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Abstract: Despite the growing concern over the potential biological impact of nanoparticles (NPs) in the aquatic environment, little is known about their interactions with other pollutants. In the marine mussel Mytilus galloprovincialis exposure to n-TiO2, one of the most widespread type of NPs in use, in combination with 2,3,7,8-TCDD, chosen as model xenobiotic, was shown to affect different biomarkers, with distinct effects depending on cell/tissue and type of response. In this work, the interactive effects of n-TiO2 and TCDD at the tissue level were further investigated in mussel digestive gland using an integrated approach transcriptomics/ immunohistochemistry. Mussels were exposed to n-TiO2 (100 µg L-1) or to 2,3,7,8-TCDD (0.25 µg L-1), alone and in combination, for 96 h. Transcriptomic analysis identified 48 DEGs, 49 DEGs and 62 DEGs in response to n-TiO2, TCDD and n-TiO2/TCDD, respectively. Transcription of selected genes and expression of target proteins of interests (tubulin) were verified by qPCR and by immunolabelling, respectively. Tissue TCDD accumulation was evaluated by immunofluorescence with an anti-dioxins antibody.
GO term analysis revealed distinct biological processes affected in different experimental conditions, with n-TiO2 mainly up-regulating cytoskeletal genes, and TCDD endocrine and signal transduction related processes. Exposure to the mixture involved transcriptional changes common to individual treatments, and identified a newly generated process, response to chemical stimulus. The results demonstrate both distinct and interactive effects of n-TiO2 and TCDD in mussel digestive gland at the molecular and tissue level, identify the main molecular targets involved, and underline how exposure to the n-TiO2/TCDD mixture does not result in increased TCDD accumulation and overall stressful conditions in the tissue. These represent the first data on transcriptional responses of marine invertebrates to exposure not only to n-TiO2 as a model of NP, but also to a legacy contaminant like dioxin.

Dear Prof. Domingo,

I send you the manuscript "Combined effects of n-TiO_2 and 2,3,7,8-TCDD in *Mytilus galloprovincialis* digestive gland: a transcriptomic and immunohistochemical study", to be considered for publication in Environmental Research.

All of the authors have read and approved the paper and it has not been published previously nor is it being considered by any other peer-reviewed journal.

Looking forward to hearing from you

Sincerely yours

Laura Canesi

Highlights

- Interactive effects of n-TiO_2 and TCDD were investigated in Mytilus digestive gland
- Responses were evaluated by a transcriptomics/immunohistochemistry approach
- Distinct and interactive effects were observed at molecular and tissue level
- Exposure to n-TiO_2/TCDD did not result in increased stressful conditions
- First data on transcriptional responses of marine invertebrates to a NP/POP mixture

Combined effects of n-TiO_2 and 2,3,7,8-TCDD in *Mytilus galloprovincialis* digestive gland: a transcriptomic and immunohistochemical study.

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Abstract

Despite the growing concern over the potential biological impact of nanoparticles (NPs) in the aquatic environment, little is known about their interactions with other pollutants. In the marine mussel *Mytilus galloprovincialis* exposure to n-TiO_2, one of the most widespread type of NPs in use, in combination with 2,3,7,8-TCDD (TCDD), chosen as model organic xenobiotic, was shown to affect different biomarkers, with distinct effects depending on cell/tissue and type of response. In this work, the interactive effects of n-TiO_2 and TCDD at the tissue level were further investigated in mussel digestive gland using an integrated approach transcriptomics/ immunohistochemistry. Mussels were exposed to n-TiO_2 (100 μg L^{-1}) and to TCDD (0.25 μg L^{-1}), alone and in combination, for 96 h. Transcriptomic analysis identified 48 DEGs, 49 DEGs and 62 DEGs in response to n-TiO_2, TCDD and n-TiO_2/TCDD, respectively. GO term analysis revealed distinct biological processes affected in different experimental conditions. n-TiO_2 mainly up-regulated cytoskeletal genes, and TCDD endocrine and signal transduction related processes. Co-exposure induced transcriptional changes common to individual treatments, and identified a newly generated process, response to chemical stimulus. Transcription of selected genes and expression of target proteins of interests (tubulin) were verified by qPCR and by immunolabelling, respectively. Tissue TCDD accumulation was evaluated by immunofluorescence with an anti-dioxins antibody.

