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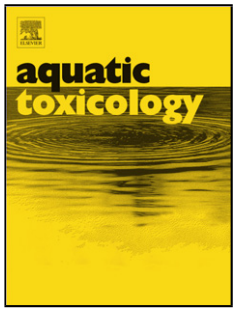

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Advances on assessing nanotoxicity in marine fish - the pros and cons of combining an *ex vivo* approach and histopathological analysis in gills

C.L. Mieiro[1,2,a,*], M. Martins[3,4,a], M. da Silva[3], J.P. Coelho[1], C.B. Lopes[5], A. Alves da Silva[2], J. Alves[2], E. Pereira[6], M. Pardal[2], M.H. Costa[3], M. Pacheco[1]

[1] Department of Biology & CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[2] Centre for Functional Ecology – CFE, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal

[3] MARE & Department of Environmental Sciences, Faculty of Sciences and Technology, University Nova of Lisbon, 2829-516 Caparica, Portugal

[4] UCIBIO, REQUIMTE, Department of Chemistry, Faculty of Sciences and Technology, University Nova of Lisbon, 2829-516 Caparica, Portugal

[5] Department of Chemistry & CICECO, University of Aveiro, 3810-193 Aveiro, Portugal

[6] Department of Chemistry & CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[a]These authors contributed equally to this work

[*]Corresponding author

Highlights

- Widespread use of nanoparticles in marine waters is a key environmental concern
- Environmentally realistic concentrations of TiO_2 Np induced moderate toxicity in gills
- After 2h, gills demonstrated adaptive responses, while after 4h recovery was clear
- Simplified *ex vivo* assays are reliable for assessing nanotoxicity in marine fish
- The NPs legal and logistical constraints are minimized with this approach

Abstract

The need to overcome logistic and ethical limitations of *in vivo* nanotoxicity evaluation in marine organisms is essential, mostly when dealing with fish. It is well established that medium/solvent conditions affect dispersion and agglomeration of nanoparticles (NPs), which represents a constraint towards a solid and realistic toxicity appraisal. In this way the pros and cons of an *ex vivo* approach, using a simplified exposure medium (seawater) and addressing gills histopathology, were explored. The nanotoxic potential of environmentally realistic concentrations of titanium dioxide NPs (TiO_2 NPs) was also assessed, disclosing the morpho-functional effects on the gills and the possible uptake/elimination processes. Excised gills of the Senegalese sole (*Solea senegalensis*) were directly exposed in artificial seawater to 20 and 200 $\mu g.L^{-1}$ TiO_2 NPs, for 2h and 4h. Semi-quantitative and quantitative histological analyses were applied. The normal morphology of the gill's epithelia was only slightly altered in the control, reflecting protective mechanisms against the artificiality of the experimental conditions, which, together with the absence of differences in the global histopathological index (I_h), corroborated that the gill's morpho-functional features were not compromised, thereby validating the proposed *ex vivo* approach. TiO_2 NPs

induced moderate severity and dissemination of histopathological lesions. After 2h, a series of compensatory mechanisms occurred in NP treatments, implying an efficient response of the innate defense system (increasing number of goblet cells) and effective osmoregulatory ability (chloride cells proliferation). After 4h, gills revealed signs of recovery (normalization of the number of chloride and goblet cells; similar I_h), highlighting the tissue viability and effective elimination and/or neutralization of NPs. The uptake of the TiO_2 NPs seemed to be favored by the higher particle sizes. Overall, the proposed approach emerged as a high-throughput, reliable, accurate and ethically commendable methodology for nanotoxicity assessment in marine fish.

Keywords: *ex vivo*; TiO_2 nanoparticles; marine fish; realistic concentrations; gills;histopathology.

1. Introduction

The distinctive physicochemical characteristics of nanoparticles (NPs) lead to their application into a wide range of fields and their fast dissemination into the environment (Khan et al. 2017). The discharges of NPs in surface waters will end up in marine systems being likely to affect its wildlife. Concomitantly, NPs specifically designed for marine applications, such as remediation and antifouling coatings, are directly introduced in these systems (Matranga & Corsi 2012; Corsi et al. 2014). Despite the evident susceptibility of the marine environment to NPs, the number of available studies evaluating the nanotoxicity on saltwater organisms, and especially in fish, is still limited (Baker et al. 2014). This paucity cannot be dissociated from the complexity of

processes occurring at environmental matrices and at organism-environment interfaces dictating the NPs behavior and bioavailability. Thus, improved methodologies are still required, more reflective of modern scientific practices and optimized in terms of suitability, reproducibility, simplicity, ethical use of animals and realism of NPs exposure profile (*e.g*. medium/solvent, concentrations and particle sizes).

In vivo studies are suitable to directly understand the overall response of the organism to contaminants (Clift et al. 2010). Nevertheless, the number of studies addressing *in vivo* nanotoxicity in marine fish is scarce (Wong & Leung 2013; Torre et al. 2015; Nigro et al. 2015). This scarcity is closely related to NPs' physicochemical properties, in particular those related with their ability to aggregate, flocculate and to heteroaggregate in seawater (Corsi et al. 2014). In addition to these limitations, *in vivo* studies may produce a massive amount of undesirable contaminated water and have ethical constraints regarding the use of fish as experimental models. In contrast, *in vitro* studies, performed in controlled environment, provide reduced variability between experiments, being faster and easier to operate. Besides having minor ethical restrictions, they also avoid interactive systemic effects, being thus, suitable for preliminary screening assays (García & Díaz-Castro 2013; Dias et al. 2017; Kumar et al. 2017). In fact, *in vitro* approaches have massively promoted the understanding of toxicity mechanisms (Ghallab 2013), including within the framework of nanotoxicity (Shukla et al. 2011; Picchietti et al. 2017; Lammel & Sturve 2018). However, they reveal limitations concerning the relationships between different cell types and xenobiotic metabolism, as well as regarding the lack of exact cellular

conditions, since isolated and cultivated primary cells regularly differ substantially from the corresponding cell type in the organism, which can lead, for instance, to changes in gene expression rates (Ghallab 2013). Thus, difficulties in extrapolating to adverse effects *in vivo* are frequently invoked (Dias et al. 2017; Drasler et al. 2017).

In this way, explant/organ cultures appear as a balanced model and a connecting link between the *in vivo* and *in vitro* studies, being commonly referred to as the *ex vivo* studies (Singh et al. 2018). The *ex vivo* approach refers to a methodology performed (out of the organism) on a surgically removed organ, or organ/tissue sections, with minor changes to the natural conditions (Singh et al. 2018). Despite that this type of assays only allow short-term exposures, it demonstrated a number of advantages regarding *in vitro* studies, such as providing more comparable responses to *in vivo* circumstances, as a result of a substantial preservation of organ's architecture, morpho-functional relations between adjacent cells, and thus, better conserving the metabolic processes (Dušinská et al. 2017). Plus, *ex vivo* approach complies with the 3Rs principles, allowing reducing considerably the number of animals to use (Valant & Drobne 2011). Surprisingly, this methodology has been scarcely applied to evaluate nanotoxicity, with few studies addressing human health issues (Meiring et al. 2005; Nassimi et al. 2009; Brun et al. 2014) and, as far as our knowledge goes, has never been adopted in fish. In fact, in the context of environmental health, *ex vivo* assays were only tested and validated in the hepatopancreas of an isopod (*Porcellio scaber*), demonstrating to be useful and highly sensitive for a fast screening of the nanotoxic potential (Valant & Drobne

2011).

