	690 ± 21	2154 ± 66	113 ± 4	2.8 ± 0.1	1.8 ± 0.1

TA – Total Alkalinity; pCO_2 – partial pressure of CO_2; $\Omega_{Ar/Ca}$ – aragonite/calcite saturation states
CO_2 constant, Mehrback et al. (1973) refit by Dickson and Millero (1987)
$KHSO_4$ system, Dickson (1990); Total Boron Lee et al. (2010)
* modeled result for the control condition (salinity 30 PSU and pH 8.0)

Changes brought by acidification also produce variations in other substances present in medium, many of anthropogenic source such as contaminants. Metal based contaminants, for example, have been noted to be increasingly soluble at lower pH levels (de Orte et al. 2014; Ivanina and Sokolova 2015) while variations to the nanoparticle aggregation have also been observed in different metal based nanoparticles (Kadar et al. 2010; Xia et al. 2018). These changes in the aggregation are of utmost importance, has it has already been noted that larger aggregates may lead to increased bioavailability (Ward and Kach 2009) and henceforth potentially more damage towards the organisms.

Acidification effects on marine biota is a theme widely explored by researchers, both considering the lower pH as a single stressors (Cummings et al. 2011; Zhao et al. 2017; Melzner et al. 2020) or when considering the iterative effect with pollutants (Munari et al. 2018; Nardi et al. 2018; Shi et al. 2019). Specifically, for the case of $nTiO_2$, some reports have been trying to reach a consensus regarding how its presence in the coastal/estuarine areas under ocean acidification stress have affected the organisms. Xia et al. (2018) has noticed an increased accumulation of Ti and oxidative stress in microalgae (*C. vulgaris*) under low pH, while Hu et al. (2017) and Huang et al.

(2018b) also observed cumulative damage towards mussels (*M. Coruscus*). Recently, Kong et al. (2019)observed increasing impairment of digestive enzymes of *M. Coruscus* effects of acidification and $nTiO_2$ exposure on the.

This iterative effect of $nTiO_2$ under low pH conditions however it is not a unique addition of two unrelated stressors towards marine organisms. $nTiO_2$ isoelectric point has been explored in the literature and most common $nTiO_2$ powders report values of c.a. 6 (Keller et al. 2010; Honda et al. 2014) while a more exhaustive study reported values for commercially available nanopowders between 3 and 7.5 (Gumy et al. 2006). These values directly report to charge status of the nanoparticles in the medium, being neutral at pH of similar values of the isoelectric point. At this status the electrostatic repulsions between nanoparticles are at their minimum and thus coalescence is facilitated. As such, as pH of seawater is lowered $nTiO_2$ will get closer to its isoelectric point, leading to the formation of bigger aggregates and thus potentially increasing its bioavailability and toxicity.

In the present study, both Ti^{4+} and Degussa P-25 $nTiO_2$ were used as contaminant stressors in combination with a pH decrease of 0.4, intending to assess how the biochemical fitness parameters varied. Table 22 presents the bulk obtained data and its significant differences, while the PCO analysis (Fig. 36) was used to better understand and correlate the meaningful parameters (represented as vectors in the image).