The results demonstrate both distinct and interactive effects of n-TiO_2 and TCDD in mussel digestive gland at the molecular and tissue level, identify the main molecular targets involved, and underline how exposure to the n-TiO_2/TCDD mixture does not result in increased TCDD accumulation and overall stressful conditions in the tissue. These represent the first data on transcriptional responses of marine invertebrates to exposure not only to n-TiO_2 as a model of NP, but also to a legacy contaminant like TCDD.

Key words: *Mytilus, n-TiO_2, dioxin, transcriptomics, immunohistochemistry*

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1. Introduction

Due to the increasing production of nanoparticles (NPs) and their potential release in the aquatic environment, evaluation of their biological impact on aquatic organisms represents a major concern. Based on their exceptional physicochemical properties, NPs are likely to interact (i.e. adsorption) with other pre-existing contaminants, thus possibly affecting their bioavailability/uptake and consequent biological effects (Hartmann and Baun, 2010, 2012; Matranga and Corsi, 2012). Evidence obtained so far in aquatic organisms indicate complex and often unexpected interactive responses of NPs with other pollutants, mainly depending on type of NP and contaminant and the endpoint measured, as well as differences in bioaccumulation; however, little information is available on the possible combined effects of NPs and other contaminants, including persistent organic xenobiotics, in marine organisms (reviewed in Canesi et al., 2015).

Suspension feeding bivalve mollusks have been shown to represent a significant target for different types of NPs (Baun, 2008; Canesi et al., 2012; Corsi et al., 2014). In the marine bivalve *Mytilus galloprovincialis*, the possible interactive effects of titanium dioxide (n-TiO_2) and 2,3,7,8-tetrachlorodibenzo-p-dioxins (2,3,7,8-TCDD), chosen as model NP and organochlorine contaminant, respectively, have been recently investigated (Canesi et al., 2014). A wide range of biomarkers, from molecular to tissue level, were evaluated, in hemocytes, gills and digestive gland, and distinct interactive effects were observed, depending on cell/tissue and type of measured response (Canesi et al., 2014). In the digestive gland, n-TiO_2 and TCDD showed combined effects on different aspects of lysosomal physiology; however, no interactions were observed on transcription of common stress-response and antioxidant genes, and the possible molecular targets for either contaminant, alone and in combination, were not identified.

N-TiO_2 is one of the most widely used NPs, with predicted levels in the aquatic environment at low μg L^{-1} concentrations (Robichaud et al., 2009; Sun et al., 2014), as well as one of the most extensively studied metal oxide NPs from the perspective of ecotoxicity (Menard et al., 2011). Dioxins, considered one of the most hazardous organochlorine compounds, are ubiquitous environmental contaminants, that persist and bioaccumulate through aquatic food chains (Schecter et al., 2006; Domingo and Bocio, 2007; US EPA, 2010), and are generally present in bivalve tissues at pg/g concentrations (Wade et al., 2014; Cano-Sancho et al., 2015). In the light of the environmental relevance of both n-TiO_2 and TCDD as emerging and legacy contaminants, respectively, understanding their effects, alone and in combination, in marine invertebrates deserves further attention.

In this work, a transcriptomics/immunohistochemical approach was utilized in order to elucidate the mechanisms underlying the responses to n-TiO_2 and TCDD exposure previously observed in *M. galloprovincialis* digestive gland (Barmo et al., 2013; Canesi et al., 2014). Mussels were exposed to n-TiO_2 (100 μg L^{-1}) or to 2,3,7,8-TCDD (0.25 μg L^{-1}), alone and in combination, for 96 h, in the same experimental conditions previously described (Canesi et al., 2014). Transcriptomic analysis was performed using a *M. galloprovincialis* cDNA microarray (Venier et al., 2006) previously utilized to evaluate the effects of contaminant mixtures (Dondero et al., 2010, 2011; Canesi et al., 2011). Transcription of selected genes was also quantified by qPCR and expression of target proteins of interests (tubulin) was evaluated by immunolabelling. Accumulation of TCDD was evaluated in digestive gland tissue sections by immunofluorescence analysis using an anti-dioxins antibody.

2. Materials and Methods

2.1 Characterization of n-TiO_2 primary particles, agglomeration of n-TiO_2 standard suspensions in ASW and interactions between n-TiO_2 and TCDD in ASW.