Histopathological changes in fish have been considered a suitable biomarker for sub-lethal exposures to several environmental contaminants (Oost et al. 2003; Au 2004) and recommended for monitoring programs (ICES 2011). This is mainly related with the higher organizational level of response (cellular, tissue and organ), which provides more direct information on the real health condition of the organisms (Au 2004). In line, the evaluation of the nanotoxic potential has been successfully addressed in freshwater fish through histopathological analysis, pointing gills as a critical target of NPs (Federici et al. 2007; Ostaszewska et al. 2015; Abarghoei et al. 2016; Mansouri et al. 2017; Kumar, Krishnani & Singh 2018; Shobana et al. 2018). Indeed, gills are responsible for several vital functions (gas-exchange, osmoregulation and excretion) in fish, comprising a very large surface area with permanent and direct contact with waterborne contamination (Au 2004), which substantially determines its vulnerability (Martins et al. 2016; Salamat & Zarie 2016).

Ex vivo model systems, namely in biomedical contexts, usually require appropriate and complete culture mediums, whose complex composition may exacerbate the technical constraints related to NPs properties and behavior above mentioned. Interestingly, gills, as a fish-water interface organ, are persistently exposed to the outside environment, which supports the formulation of a hypothesis towards the simplification of the assay conditions to the limit, that means assuming seawater as exposure/culture medium. Once guaranteed reliability and accuracy of this simplified approach, some practical and ethical constraints on nanotoxicity screening in marine fish can

be overcome.

Considering the attributes inherent to *ex vivo* studies, it is worth highlighting that its added value can be maximized when adopting organ-specific endpoints (like those assessed in histopathology), as cells normal microenvironment and the cell-cell interactions are expected to be preserved, improving the realism of the approach.

Hence, the present work was designed with the aim of evaluating the pros and cons of an *ex vivo* approach combined with gills histopathological analysis as a step toward overcoming practical and ethical constraints on nanotoxicity assessment in marine fish. As a subsequent aim, it is intended to disclose the toxicity potential of TiO_2 NPs [the most relevant NPs in terms of humans and environmental exposure, based on their estimated releases and uses (Keller et al., 2013)] as well as some mechanisms of action, with emphasis on the uptake process and morpho-functional effects on the gills. Gills portions of a marine flatfish (*Solea senegalensis*) were short-term exposed to environmentally realistic levels of TiO_2 NPs under a simplified *ex vivo* protocol, and histopathological alterations were identified and scored.

2. Materials and Methods

2.1. Preparation and characterization of titanium dioxide suspensions

Titanium dioxide nanopowder (TiO_2 NPs), namely Aeroxide®P25 (declared purity ≥ 99.5% CAS# 13463-67-7) was supplied by Sigma-Aldrich. Crystalline phase and crystallite size were identified by the X-ray diffraction technique - XRD using a Philips X'Pert X-ray diffractometer equipped with a Cu Kα monochromatic radiation source ($\lambda\alpha_K$ = 1.4060 nm). Stock (4 $mg.mL^{-1}$)

and working (20 and 200 µg.L^{-1}) suspensions of TiO2 NPs were prepared with artificial seawater (ASW; Tropic Marin) by sonication with an ultrasonic processor (Sonics vibra cell), for 20 minutes at 100W, with 5:1 pulses on/off. The dispersion was performed in an ice bath. The behavior of TiO_2 NPs in exposure suspensions was evaluated by dynamic light scattering (DLS) using a ZetasizerNano ZSP (Malvern Instruments Ltd.) particle size analyzer and the TiO_2 NPs structure was confirmed by scanning transmission electron microscopy (STEM), using a JEOL 2200FS, JEOL Ltd., Japan model.

2.2. Maintenance of fish

Juvenile hatchery-brood Senegalese sole (*Solea senegalensis*) (approximately 140 g wet weight) was acquired from a local aquaculture (Aquacria Piscícolas SA, Portugal). At the laboratory, fish were kept for 2 weeks to acclimation in a closed-system recirculation arrangement of polyvinyl tanks containing 25 L of ASW. Fish were fed daily with species-specific commercial pellets and maintained under a natural photoperiod. A daily 25% water renewal was performed to maintain constancy of the parameters: salinity 35, temperature 15 ± 0.9 °C, pH 7.7 ± 0.7, dissolved oxygen 7.7 ± 0.7 mg.L^{-1} and unionized ammonia (NH_3) < 0.04 ± 0.02 mg.L^{-1}.

The choice of a flatfish species (*S. senegalensis*) relied on its suitability as indicator species of contamination in both field and laboratory trials, and on the easiness to maintain in laboratory (Costa et al. 2009; Martins et al. 2015).

2.3. *Ex vivo* experimental design

An *ex vivo* assay was performed with excised gills using environmental

realistic concentrations of TiO_2 NPs, *viz.* 20 (NP1) and 200 (NP2) $\mu g.L^{-1}$ (Gottschalk et al. 2013). Sixteen fish were sacrificed according to the European Union guidelines concerning the protection and animal welfare (Directive 2010/63/EU) and under supervision of a member of staff authorized by the competent authorities. Thereafter, gills of each fish (total of 8 branchial arches) were gently isolated. Immediately after excision, 2 branchial arches per fish were placed in fixative (see point 2.5.) and assumed as time zero (t_0). The remaining 6 arches were distributed (two arches per condition) directly into ASW (salinity 35), the control condition (Ct), and into the NPs suspensions (prepared in ASW; salinity 35) NP1 and NP2. Then, *ex vivo* exposure (performed in 4 mL tubes) was carried out for 2 and 4 hours, under constant gentle shaking (Figure 1).

2.4. Histological procedures

Gills preparation for histological analyses was performed according to Martins et al. (2016). Briefly, samples were fixed in Bouin-Hollande for 48h. Afterwards, the samples were dehydrated with a progressive series of ethanol (95% and 100% v/v) followed by an intermediate impregnation with xylene and then, embedded in paraffin. Gills sections, of 5 µm thickness, were obtained using a JUNG RM 2035 microtome (Leica Microsystems) and, at least 16 sections per slide were obtained. The slides were dewaxed and rehydrated with progressive series of xylene (100%, 95% and 70% v/v), ethanol and distilled water. Afterwards, the slides were stained with Hematoxylin and counterstained with alcoholic eosin (H&E stain) for broad structural analyses and with standard Tetrachromic procedure, combining

Alcian blue (30 min), Weigert's haematoxylin (10 min) and Van Gieson's dye (6 min), for the detection of mucous substances and chloride cells respectively (Costa et al. 2012; Martins et al. 2016). Acridine Orange fluorochrome (40 min) was also applied to identify mitochondria in chloride cells (Costa et al. 2012). For the detection of metallic deposits, histological slides were stained with Neutral Red (NR) (10 min) to enhance contrast of metallic deposits (Fernandes et al. 2017). All slides were dehydrated in ethanol, cleared in xylene and mounted with dibutylphthalate polystyrene and xylene (DPX) resinous media (BHD, Pool, UK). Four slides were prepared for each sample and staining procedure. A DMLB microscope, equipped with a DFC480 digital camera (Leica Microsystems), was used for the microscopic analysis. Image processing and analysis was performed with the software Image J (Schneider et al. 2012).