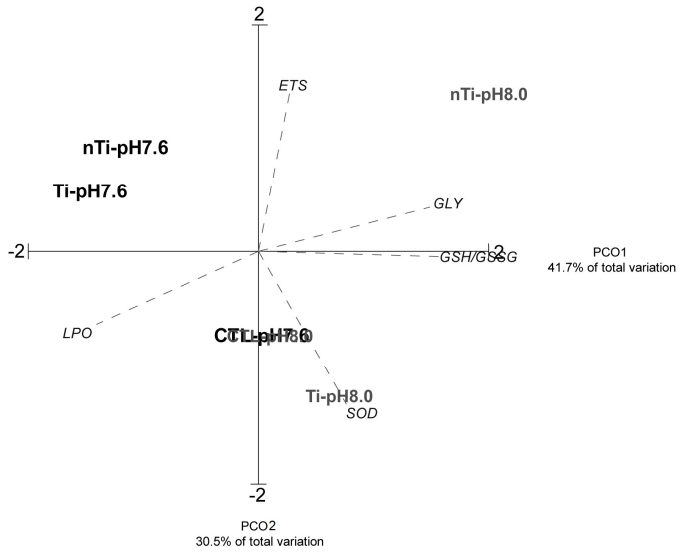

Fig. 36 – Principal component ordination analysis of biomarkers for the acidification experiment

(Control organisms (CTL), Ti^{4+} exposed organisms (Ti) and $nTiO_2$ exposed organisms (nTi) at pH 7.6 (Black) and pH 8.0 (Grey))

PCO analysis shows that PCO1 represents 42% of the total variation, clearly separating contaminated organisms exposed to different pH levels, with organisms under control pH at the positive side and organisms exposed to low pH level at the negative side. Since non-contaminated organisms under both pH levels were grouped together in the central area this means that pH itself was not causing significant effects to bivalves and thus presented similar responses. There was however a synergistic effect of pH and contaminants, evidenced by the high correlation between (negative) PCO1 and LPO ($r = 0.85$), clearly revealing increased cellular damage in organisms exposed to these conditions. On the other side, high correlation was observed between

(positive) PCO1 with GLY content and GSH/GSSG levels (r = 0.89 and 98, respectively). This correlation indicates that under exposure conditions, clams showed higher energy reserves and better oxidative status at control environmental conditions. PCO2, representing 30 % of total variation, separated individual according to their metabolic rates in the positive area (r = 0.84) and SOD activity in the negative area (r = 0.80). This was of significance for clams under control environmental conditions, in higher metabolism was linked to $nTiO_2$ exposure while higher SOD activity was prominent in Ti^{4+}.

Through this statistical tool it was possible to notice that the clams of both control groups, i.e. no contamination at pH 8.0 and pH 7.6, exhibited similar biochemical performances, which was also found in similar works with *R. philippinarum* (Velez et al. 2016b; De Marchi et al. 2017).

At control environmental conditions, organisms exposed to $nTiO_2$ showed higher correlation to both ETS and GLY, which could be due to the mechanistic requirements for the antioxidant defenses (Sokolova et al. 2012). However, as shown in other studies, $nTiO_2$ was able to suppress SOD activity (Girardello et al. 2016; Huang et al. 2018b; Xia et al. 2018) which was also observed in present situation. As such other mechanisms have been activated, probably CAT and/or GPx, and, while not measured in this work, it was observed in the other studies of Ti exposure (Canesi et al. 2010; Huang et al. 2018b). In the case of Ti^{4+} exposure, in accordance to previous findings (Monteiro et al. 2019b), ETS activity was lowered after 28 days of Ti exposure, attributed to defensive response to avoid intake of extraneous substances (Gosling 2003; Anestis et al. 2007). Contrary to previous findings for mussels (Monteiro et al. 2019a; Monteiro et al. 2019b), Ti^{4+} didn't significantly reduce SOD activity of clams.

At lower pH the burden on the clams was clearly higher, with higher cellular damage (LPO) and lower redox status (GSH/GSSG) when compared to their respective counterparts at regular pH, which was also observed for *M. coruscus* (Huang et al. 2018b). Metabolic activity wasn't influenced by the presence of Ti^{4+} while it increased in the presence of $nTiO_2$, and in the literature it was found that seawater acidification and different contaminants may produce conflicting results in ETS activity of bivalves (Freitas et al. 2016; De Marchi et al. 2017). The energetic reserves were however significantly depleted under the pressure of both contamination and seawater acidification, also observed in the previously mentioned studies. The observed metabolic and energetic parameters may indicate a status of intensive energy requirements, most likely due to the activation of antioxidant defenses (Sokolova et al. 2012). However, it was noticeable an adverse effect of severe SOD suppression for both contaminants, which has been noted for TiO_2 contaminants (Girardello et al. 2016; Monteiro et al. 2019b) and it can be magnified under lower pH levels (Huang et al. 2018b). Since antioxidant defense are impaired under the cumulative effect of both stressors, the obtained higher values for damage markers can be explained, results which are also supported by other researchers for bivalves under similar situation albeit using different contaminants (Velez et al. 2016c; De Marchi et al. 2017; Huang et al. 2018b; Huang et al. 2018a).