Nanosized Titanium Dioxide (n-TiO_2), Aeroxide® P25 namely Aeroxide © (declared purity of 99.9%) was kindly provided from Eigenmann & Veronelli (Milan, Italy). The obtained batch was characterized by a combination of analytical techniques (HR-TEM, TEM-EDX, XRD, HR-TEM-SAED, BET, ICPMS, etc.) as previously described (Barmo et al., 2013). Stock suspensions of n-TiO_2 were freshly prepared in filtered artificial sea water-ASW (ASTM 2004) at 10 mg L^{-1}, sonicated for 15 min at 100 W, 50% on/off cycle while cooling the dispersion in an ice bath, with a UP200S Hielscher Ultrasonic Technology (Teltow, Germany). Size distribution of n-TiO_2 suspensions were evaluated by Dynamic Light Scattering (DLS) analysis performed with a Submicron Particle Sizer Nicomp 370 (Santa Monica, Ca, USA) equipped with a 35 mW He-Ne laser, 632.8 nm laser diode and photodiode detector set at 90 °C as previously described (Brunelli et al., 2013). The obtained results, previously reported in Canesi et al. (2014) and Della Torre et al., (2015), are summarized in Table S1. Size distribution of n-TiO_2 by TEM analysis ranged approximately from 10 to 65 nm (27 nm average), with shape partly irregular and semi-spherical. The main crystallographic phases were confirmed to be anatase and rutile (4:1 ratio), in accordance with the manufacturer's data. BET analysis indicated a specific surface area of 61±0.2 m^2/g, a pore size of 0.5±0.1 ml/g and a bimodal pore size distribution in the 2-4 and 10-90 nm size range, respectively. According to these results, the selected n-TiO_2 sample could be classified as mesoporous. DLS analysis of a n-TiO_2 suspension (100 μg L^{-1} in ASW), indicated the general

formation of agglomerates, starting immediately after n-TiO_2 addition (180 ±21 nm), and whose average size increased after 25 h (207 ±26 nm) and 50 h (304 ±38 nm).

Chemical interaction between n-TiO_2 and 2,3,7,8- TCDD in ASW and in the presence of vehicle DMSO (0.001‰) was investigated by UV-Vis adsorption spectroscopy and Nuclear Magnetic Resonance (NMR) spectroscopy as described in Della Torre et al. (2015), indicating no interaction between nano-TiO_2 and TCDD in ASW exposure medium.

2.2. Animals and treatments

Mussels (*Mytilus galloprovincialis* Lam.) 4–5 cm long, purchased from an aquaculture farm (Arborea-OR, Italy), were kept for 3 days in static tanks containing 1 L ASW mussel^{-1} at 16°C and daily fed with 30 mg mussel^{-1} Marine Liquifry (Interpet, England). Sea water was changed daily.

Exposure experiments were performed as previously described (Canesi et al., 2014). Stock suspensions of n-TiO_2 in ASW were prepared by sonication as for DLS analysis and immediately spiked in the tanks in order to reach the desired concentration. TCDD (Wellington Laboratories, Ontario, Canada) purchased in dimethyl sulfoxide (DMSO) (32.2± 1.6 mg mL^{-1}) was suitably diluted in ASW and spiked in the tanks to reach the desired concentration. Mussels (at least 15 mussels in quadruplicate for each condition) were exposed for 96 h to either n-TiO_2 at 100 µg L^{-1} nominal concentration levels, or TCDD 0.25 µg L^{-1} and to both n-TiO_2 and TCDD at the same concentrations. Two parallel groups of control (untreated) and vehicle-treated (0.001 % DMSO) mussels were kept in clean ASW. Sea water was changed each day before addition of the contaminants. Animals were not fed during the experiments. No mortality was observed in different experimental conditions.

After treatments, digestive glands were rapidly removed and frozen for subsequent analyses. For transcriptomics, tissues were kept at – 20 °C in a RNA preserving solution (RNA Later, Sigma-Aldrich); for histochemistry, tissues were mounted on aluminum chucks, frozen in super-cooled n-hexane and stored at – 80 ° C.

2.3 Histological analysis

Frozen digestive gland sections (10 µm) of ten mussels from each exposure condition were cut by cryostat (Leica CM3050) and flash-dried by transferring them onto poly-L-lysine-coated microscope slides at room temperature. After fixation (4% paraformaldehyde-PFA in phosphate buffer saline-PBS, pH 7.2, 20 min), sections were stained with hematoxylin and eosin (Chan, 2014) or Fontana-Masson (for melanin staining), mounted in DPX and then viewed under 400 × magnification by a Axiolab photo-microscope (Zeiss).

2,4 Immunofluorescence analysis

Digestive gland tissue sections obtained as described above were fixed in PFA solution (4% in PBS, pH 7.2) for 20 min at 20 ± 1 °C.