2.5. Gills histopathology criteria

A screening of the alterations observed in each slide was performed, providing the basis for the subsequent histopathological analysis. Semi-quantitative histopathological condition indices were estimated for each sample, according to the weighted indices of Bernet et al. (1999) and Costa et al. (2013). Each histopathological index considered the relative biological significance (weight) and a different degree of dissemination (score) of each alteration within the studied organ. The weights of alterations w varied between 1 (low severity) and 3 (high severity), and the score varied from 0 (absent alteration) to 6 (diffuse alteration). The respective pathological alterations were categorized into three reaction patterns (Table 1):

inflammatory responses, regressive (functional loss) and progressive alterations (altered function) (for further information, see Martins et al. 2016). The global histopathological index (I_h) was estimated for each sample according to the following formula:

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

where I_h is the histopathological condition index for the individual h, w_j is the weight of the j^{th} histopathological alteration, a_{jh} is the score attributed to the h^{th} individual for the j^{th} alteration and M_j is the maximum possible score for the j^{th} alteration. The equation's denominator normalizes I_h to a value between 0 and 1. Besides I_h, the different reaction patterns were also estimated in partial indices: I_1 (inflammatory responses), I_2 (regressive changes) and I_3 (progressive alterations).

In order to assess the accuracy of the observation, a blind review was performed at the end of the histopathological analyses in 25% of the samples, within which we found an average of 12.8% of error in all samples analysed.

Quantitative analyses were performed by counting the chloride cells and goblet cells (mucocytes) per interlamellar space and metal deposits per histological section. Data were expressed as mean number of each gill cell metrics per interlamellar space, while 100 metal deposits were counted per histological section.

2.6. Statistical analysis

To analyze the effects of treatment per exposure moment (independent variable) on the condition index based on the qualitative histopathology and

semi-quantitative histopathological condition indices (dependent variables), linear models (LM) were used. Since both condition index and semi-quantitative histopathological condition indices values range between 0 and 1, they were logit transformed to achieve an approximation of a normal distribution and to reduce heterogeneity (Warton and Hui 2011). Considering the possibility of the global histopathological index to be correlated with the semi-quantitative histopathological condition partial indices, a Pearson correlation analysis was performed.

To analyze the effects of treatment per exposure moment (independent variable) on quantitative histopathology (dependent variables), generalized linear models (GLM) with Poisson distribution and log link function were used.

For each dependent variable, pairwise multiple comparisons were performed using sequential Bonferroni correction. Model validation was performed for each LM and GLM, on the residuals by checking heteroscedasticity, normality, and influential observations.

The results were expressed as estimated mean and 95% confidence intervals. All the statistical tests were considered significant when $P < 0.05$. The statistical analyses were performed using IBM.SPSS®, version 23.

3. Results

3.1. Titanium dioxide nanoparticles characterization

The XRD analysis demonstrated the presence of two crystalline phases: anatase (86.8%) and rutile (13.2%). Typical spheroid irregular shape of TiO_2 NPs (Aeroxide© P25) and primary size of a mean particle diameter of 17.74 ± 0.43 nm was confirmed by STEM (Fig. 2). The histogram of different

sizes of TiO_2 NPs showed a bimodal distribution, with 50% of the nanoparticles analyzed having a size ranging between 14 and 18 nm, 9% ranging from 20 to 24 nm, and 9% around 28 and 32 nm. DLS analysis of the TiO_2 NPs stock suspension (4 $mg.L^{-1}$) in ASW revealed the presence of agglomerates (558.1 ± 11.6 nm), with values of polydispersity index (PdI) of 0.394 ± 0.043. The average size of the agglomerates of TiO_2 NPs in the tested solutions (in ASW), provided by the average DLS hydrodynamic diameters, were 815.7 ± 11.3 (0.615 ± 0.0011 PdI) and 513 ± 5.6 nm (0.474 ± 0.054 PdI) at 20 and 200 $\mu g.L^{-1}$, respectively.

3.2. Gills histopathological alterations and condition indices

Gills from t_0 condition exhibited the normal morphology of a juvenile marine teleost (Fig. 3A), displaying well-defined lamellae attached to filaments, as reported in Costa et al. (2010) for the same species. Gills epithelia contained pavement cells, goblet cells (mucocytes) and chloride cells (mitochondria-rich).

Gills from the control group (only submitted to ASW) presented slightly to moderate alterations in comparison to t_0, namely disruption of the lamellae structure ($F_{(3,25)} = 4.699$, $P = 0.01$ for 2h and $F_{(3,25)} = 9.148$; $P < 0.001$ for 4h) and epithelial lifting ($F_{(3,25)} = 29.477$, $P < 0.001$ and $F_{(3,25)} = 33.490$, $P < 0.001$ for 2h and 4h, respectively) for both experimental moments (Fig. 3B). After 4h, control gills also presented interlamellar hyperplasia of the epithelial cells (from now on referred as hyperplasia) relatively to t_0 ($F_{(3,25)} = 3.795$, $P = 0.023$). Other alterations such as infiltration of inflammatory cells and

apoptosis presented low dissemination, varying between 0 and 2, being similar between t_0 and control for both exposure moments.

Histopathological alterations observed in NPs treatments displayed higher severity and dissemination (Fig. 3C), among which changes in the lamellae structure and goblet cell hypertrophy were the most prominent. After 2h, NP1 presented higher incidence of alterations in the lamellae structure than control ($F_{(3,25)}$ = 4.699, P = 0.01), whereas after 4h they were more frequent at both NPs treatments (NP1 ≈ NP2 > Ct; $F_{(3,25)}$ = 9.148, P < 0.001). An increase of goblet cells hypertrophy (Fig. 3D) was observed in both TiO_2 NPs treatments in relation to the control after 2h of exposure ($F_{(3,25)}$ = 6.673, P = 0.002), while after 4h they increased in NP2 relatively to NP1, though not signalizing differences with the control ($F_{(3,25)}$ = 4.084, P = 0.017). Metal deposits were found in gills macrophages (forming black deposits) in both TiO_2 NPs treatments (Fig. 3E). Chloride cells autolysis was also detected, but with similar distribution among t_0, control and NPs treatments for both exposure moments.