In conclusion, seawater acidification clearly affected the interaction between the organisms and the contaminants by which their biochemical responses showed different tendencies. In either case of the climate scenarios, the impact of the contaminants on the organisms were sublethal however seawater acidification magnified the impact of both contaminants through both energetic stress and antioxidant defense suppression.

Table 22 – Biochemical parameters in clams in the experiment of exposure to Ti^{4+} and $nTiO_2$ at different pH

Condition		GLY	PROT	LPO	ETS	SOD	GSTs	GSH/GSSG
		mg g^{-1} FW	mg g^{-1} FW	mmol MDA g^{-1} FW	nmol min^{-1} g^{-1} FW	U g^{-1} FW	U g^{-1} FW	-
CTL	pH 8.0	1.94 ± 0.81 (a; A)	6.65 ± 1.29 (a; A)	8.42 ± 0.84 (a; A)	22.99 ± 8.27 (a; A, B)	1.07 ± 0.35 (a; A)	0.23 ± 0.04 (a; A)	3.55 ± 0.84 (a; A)
	pH 7.6	2.93 ± 0.77 (a; A)	9.36 ± 1.61 (b; A)	9.11 ± 1.05 (a; A)	18.59 ± 5.64 (a; A)	0.70 ± 0.13 (b; A)	0.26 ± 0.06 (a; A)	4.01 ± 0.93 (a; A)
Ti	pH 8.0	2.64 ± 1.19 (a; A, B)	8.31 ± 1.03 (a; B)	8.26 ± 0.51 (a; A)	14.60 ± 5.27 (a; A)	0.99 ± 0.24 (a; A)	0.25 ± 0.04 (a; A)	4.36 ± 0.69 (a; A, B)
	pH 7.6	1.28 ± 0.30 (b; B)	6.36 ± 0.83 (b; B)	8.91 ± 2.02 (a; A)	19.46 ± 6.29 (a; A)	0.28 ± 0.12 (b; B)	0.26 ± 0.04 (a; A)	2.80 ± 0.70 (b; B)
nTi	pH 8.0	4.11 ± 2.01 (a; B)	6.51 ± 1.17 (a; A)	7.20 ± 0.64 (a; B)	32.48 ± 11.75 (a; B)	0.54 ± 0.15 (a; B)	0.27 ± 0.05 (a; A)	5.25 ± 1.00 (a; B)
	pH 7.6	2.18 ± 0.55 (b; A)	8.66 ± 1.35 (b; A)	8.78 ± 0.98 (b; A)	32.06 ± 5.23 (a; B)	0.32 ± 0.08 (b; B)	0.23 ± 0.01 (a; A)	2.77 ± 0.35 (b; B)

Lower-case letters indicate difference between pH within the same exposure conditions (p = 0.05)

Upper-case letters indicate differences between exposure conditions for the same pH (p = 0.05)

Effect of temperature

Temperature may affect the bioavailability of metal contaminants (Ansari et al. 2004) through variations of solubility in tandem with other properties (e.g. pH and salinity). In the case of Ti the inherent low solubility may not be impacted in the narrow range of working parameters (18 ºC – 21 ºC), as study found greater correlation between Ti content in seawater and dust sources than to the abiotic parameters such has temperature (Dammshäuser et al. 2011). For $nTiO_2$, increasing temperatures (10 ºC to 65 ºC) was followed by greater agglomerate formation and sedimentation rates within the same temperature range (Lv et al. 2016). Studies with other nanoparticles indicated that lowering the viscosity of the medium, and reducing the interfacial energy barrier between particles, may be the reason of such effects (Chen et al. 2012; Yung et al. 2017).