Immunofluorescent anti-tubulin staining: after fixation, sections were washed three times in PBS (5 min) and incubated in a permeabilisation and blocking solution (0.5% Triton X-100, 2% bovine serum albumin-BSA, 0.5% goat serum in PBS) for 1 h at 20 ± 1 °C. After rinsing, sections were incubated with the primary antibody (rabbit polyclonal to tubulin, Abcam, 1/100 in PBS containing 1% BSA and 0.05% Triton X-100) overnight at 4 °C, and then in the secondary antibody i.e. goat anti-rabbit IgGH&L (Chromeo™ 488) (Abcam) (1/100 in 1% BSA and 0.05% Triton X-100 in PBS) for 1 h at 20 ± 1 °C in the dark. Sections were then rinsed in PBS, stained with DAPI (DNA-specific fluorescent probe) and then mounted in Mowiol mounting medium (Cold Spring Harb Protoc, 2006).

Labelling with the monoclonal mouse antibody anti-dioxins, highly specific for TCDD (monoclonal mouse anti-dioxins antibody from Cosmo Bio Co., Ltd.) was performed as described in Sforzini et al. (2014). Sections prepared as described above were incubated in a permeabilization and blocking solution (0.5% Triton X-100, 2% BSA, 0.5% rabbit serum in PBS) and then with the primary antibody (anti-dioxins antibody 1/100 in PBS containing 1% BSA and 0.05% Triton X-

100) overnight at 4 °C in a moist chamber. Sections were the washed three times in PBS (5 min) and the secondary antibody was applied, i.e. polyclonal rabbit to mouse IgG (FITC) (Abcam) (1/100 in 1% BSA and 0.05% Triton X-100 in PBS) for 1 h at 20 ± 1 °C in the dark. Finally, sections were counterstained with DAPI and mounted.

Controls for non-specific staining included sections that were processed in the absence of the secondary antibodies. Slides were viewed under 400 x magnification by an inverted photo-microscope (Zeiss Axiovert 100M connected to a digital camera Zeiss AxioCamMRm) equipped for fluorescence microscopy using FITC and DAPI emission filters. Images were analysed using an image analysis system (Scion Image) that allowed for the quantification of the mean fluorescence intensity. Data were analyzed by the non-parametric Mann-Whitney U-test.

2.5 Microarray hybridization and analysis

Competitive dual-color microarray hybridization was performed with the Mytarray V1.1 platform (Venier et al., 2006); fluorescent-labeled cDNA probes were obtained by direct labeling in the presence of modified Cy3- and Cy5-dCTP (Perkin Elmer). The procedure was carried out as described by Dondero et al. (2011) using 0.5 μg of an anchored oligodT (19) VN. Total RNA was extracted from digestive gland using acid phenol-chloroform precipitation according to Chomczynski and Sacchi (1987), with TRI-Reagent (Sigma-Aldrich). RNA was further purified by precipitation in the presence of 1.5 M $LiCl_2$, and the quality of each RNA preparation was confirmed by UV spectroscopy and TBE agarose gel electrophoresis in the presence of formamide, as described in Banni et al. (2011). Laser scanning of microarrays was performed with an Agilent G2565CA scanner (Agilent Technologies, Inc., USA) at 5-μm resolution. Sixteen-bit TIFF images were analyzed with Genepix 6.0 (Axon) to extract raw fluorescence data from each spot.

The experimental design accounted for a complete "triangular loop" in which each RNA sample from the tissue of mussels exposed to chemicals (n-TiO_2, TCDD and mixture) was

hybridized with RNA from control mussels. Each experimental condition had at least four biological replicates of RNA samples from single individual female animals using the day-swap procedure, for a total of 12 experiments.

Computational and statistical analysis of microarray data was performed using the Linear Mode for Microarray Analysis software (Smyth, 2004). Offset background subtraction, loess normalization, and least-squares regression were employed, along with moderated t-tests and empirical Bayes statistics. Gene expression was considered to be significantly different in the test condition versus the reference condition when the log-odd value (B) was higher than 0. The analysis procedure was carried out essentially as described in Dondero et al. (2011). Microarray data were clustered with the Genesis software (D'haeseleer, 2005; Sturn et al., 2002).