The global histopathological condition index (I_h) was similar between t_0 and control, for both exposure moments (Fig. 4A). However, after 2h, gills exposed to NP2 showed a higher I_h score in relation to control ($F_{(3,25)}$ = 5.626, P= 0.004). After 4h, no differences in the I_h values were found among NPs treatments and control. I_1 I_2 and I_3 were correlated with I_h (I_1 r = 0.532, P < 0.001; I_2: r = 0.785, P < 0.001; I_3: r = 0.714, P < 0.001) and thus, exhibited similar variation patterns. The Pearson correlation analysis showed the highest correlations between I_h- I_2 and with I_h- I_3 (I_2: r = 0.785, P < 0.001; I_3:

r = 0.714, P < 0.001). I_1 and I_2 were also positively correlated (r = 0.485, P < 0.001).

3.3. Quantitative histopathological measures

Quantitative histopathological measures demonstrated that the number of chloride cells was similar between t_0 and control, for both exposure moments. However, after 2h, the number of these cells significantly increased in both NPs treatments in relation to control, being the highest number registered for NP1 (Ct < NP2 < NP1; Wald-$\chi^2_{(3)}$ = 136.871; P < 0.001) (Fig. 5A). After 4h of exposure, significant differences in the number of chloride cells were only observed between TiO_2 NPs concentrations, with higher incidence at NP1 (NP2 < NPs1; Wald-$\chi^2_{(3)}$ = 11.461, P = 0.009) (Fig. 5A).

Relatively to the number of goblet cells, and similarly to chloride cells, no differences were observed between t_0 and control after 4h. The number of goblet cells significantly increased in NP1 after 2h when comparing with control and NP2 (Ct ≈ NP2 < NP1) (Wald-$\chi^2_{(3)}$ = 91.355, P < 0.001) (Fig. 5B). Conversely, the number of goblet cells decreased in both TiO_2NPs treatments comparing to control after 4h (NP1 ≈ NP2 < Ct; Wald-$\chi^2_{(3)}$ = 69.691, P < 0.001) (Fig. 5B).

The presence of metal deposits (Fig. 5C) was similar between t_0 and control, for both exposure moments. Yet, after 2h, the occurrence of these deposits was higher at NP1 relatively to both control and NP2 (Ct ≈ NP2 < NP1), while after 4h metal deposits were found in higher extent in NP2 in comparison to control and NP1 (Ct ≈ NP1 < NP2) (Wald- $\chi^2_{(3)}$ = 66.353, P < 0.001; Wald- $\chi^2_{(3)}$ = 27.475, P < 0.001).

4. Discussion

4.1. Appraising the accuracy of the *ex vivo* approach and identifying the pros and cons for assessing nanotoxicity in marine fish

The accuracy of the simplified *ex vivo* protocol, as proposed in this work and involving fish gills, greatly depends on the demonstration of the preservation of normal histological features in the unexposed group (control), as a function of time, taking t_0 profile as reference. In this direction, after the *ex vivo* assay (2h and 4h) control gills exhibited moderate alterations, mostly regressive and easily reversible (Table 1; Bernet et al. 1999), in comparison with the normal morphology exhibited at t_0. Progressive alterations were also found in control after 4h, namely hyperplasia, but this alteration is considered reversible in most cases (Bernet et al. 1999). Plus, an increase in the number of goblet cells in control relatively to t_0 was observed after 2h. Epithelial lifting and hyperplasia are the most commonly reported histopathological alterations after exposure to stressors (both contaminants and water physicochemical properties) (Good et al. 2010; Martins et al. 2016; Mansouri et al. 2016; Kumar, Krishnani & Singh 2018). Moreover, the epithelial lifting is considered as one of the first histopathological responses in fish gills (Santos et al. 2014) and has been suggested as an adaptive strategy of the organism to preserve its homeostasis (Mallatt 1985) together with hyperplasia and goblet cell proliferation, since all increase the distance between external media and blood (Mallatt 1985; Costa et al. 2009; Martins et al. 2016). Despite the presence of these alterations in control gills, the overall structure and function

of the organ did not seem compromised by the current *ex vivo* conditions. This is a *sine qua non* condition that was ensured by the proposed protocol.

The presence of mild histological alterations (suboptimal conditions) has also been found in fish from uncontaminated scenarios (reference/control conditions) (Costa et al. 2011; Hawkins et al. 2015). For instance, Costa et al. (2011) suggested that in *in vivo* laboratory experiments an undetermined number of variables produces experimental background noise in control groups (merely reflecting protective mechanisms against the artificiality promoted by the experimental conditions) but without compromising the global outcomes of the assay. The shift from optimal water conditions (*e.g.* alteration of the oxygen supply) induces specialized physiologic adaptations. Likewise, as a result of fish aerobic metabolism, carbon dioxide (CO_2) is produced and excreted through the gills into the surrounding media (Good et al. 2010). The release of CO_2 into the medium may have induced acidosis, which, in turn, could lead to epithelial lifting and hyperplasia (Fergunson 1989), as currently observed in control groups.

Notwithstanding the presence of some moderate reversible alterations, the histopathological condition indices, and particularly I_h, were similar between control and t_0. The results provided by the histopathological indices were reported as suitable to the evaluation of the biological risk, independently of the methodological approach (Bernet et al. 1999; Martins et al. 2016; Costa et al. 2011). This information is more integrative since it combines both the dissemination of the lesions and their biological importance, accounting for the inter-individual and assay variations, being thus a valuable proxy of the real condition of the organism. Thus, the

presence of moderate alterations in control gills merely reflects an efficient protective measure against the artificiality induced by the exposure conditions, which corresponds to deviations of the normal conditions but without compromising the applicability of the *ex vivo* assay. This evidence is corroborated by the absence of differences when evaluating the alterations altogether and with the respective condition index (dissemination and severity). Moreover, the higher prevalence of other histopathological alterations in NPs exposed groups in comparison with control validates both the tissue responsiveness under the *ex vivo* conditions and the suitability of the gills to reflect the NPs toxic effects (as demonstrated in the next sections of the discussion), thereby reinforcing the consistency of the approach.

Considering the presence of negligible disturbances (moderate and easily reversible changes) in the control, and the absence of differences in the histopathological indexes at exposure moments of 2h and 4h, it can be assumed that both times are appropriate for this *ex vivo* approach. The presence of easily reversible changes in most cases, together with the sporadic occurrence of the progressive ones, indicates that the tissues maintained their ability to multiply, which is only achieved whenever the essential conditions are guaranteed. For these reasons, the present *ex vivo* approach appears as a valid alternative for histopathological evaluations using seawater as medium and without requiring aseptic conditions.

The main advantages of this methodology are related with the high-throughput regarding time of exposure and sample processing, as well as with the reduction of the wastewater contaminated with NPs (it allows a reduction of at least 500 times in the amount of wastes) and the number of animals

necessary. In fact, with the current approach only 16 fish were sacrificed, when compared to a total of 64 that would have been required to replicate this experimental design in an *in vivo* assay, composed of 4 conditions (t_0, control, 20 and 200 µg. L^{-1}) and two exposure moments (2h and 4h). Performing the *ex vivo* assay with a simplified medium, such as seawater, has proved to be suitable for short-term assays, presenting advantages over both *in vivo* and *in vitro* assays. The reduced volume of medium needed as well as its simplicity, mitigates the common aggregation/agglomeration problems associated to NPs in both type of assays. Furthermore, this is a cost-effective and easy to replicate technique, but limited to a short time frame, since the removal of the organs/tissues from the organism, sooner or later, lead to the degradation of their metabolic/physiological processes and degenerative changes of tissue microstructure.