In the case of marine biota, the bivalve community has been a staple proxy for awareness towards the negative impacts of the increasingly higher surface water temperatures. Biological alterations and oxidative responses has been used as a tool to identify the vulnerabilities onset by this stressor (Resgalla Jr et al. 2007; Anestis et al. 2010; Wang et al. 2015; Velez et al. 2017; Andrade et al. 2019b). Furthermore, under thermal stress bivalves have been noted to respond differently to contaminant stressors (Verlecar et al. 2007; Ivanina et al. 2009; Attig et al. 2014; Pirone et al. 2019), including nanoparticles (Falfushynska et al. 2015; Andrade et al. 2019a).

The present study modulates the biochemical responses of clams under a 4 ºC increase and two Ti(IV) contaminants, soluble titanium standard (Ti^{4+}) and nanopowder ($nTiO_2$), with full data presented in Table 23. Principal coordination analysis (Fig. 37) helps evidencing the trends brought by the variations in biochemical responses, correlated by the vectors representing each of the relevant parameters.

First analysis of the PCO indicates that vectors correlate c.a. 78 % of total variation, divided between the horizontal axis (PCO1) and vertical axis (PCO2) explaining, respectively, 47 % and 31 % of the total variation, clearly separating organisms under control temperature at the positive side of PCO2 from organisms in the negative side at PCO2 negative side Horizontal axis correlates better with metabolic (ETS; r = 0.91) and enzymatic (SOD and GSTs; r = 0.92 and 0.90, respectively) responses while cellular redox status (GSH/GSSG) and energy reserves (PROT) tend more for PCO 2 (r = 0.93 and 0.76, respectively).

From the perspective of the metabolic alterations, it was observed a clear distinction between control conditions in each of the temperatures. Temperature rise increased led to the increase in metabolic activity of clams, which albeit supported by some authors (Bielen et al. 2016; Solan and Whiteley 2016; Pirone et al. 2019), others point to decrease their metabolism under similar conditions (Anestis et al. 2010; Coppola et al. 2017). However, it's a common response to decrease the metabolism under both stressors (Coppola et al. 2018b; Pirone et al. 2019), concordant with the findings from this exposure assay, due to defensive mechanisms to avoid contamination by closing the valves (Gosling 2003; Anestis et al. 2007).

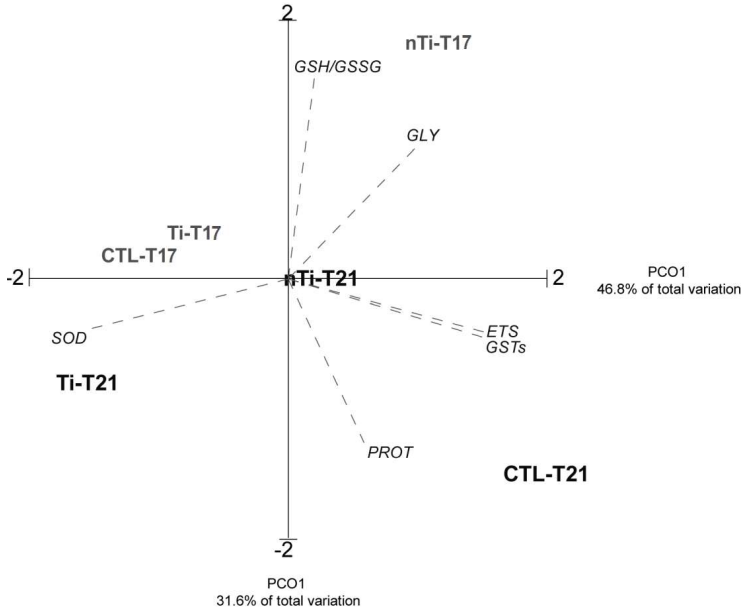

Fig. 37 – Principal component ordination analysis of biomarkers for the warming experiment (Control organisms (CTL), Ti^{4+} exposed organisms (Ti) and $nTiO_2$ exposed organisms (nTi) at 21 ºC (Black) and 18 ºC (Grey))