The present *ex vivo* approach is especially suitable when using gills as model tissue due to their permanent and direct contact with seawater in alive/breathing fish. However, the adoption of other freshly excised tissues would be also possible, though requiring higher complexity of the assay medium. Globally, the application of this methodology for nanotoxicity evaluation in marine organisms fits the goals of the environmental risk assessment (ERA) since it allows predicting both the uptake and the biological effects induced by NPs.

4.2. Elucidation of TiO_2 NPs toxicity to gills

The presence of environmentally realistic concentrations of TiO_2 NPs in the incubation medium induced moderate dissemination and severity of

histopathological lesions, mostly goblet cells hypertrophy and alteration of the lamellae structure (2h and 4h). Previous studies with TiO_2 NPs also reported the presence of these alterations in gills of freshwater fish (Federici et al. 2007; Hao et al. 2009; Mansouri et al. 2016). Nevertheless, these effects are not specific of TiO_2 NPs, being described in fish exposed to a wide variety of pollutants, including other NPs (Martins et al. 2016; Abarghoei et al. 2016; Kumar et al. 2018; Shobana et al. 2018). The severity of these lesions seemed to be related both with the concentration and with the duration of the exposure, though not linearly. While hypertrophy of the goblet cells increased in both NPs treatments relatively to control after 2h, only differences between exposed groups were observed after 4h. Concomitantly, the number of goblet cells increased in the lowest TiO_2 NPs concentration after 2h, whereas, after 4h both TiO_2 NPs-treated groups depicted lower number of these cells than control. This trend (2h and 4h) was previously observed in fathead minnows after 24h and 96h of exposure to silver NPs (Hawkins et al. 2014; Hawkins et al. 2015). Some authors suggested that, after hypersecretion of mucous, a depletion of the goblet cells might occur due to the gills incapacity to promptly produce new cells (Srivastata et al. 2014). In fact, the goblet cells in human intestines may lose part of their cytoplasm together with mucous in response to stress (Deplancke and Baskins 2001). Hence, the decline in the number of goblet cells in the current NPs treatments after 4h may reflects a refractory period following a stimulus (as depicted in the hypersecretion observed at 2h). Mucous production is recognized as a short-term protective measure for trapping NPs (Handy et al. 2008; Smith et al. 2007). Moreover, mucous also functions as ionic and osmotic regulator (Shepard 1994), suggesting that

changes in its production will affect these processes. In fact, alteration of the osmoregulatory ability of gills exposed to TiO_2 NPs may be assumed due to variations in the number of the chloride cells after 2h. Chloride cells are responsible for the regulation of the majority of gills' physiological processes (Wilson & Laurent 2002). As stated for goblet cells, the variation pattern of chloride cells mirrors a gills' response to TiO_2 NPs following 2h exposure. Previous studies indicated that the increasing number of chloride cells has been coupled to adaptive physiological responses (Giari et al. 2008; Martins et al. 2016; Macirella & Brunelli 2017) and to excretory and detoxifying functions of this type of cells (Oronsaye & Brafield 1984; Mallatt 1985). The absence of differences in the number of chloride cells between the NPs treatments and the control after 4h, probably reflects an efficient osmolarity rebalancing, despite suggesting a slightly greater challenge in the lower comparatively to the higher TiO_2 NPs concentration.

The information provided by the indices (I_h, I_1 I_2 and I_3) indicated that, after 2h, both the dissemination and the biological relevance of the alterations were higher at the highest TiO_2 NPs concentration, while, after 4h, no differences were observed between control and NPs exposed groups, suggesting a gills recovery and adaptability to realistic concentrations of TiO_2 NPs. The convergent information provided by I_h and the number of goblet and chloride cells (changes induced by NPs after 2h and recovery after 4h), reinforces the suitability of this index in providing a picture of the overall response of the organ. In addition, the correlation between the global index with I_2 and I_3 reaction patterns demonstrated that regressive and progressive alterations are the most relevant and thus, fundamental to increase the

histopathological indices. I_1 and I_2 were also positively correlated, suggesting that inflammatory alterations are concurrent with regressive alterations (Barišić et al. 2015).

The susceptibility of gills facing conditions recreating environmentally realistic contamination scenarios revealed that *in vivo*, large size (nm) TiO_2 NPs can access cells and be biodistributed through blood to other internal tissues/organs, especially liver and intestine. Hence, the presence of TiO_2 NPs in fish tissues, after diet contamination and intraperitoneal (i.p.) injection, was reported in blood, gills, liver, kidney, spleen, digestive tract, brain and muscles (Ramsden et al. 2009; Vignardi et al. 2015), inducing oxidative stress, ionoregulatory disturbances, cytotoxicity and genotoxicity (Handy et al. 2011; Vignardi et al. 2015). *In vivo* exposures also demonstrated innate and adaptive immune processes in fish gills as well as pro-inflammatory responses (Torre et al. 2015), which reinforce gills as a target of NPs and their role in protecting the organism of external contamination/stressors.

The detection of metal deposits was evaluated through the NR technique according to Fernandes et al. (2017). Despite that this methodology may not be extremely specific for metals, it increases the metallic contrast and therefore allows distinguishing the presence of dense granules, which, in the present case, suggests the uptake of TiO_2 NPs by gills. After 2h, black deposits were found in higher extent in the gills exposed to the lower TiO_2 NPs concentration, while after 4h their presence was higher at the higher TiO_2 NPs concentration. Previous data regarding TiO_2 NPs i.p. injection in a marine fish also demonstrated their uptake by the presence of dense granules, both in the cytoplasm and the nuclei of gill cells (Vignardi et al. 2015). An *in vivo*

study, with TiO_2 NPs, also found NPs aggregates inside gills epithelia as well as retained in the mucous (Moger et al. 2008). The presence of TiO_2 NPs inside and at the surface of the cells was also demonstrated in an *in vitro* study with embryonic cells of a marine fish (Picchietti et al. 2017).

The current DLS information indicated a high rate of aggregation of the TiO_2 NPs in ASW (815.7 ± 11.3 nm for the lower and 513 ± 5.6 nm for the higher TiO_2 NPs concentrations). Vignardi et al. (2015) reported similar aggregation effects (163 ± 50 nm to 830 ± 389 nm), suggesting that gills may internalize large size TiO_2 NPs. Moreover, the information provided by the presence of metal deposits, together with the information obtained by DLS, showed that the uptake was determined by the particle size, independently of the concentration (which can play an indirect role by the determination of aggregation rates). The internalization of smaller particle sizes was less efficient than bigger sizes, since the former have to reach higher densities to trigger the uptake processes. Hence, current DLS data can explain the faster uptake of NP1 depicted after 2h, while NP2 internalization was only perceived after 4h. Moger et al. (2008) also reported that small NPs require an extended period of exposure to be internalized after being retained in the mucous for longer periods.