This metabolic adaptation appears to be a successful defense mechanism for clams, as cellular damage (LPO) remains stable in regardless of stressor, thus not appearing as a meaningful vector for PCO, while redox status of the cell (GSH/GSSG) remains at baseline levels in most conditions. This is contrary to most of the work presented in the literature, in which LPO generally increases and there's an increase in the oxidative state of the cell (Attig et al. 2014; Velez et al. 2017). Nonetheless, the short-lived permanence time of Ti(IV) contaminants tendentially may reduce its toxicity, while in cases of increased temperature they may sediment faster and the instilled metabolic reduction may prevent the natural production of ROS by the mitochondria (Gibbin et al. 2017), henceforth reducing the overall impact on the internal redox system. The only unexplainable result demarks the separation of the exposure to $nTiO_2$ under control temperature, condition at which organisms show greater energy reserves (GLY) and GSH/GSSG without any variation to their metabolism and even showing the characteristic SOD inhibition (Girardello et al. 2016; Huang et al. 2018b; Xia et al. 2018).

Enzyme activity of both GSTs and SOD showed clear difference when exposed to each of the stressors or a combination of the two. The biotransformantion enzyme (GSTs) was kept at basal levels at control condition regardless of contaminant, contrary to mussel responses found on mussels in previous works (Monteiro et al. 2019b; Monteiro et al. 2019a) however similar situations were also found by other authors (Benedetti et al. 2016; Almeida et al. 2017). In the case of warming scenario, the clams under no contamination responded to the thermal stimulus by increasing the GSTs activity, as also reported by Matozzo et al. (2013) and Pirone et al. (2019), most likely in to counteract the effects of ROS release due to increased metabolism. Furthermore, in the work of Pirone et al. (2019) further replicated the trend of reducing GSTs activity under the action of both stressors (temperature and different drugs as contaminants). In the case of SOD, at control temperature its activity was tendentially lower in contaminated conditions, and while it wasn't significantly lower as in the case study with Ti^{4+} and mussels (Monteiro et al. 2019b; Monteiro et al. 2019a), the effects of $nTiO_2$ has shown similar suppressive effects as already reported in other works (Girardello et al.

2016; Huang et al. 2018b; Xia et al. 2018). When considering the situation of increased temperature, control organisms shown lower activities of this enzyme, in accordance to previous findings of Coppola et al. (2018b). Combined effect of both stressors revealed contrasting effects, with Ti^{4+} showing increased SOD activity and henceforth having a placement highly correlated with SOD vector, and $nTiO_2$ reporting near basal values. SOD inactivation under contamination due to the presence of $nTiO_2$ may be related to a magnification of their suppressive effect on this enzyme, moreover, some nanoparticles in relevant environmental concentration, both on regular and increased temperatures, have been demonstrated to have no effect on SOD activation (Marchi et al. 2018a; Andrade et al. 2019a). However, in cases of exposure to metal ions, the activation of SOD under thermal pressure is a well-documented event (Coppola et al. 2017; Coppola et al. 2018a; Pirone et al. 2019).

Overall, warming appears to have a deeper impact on the biological activity of the organisms and thus leading to different interactions with the contaminants. Particularly the metabolic increase under thermal stress may lead to cellular redox imbalance, through ROS generation through the mitochondria, while contaminants may counteract such effects by inducing slower metabolism. Particularly in Ti(IV) contaminants, due to their low residence time and thus potentially lower toxicity, their ocasional presence may thus help protecting the organisms.