The observed dissemination of TiO_2 NPs in gills tissue, including encapsulated in macrophages, suggests a high internalization ability of these NPs. It is documented that increasing NP sizes enhances its uptake by macrophages (He et al., 2010), which agrees with the current circumstances as both concentrations showed NPs size greater than 500 nm. Gustafson et al. (2015) stated that macrophages are the first and the main cells to process

NPs in all tissues, being connected to the modulation of the inflammatory and other immunological responses. In agreement, the presence of metal deposits in macrophages currently detected suggests gills adaptive responses.

The uptake of NPs is not only dependent on the particle size, but also on the cell type (Kuhn et al. 2014). In macrophages, uptake occurs either by phagocytosis or by clathrin-mediated mechanisms (Kuhn et al. 2014), and can be favored for negatively charged NPs, as TiO_2 NPs (Bolis et al. 2012, Liu et al. 2013). In non-phagocytic cells, NPs < 200 nm are internalized by clathrin-mediated endocytosis and around 500 nm by caveolae-mediated processes (Rejaman et al. 2004). An *in vivo* study with fish reported that the main uptake mechanism of TiO_2 NPs was endocytosis (Vignardi et al 2015). According to the current DLS data (particle size was > 500 nm in both TiO_2 NPs concentrations) TiO_2 NPs uptake in gills non-phagocytic cells may have occurred through caveolae-mediated processes.

Recently, it has been demonstrated that, besides the classic tissue macrophages, other cells, known as “nonprofessional phagocytes”, also have the ability to phagocytize (Atabai et al. 2007). Studies with mammary alveolar cells of mouse revealed a macrophage-like behavior, disclosing a capability to participate in the removal of neighboring apoptotic cells (Monks et al. 2005). This type of cells shares with goblet cells their epithelial nature, secretory/glandular role and apocrine functioning (*i.e*., they release the secretory materials by losing the apical portion of cytoplasm, producing extracellular membrane-bound vesicles). Though still requiring a scientific validation, this similarity legitimates theorizing a macrophage-like behavior for goblet cells. This would support the hypothesis of NPs uptake through a

phagocytic activity of the goblet cells. Indeed, Mosquera et al. (2018) described that particles > 500 nm (like those found in the present study) are internalized by phagocytosis.

The information provided by the presence of metal deposits doesn't seem to be a good proxy of the NPs' effects, as the observed response pattern is not extrapolated from the TiO_2 NPs concentration and *vice versa*. After 2h, metal deposits were found in higher extent in the gills exposed to the lower TiO_2 NPs concentration while the I_h score only increased at the higher TiO_2 NPs concentration. The possibility of uptake cannot be discarded in NP2 group after 2h, suggesting possible limitations of the NR technique in detecting metal deposits or uptake restrictions due to the particle size. After 4h, the I_h did not reflect the increasing uptake of the higher TiO_2 NPs concentration, reinforcing the possible limitations of the NR technique.

The increase of I_h score at NP2 after 2h, despite the absence of uptake evidences (through NR technique) may be related with the trapping of NPs in the mucous due to the hypersecretion above hypothesized (concomitantly to a gentle uptake process of the NP2 due to their smaller size than NP1). The retention of metals in the mucous into critical sites for toxicity (Satchell, 1984) may induce damage through contact with gills epithelia, being also a possible explanation for the higher I_h score at NP2 after 2h. The NPs retained in the mucus may also form aggregates with Mg^{2+} and Ca^{2+} present in the gill's tight junctions, preventing paracellular diffusion and increasing the contact between gills epithelia and NPs (Handy et al. 2008). Moreover, the presence of secondary toxicity (without NPs accumulation/uptake), as previously reported by Federici et al. (2007), seems to be a plausible

explanation due to the oxidizing ability of TiO_2 NPs, leading to the formation of reactive oxygen species and thus, inducing toxicity via oxidative stress. On the contrary, the absence of differences in the I_h score after 4h for NP1, may suggest gills' recovery ability by the mechanisms previously mentioned, while the absence of differences in the I_h score for NP2 suggests a weak correlation between the presence of metal deposits (detected by the NR technique) and the observed histological alterations.

The apparent recovery of the gill's morpho-functional features from 2h to 4h, after exposure to the lower TiO_2 NPs concentration, suggests NPs elimination from the tissue and, with the exception of redistribution via blood circulation (absent in this experimental model), different pathways can be considered. It may have occurred in association to cellular renewal, with subsequent loss of NPs. The cellular turnover (at normal or increased rates) of the pavement cells, which comprise circa 95% of gills' epithelial cells (Goss et al. 1995), could have contributed to the elimination of TiO_2 NPs. However, this loses some plausibility considering that gills cellular turnover occurs in a time lapse ranging from 6h to 16h (Bonga and Meij, 1989), while NPs removal took place in 2h. A direct removal through active excision processes gains likelihood. The excessive production of mucous (after 2h) may have induced its sloughing and thus leading to the elimination of NPs by that way. Handy et al. (2008) stated that sloughing of mucous promptly occurs in the presence of NPs in comparison with other contaminants. Plus, the proliferation of chloride cells (after 2h) might also have promoted the elimination of the NPs, since these cells have the ability to release contaminants (Mallat 1985).

5. Conclusions

The present gills histopathological analysis endorsed *ex vivo* assays, performed with a simplified medium (seawater), as a reliable and accurate methodology for assessing the nanotoxic potential in marine fish. The preservation of normal histological features in the unexposed gills was ensured (2h and 4h), providing sub-optimal conditions comparable to those of *in vivo* assays. The approach, as proposed, allows a high-throughput analysis, carrying logistic (*e.g*. decrease of the wastewater contaminated discharges and restraining NPs aggregation) and ethical (reduced number of the animals required) advantages in relation to the *in vivo* and *in vitro* NPs testing. Its main limitation concerns the presumable loss of viability and structural integrity of the tissue in time lapses longer than those tested.

Realistic concentrations of TiO_2 NPs induced a moderate degree of histopathological lesions in gills (inflammatory, regressive and progressive alterations). After 2h, gills showed a series of compensatory mechanisms, in particular in response to the lower TiO_2 NPs concentration (20 $\mu g.L^{-1}$), suggesting an efficient response of the innate defense system (increase in the number of goblet cells and the presence of metal deposits), as well as efficient osmoregulatory ability (increase of chloride cells). After 4h, gill epithelia exposed to the lower TiO_2 NPs concentration revealed a recovery (normalization of the number of chloride, similar I_h), pointing out the tissue viability and an effective neutralization and/or release of NPs. The uptake of the TiO2 NPs seemed to be limited by the size, with greater accumulation of larger NPs. The presence of metal deposits demonstrated a weak correlation with the observed histological effects.