Table 23 – Biochemical parameters in clams of exposure to Ti^{4+} and $nTiO_2$ at different temperatures

Condition		GLY mg g^{-1} FW	PROT mg g^{-1} FW	LPO mmol MDA g^{-1} FW	ETS nmol min^{-1} g^{-1} FW	SOD U g^{-1} FW	GSTs U g^{-1} FW	GSH/GSSG -
CTL	T 18	1,94 ± 0,81 (a; A)	6,65 ± 1,29 (a; A)	8,42 ± 0,84 (a; A)	22,99 ± 8,27 (a; A,B)	1,07 ± 0,35 (a; A)	0,23 ± 0,04 (a; A)	3,55 ± 0,84 (a; A)
	T 21	2,63 ± 0,67 (a; A, B)	9,27 ± 1,27 (b; A)	7,39 ± 0,53 (b; A)	44,39 ± 14,02 (b; A)	0,5 ± 0,14 (b; A)	0,31 ± 0,05 (b; A)	2,70 ± 0,55 (a; A)
Ti	T 18	2,64 ± 1,19 (a; A, B)	8,31 ± 1,03 (a; B)	8,26 ± 0,51 (a; A)	14,60 ± 5,27 (a; A)	0,99 ± 0,24 (a; A)	0,25 ± 0,04 (a; A)	4,36 ± 0,69 (a; A, B)
	T 21	2,12 ± 0,65 (a; B)	7,63 ± 0,88 (a; B)	7,28 ± 1,01 (a; A)	18,79 ± 4,43 (a; B)	1,57 ± 0,43 (b; B)	0,23 ± 0,02 (a; B)	2,81 ± 0,66 (b; A)
nTi	T 18	4,11 ± 2,01 (a; B)	6,51 ± 1,17 (a; A)	7,20 ± 0,64 (a; B)	32,48 ± 11,75 (a; B)	0,54 ± 0,15 (a; B)	0,27 ± 0,05 (a; A)	5,25 ± 1.00 (a; B)
	T 21	3,42 ± 0,61 (a; A)	7,57 ± 0,63 (a; B)	8,07 ± 0,8 (a; A)	28,64 ± 5,45 (a; C)	0,68 ± 0,18 (a; A)	0,24 ± 0,02 (a; B)	3,10 ± 0,24 (b; A)

Lower-case letters indicate difference between temperature within the same exposure conditions (p = 0.05)

Upper-case letters indicate differences between exposure conditions for the same temperature (p = 0.05)

Encompassed environmental parameters analysis

Each of the environmental parameters' variations have been analyzed isolatedly and were noticed and discussed the unique trends when correlated to Ti(IV) exposure. As a final study, the encompassed data of the complete multivariate assay has been put through a PCO analysis (Fig. 38).

Its readily observable that 76.2 % of total variation has been explaining, with major contribution of PCO1 (horizontal axis) representing 62.7 % of said variation. PCO1 shows high correlation to ETS, SOD, GSTs and GSH/GSSG in the negative side (r = 0.87, 0.87, 0.92 and 0.79, respectively), zone demarked by the presence of clams in a low salinity environment. At the positive side of PCO1 are located the clams in high salinity environment, showing considerable correlation to LPO and PROT (r = 0.72 and 0.76, respectively). In the middle area of PCO1, are located the remaining studied conditions consisting of the environmental control (salinity 30 PSU, pH 8.0 and 18 ºC), as well as the acidification (pH 7.6) and warming (T 21) experiments. PCO2, the vertical axis, represents considerably less of the total variation, 13.5 %, showing only high correlation towards the positive end with GLY content (r = 0.85). Due to its limited representativeness, no additional groups of samples were noticeable, with most of the centroid samples forming a single entity.

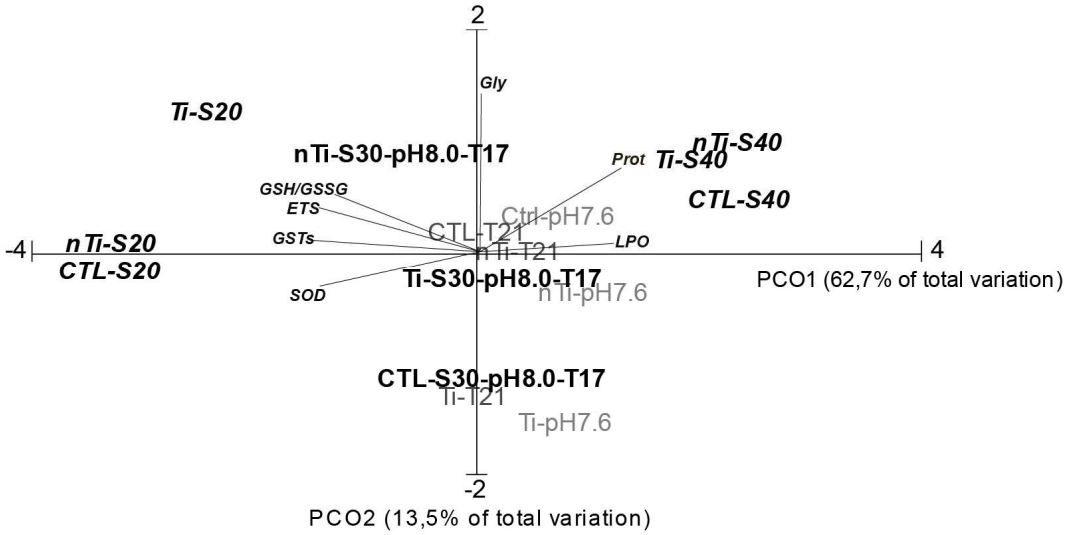

Fig. 38 – Principal component ordination analysis of biomarkers for encompassed data of multivariate assay

(Control organisms (CTL), Ti^{4+} exposed organisms (Ti) and $nTiO_2$ exposed organisms (nTi)
salinity 20 PSU (S20), 30 PSU (S30), 40 PSU (S40); pH 8.0 and pH 7.6; temperature 18 ºC (T18) and 21 ºC (T21)
Bold Black –environmental control; Bold Italic – Salinity shifts; Light Grey – Acidification; Dark Grey – Warming)

This graphical representation is rather similar to the previously presented when studying the salinity shifts, with discussion regarding its effects already elaborated and discussed. Thus, when considering the complete scale of the studied environmental parameters variations, salinity comes as the major influence over clams' biochemical responses. The presence of Ti(IV) at recently measured load out levels may induce slightly enhanced oxidative stress markers to marine biota under stable environmental conditions. However, when considering transitional water systems, in which salinity fluctuates cyclically, the presence of Ti(IV) can be mostly disregarded as the observed responses may come from the natural adaptative defense mechanisms to the environmental changes.

Chapter 4

Conclusion

4. Conclusion

Among the different nanomaterials, titanium dioxide is one of the most commercialized in different types of products, ranging from common household to high-end items (Robichaud et al. 2009; Johnson et al. 2011; Haider et al. 2017). Stemming from this widespread use, different studies have focused on its fate in the environment (Baker et al. 2014; Bundschuh et al. 2018). Enrichment of titanium in effluents has been reported (Westerhoff et al. 2011), and as such, the aquatic system has been noted as the ultimate sink of nanomaterials such as TiO_2. (Gottschalk et al. 2009; Vale et al. 2016). Consequently, its interactions within the ecosystem need further research.

Focusing on the estuarine environment, the present research work first goal was to provide new information regarding the dynamics of titanium. The so far reported "low solubility and fast precipitation in water" was confirmed in present work. Ti concentrations decreased sharply in the first 24 hours subsequent to the spiking and elimination from the dissolved fraction (< LoQ, 2 $\mu g\ L^{-1}$) was observed after 72 hours. Such low Ti contents are indicative of its fast removal to the particulate fraction.