The information provided by the I_h reflected a holistic and more accurate perspective of the organ's condition, while quantitative histology reflected cellular specificities, complementing each other.

Environmentally realistic concentrations of TiO_2 NPs induced moderate toxicity in marine fish gills, which also displayed an adaptive capacity to cope with this challenge.

conflict of interest

The authors declare no conflict of interest .

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Figure 1. Schematic representation of the experimental set-up used in the *ex vivo* assay. Gills were excised from eight fish and the branchial arches (8) were randomly distributed (two per condition) for t_0 and through treatments: control (Ct), 20 (NP1) and 200 (NP2) µg.L^{-1} TiO_2 NPs. This set-up was used in the two independent exposure moments (2h and 4h).

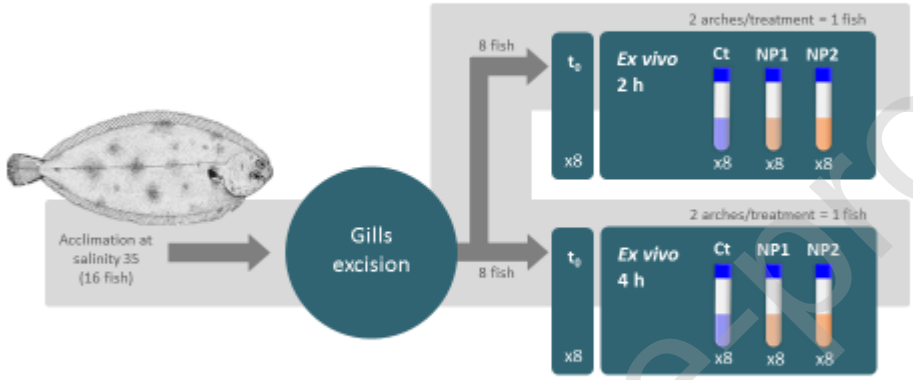

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Figure 2. Scanning transmission electron microscope (STEM) image of titanium dioxide nanoparticles (TiO_2 NPs) suspension in artificial seawater (scale 100 nm) and their size distribution histogram.

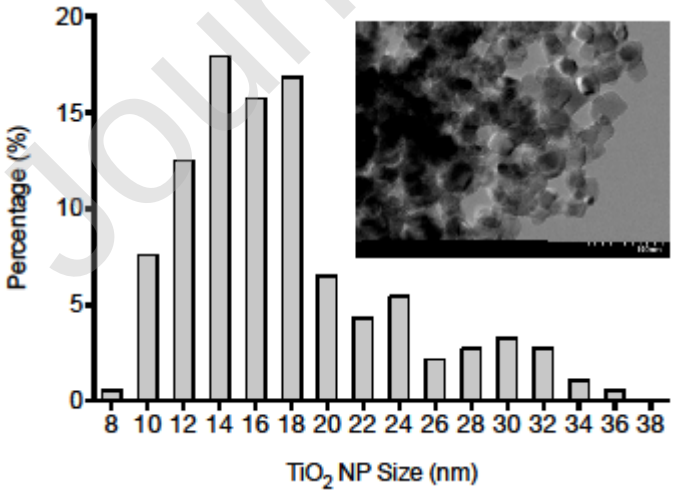


Figure 3. Gill histopathological sections of *S. senegalensis* stained with Tetrachrome (A, B, C, D) and Neutral Red (E) stains, depicting the overall aspect of the organ morphology obtained from: (A) t_0 (gill excised and immediately fixed), exhibiting normal gill filament (fl), lamella (lm), pillar cells (pc), pavement cells (pv), chloride cells (cc) and goblet cells (gc); (B) control samples, exhibiting epithelial lifting (white arrowhead) and structure alterations (black arrow); (C) 200 µg.L^{-1} TiO_2 NPs samples after 4h, showing epithelial lifting (white arrowhead) and chloride cells autolysis (cca); (D) 200 µg.L^{-1} TiO_2 NPs samples after 2h, displaying hypertrophied goblet cells (black arrowhead); (E) 20 µg.L^{-1} TiO_2 NPs samples after 4h presenting metal deposits (md) forming black spots.

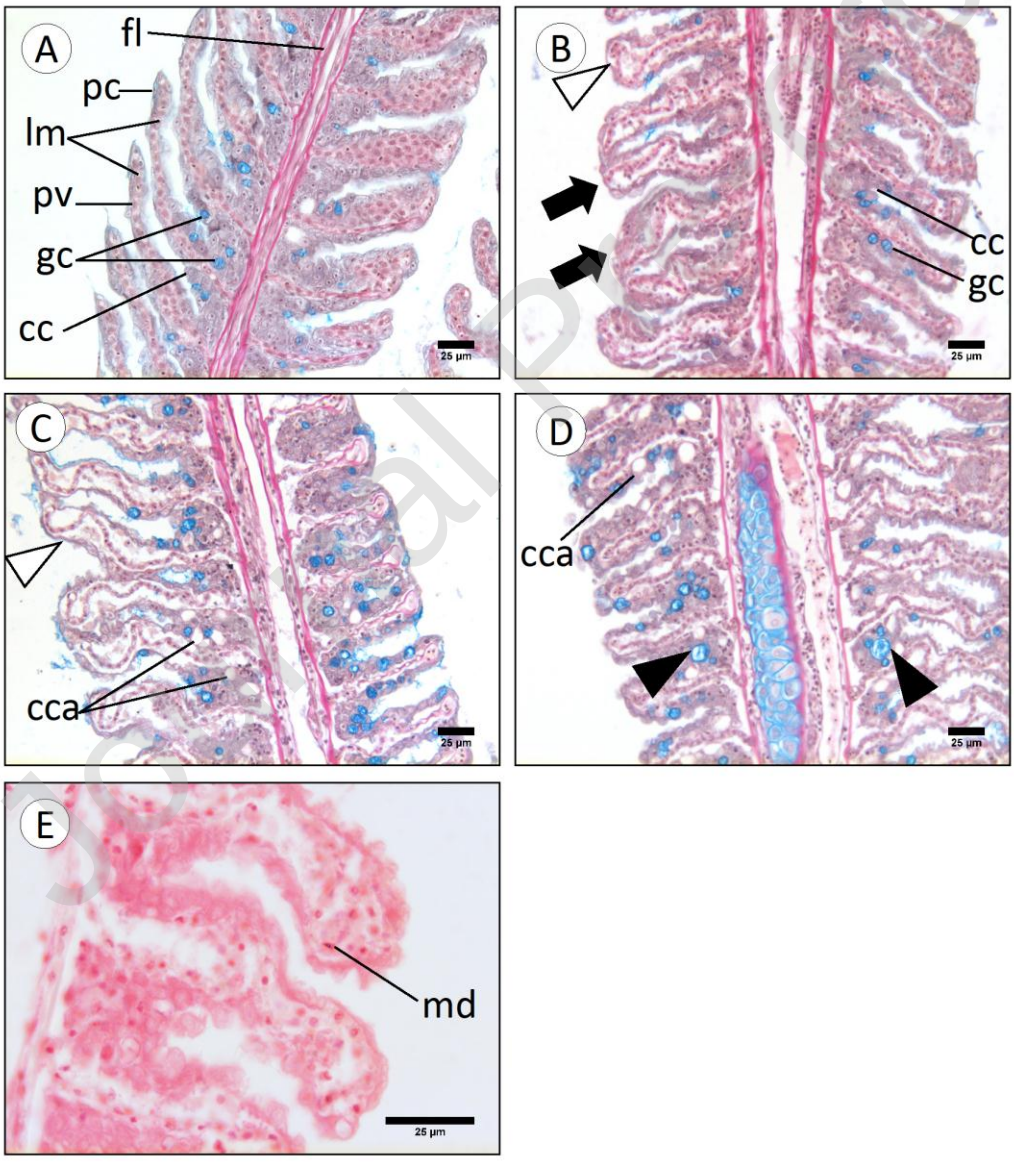


Figure 4. Histopathological indices (global histopathological index and indices for each reaction pattern) in gills from control (Ct), 20 (NP1) and 200 (NP2) µg.L^{-1} TiO_2 NPs treatments, after 2 and 4h, and t_0 (gills excised from fish and immediately fixed). A - Global histopathological index (I_h), B - Circulatory disturbances/Inflammatory response (I_1), C - Regressive alterations (I_2) and D - Progressive alterations (I_3). Different lower-case letters denote significant differences among control and NPs treatments ($P < 0.05$). Columns correspond to mean, and error bars represent the standard error.

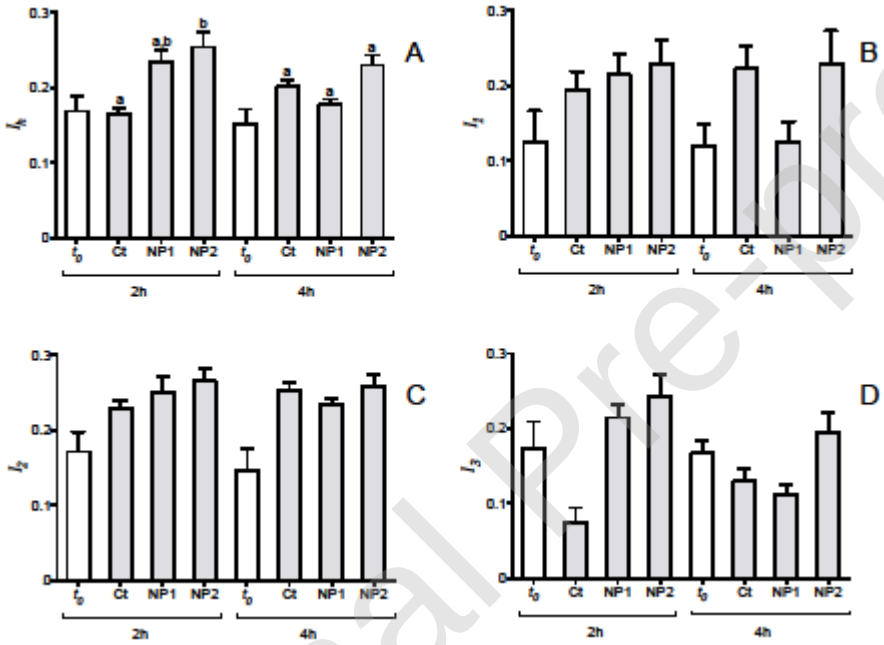
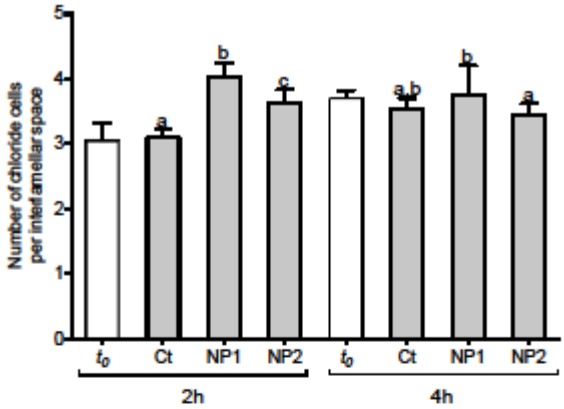
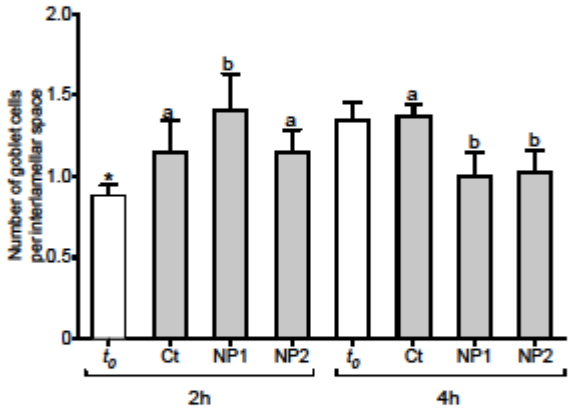


Figure 5. Comparison of the number of chloride cells (A) and goblet cells (B) per interlamellar space, as well as metal deposits (C) per section, in gills from control (Ct), 20 (NP1) and 200 (NP2) µg.L^{-1} TiO_2 Np treatments, after 2h and 4h, and t_0 (gills excised from fish and immediately fixed). Different lower-case letters denote significant differences among control and NP treatments ($P < 0.05$). * denotes differences between t_0 and control ($P < 0.05$). Columns correspond to mean, and error bars represent the standard error.





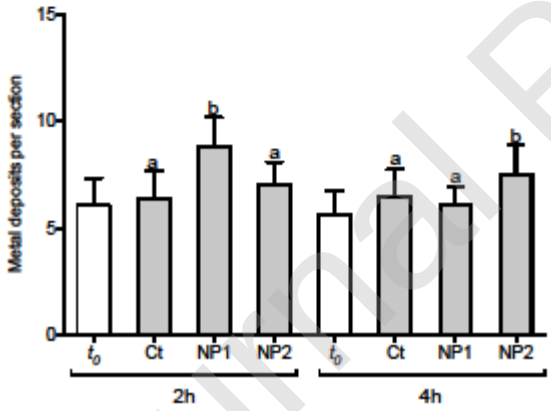


Table 1. Histopathological alterations observed in the gills *S. senegalensis*, and their respective condition weights, after the *ex vivo* experiment.

Reaction Pattern	**Histological alterations**	**Weight**
1. Inflammatory responses	Infiltration of inflammatory cells	1[b]
2. Regressive	Epithelial lifting	1[b]
	Structure alterations of lamellae	1[b]
	Goblet cells degeneration	2[b]
	Chloride cells autolysis	2[c]
	Apoptosis	2[b]
3. Progressive	Epithelial cell hypertrophy	2[b]
	Chloride cell hypertrophy	2[a]
	Interlamellar hyperplasia of the epithelial cells	2[a]

[a] Bernet et al., 1999; [b] Costa et al., 2009; [c] Martins et al., 2015