The low residence time of titanium in the dissolved fraction of seawater is of utmost importance when factoring the interaction with marine organisms. Most likely, when Ti^{4+} was introduced into the aquaria of the present study, organisms were only briefly exposed to the added concentration in ionic form. As titanium reacted with oxygen, Ti^{4+} was gradually converted into the colloidal TiO_2 or $TiO(OH)_2$. When TiO_2 was used as a primer for contamination, only the colloidal form should be considered due to its low solubility. Presumably, as colloidal form new aggregates, sedimentation of larger particles act as driver for its removal from the dissolved phase, and thus lowering its timeframe of bioavailability. This hypothesis is in-line with previous reports of $nTiO_2$ behavior in simplified mediums and natural waters (Suttiponparnit et al. 2010; Sillanpää et al. 2011). The tendency of Ti sedimentation reduces the bioavailable for organisms of the water column. Low residual content in mussel tissues (2 – 4 $\mu g\ g^{-1}$) exposed to spiked solutions was in line with the dynamic of Ti in oxygenated seawater. Total titanium in tissues was measured in individuals 24 hours after being estimated almost complete removal of Ti from the spiked solution. Furthermore, organisms exposed to the highest spiked solution (100 $\mu g\ L^{-1}$) showed slight enrichment of this element in tissues, evidencing an effective intake of Ti and subsequent depuration.

Despite the short permanence of Ti in bivalves' biological responses have been registered. Mussels exposure to Ti(IV) exhibited significant biochemical alterations, most being dose dependent. Metabolic reduction (ETS) accompanied by energy reserve increase are characteristic of self-defense mechanisms by lowering filtrations rate. In the case of TiO_2 both metabolic and energy reserves remained nearly unchanged, potentially due to a lower permanence in the dissolved fraction. Nonetheless, enzymatic activities were altered, with GPx and GSTs being increasing in activity for both Ti and TiO_2 and SOD being mostly inhibited with Ti^{4+}. Cellular damage was found to be sub-lethal and independent of the chemical form of Ti introduced in the aquaria. Studies under post-exposure conditions revealed that organisms recovered in two weeks. Under increased temperature, mussels revealed an increased baseline for damage markers. Furthermore, biochemical responses to rutile powder under warming conditions revealed two major differences: i) altered

metabolism, with higher baseline lowering with increased concentration of TiO_2; ii) SOD activation along exposure gradient.

Environmental conditions, such as salinity, temperature and pH, fluctuate over short to seasonal time scales in estuaries and coastal lagoons. These variations could affect the contaminants availability and the behavior of organisms, eventually creating iterative effects between both. Obtained results have shown the clams' responses to Ti^{4+} or the $nTiO_2$, as well as to environmental conditions. Salinity shift impact on biochemical performance was found to dwarf the effects of Ti, with lower metabolism and increased LPO being noted along the salinity gradient. Iterative effects of Ti and lower pH increased the cellular damage (LPO) and lower redox balance (GSH/GSSG). Temperature was found to have an impact on baseline metabolic activity (ETS) of clams and Ti didn't show any increased cellular damage (LPO). Multivariate analysis to the encompassed data showed a deeper relation between biochemical responses and salinity variation than any other parameters, Ti, temperature or pH. This means that in land-ocean transitional systems salinity regulates the biochemical responses and dissolved Ti appears to be mostly irrelevant under current levels of exposure. Despite these environmental parameters have been used as proxy of climatic change scenarios, variation of those properties is intrinsic in tidally dominated estuarine systems, and organisms are adapted to those short-time scale fluctuations. Associated biochemical responses appear to mask potential and reversal impact due to the enhanced availability of Ti.

Chapter 5

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5. References

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