MIAMI-compliant microarray data, including a detailed description of the experimental design and each hybridization experiment, were deposited in the Gene Expression Omnibus with identifier "GSE69867". The following link provides access to the deposited data http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69867

2.6 Functional genomics analysis

Functional characterization of mussel genes represented on the microarray was based on Gene Ontology (GO) annotation and was carried out with Blast2GO (Conesa et al., 2005) using default parameters. Briefly, 1,673 mussel sequences with EMBL IDs were subjected to the annotation analysis; 880 sequences had no BLASTX hits (Altschul et al., 1990), while another 63 sequences did not map to GO terms. Putative annotation for 873 mussel sequences was established based on GO terms for the first 20 BLASTX hits or based on protein domains obtained from Inter Pro Scan (Altschul et al., 2005; Banni et al., 2011). GO term enrichment was evaluated with hypergeometric statistics ($p<0.05$); the distribution of GO terms in each set of interest was compared against the set reflecting the entire microarray sequence catalogue.

2.7 qRT-PCR

qRT-PCR was carried out with the same RNA extract used for microarray hybridization. Relative mRNA abundances of the mussel genes encoding for dynein light chain 2 (AJ516886), beta tubulin (AJ516796), beta-n-acetylhexosaminidase (AJ623463), matrilin 2 (AJ625256), heat shock protein 90 (AJ625915) fk506-binding protein (AJ624969). Probes and primer pairs (Table S2) were designed using Beacon Designer v3.0 (Premier BiosoftnmInternational, Inc.). All primers and dual-labeled Taqman probes were synthesized by MWG-Biotech Gmbh (Germany).

cDNA (25 ng RNA reverse-transcribed to cDNA) was amplified in a CFX384 Real-Time PCR detection system (Bio-Rad Laboratories) with iQTM Multiplex Power mix (Bio-Rad Laboratories) according to the manufacturer's instructions for the triplex protocol. All multiplex combinations accounted for the following dual fluorescence tags: 6-carboxyfluorescein/Black Hole (BH) 1, 6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein/BH1, and Texas Red/BH2. Briefly, cDNA was amplified in the presence of 1X iQTM Multiplex Power mix, 0.3 µM each primer, and 0.1 µM each probe (Table S2) in a final volume of 10 µL. Relative expression data were geometrically normalized to18S rRNA (L33452), an invariant actin isotype (AJ625116), and ribosomal proteinriboL27(AJ625928), which were selected from a list of genes whose expression did not vary over more than 50 conditions (including toxic treatments, stages of the life cycle, and various tissues) (Negri et al., 2013). A specific duplex Taqman assay was developed to amplify 0.25 ng of RNA reverse-transcribed to cDNA in the presence of 0.1 µM of each dual-labeled probe (hexachlorofluorescein/BH1 for actin and Texas Red/BH2 for 18S rRNA) and 0.1 µM and 0.4 µM of forward and reverse primer, respectively, for 18S rRNA and actin (Table S2). For all Taqman assays, the thermal protocol was as follows: 30 s at 95 °C, followed by 40 cycles of 10 s at 95 °C and 20 s at 60 °C. qRT-PCR was performed with four biological replicates and three technical replicates. The normalized expression was then expressed as relative quantity of mRNA (relative

expression) with respect to the control sample. Data are the mean ±SD of at least 4 biological samples. Statistical analyses were carried out on the group mean values using a random reallocation test (Pfaffl et al., 2002).

3. Results

3.1 Histology

Representative images of hematoxylin/eosin stained tissue sections are reported in Fig. 1 (A-D). In the digestive tubules of control mussels cells showed evident cytoplasm and regular distribution of nuclei (Fig. 1A). In samples from n-TiO_2-exposed mussels infiltrating hemocytes were observed (Fig. 1B). Much more evident alterations were observed in TCDD-exposed samples (Fig. 1C): cells with a reduced cytoplasmic volume and intracellular vacuolation, this latter in line with the previously observed increase in neutral lipids (Canesi et al., 2014). Moreover, strong hemocyte infiltration was observed. Similar alterations were observed in samples from mussels exposed to the mixture (cells with reduced cytoplasm, increased vacuolation, irregular distribution of nuclei, infiltrating hemocytes), although to a lower extent (Fig. 1D). In all exposure conditions digestive gland cells showed the presence of several small intracellular brown granules (see inset in Fig. 1B) that were mostly positive to Fontana-Masson (melanin) staining (not shown), though no difference was observed among treatments.

3.2 Transcriptomic analysis

Large-scale transcriptional profiling was performed to identify the main molecular mechanisms involved in the response of mussels to n-TiO_2, TCDD and their mixture. Using a 1.673-feature cDNA microarray, we initially generated transcriptome profiles for digestive glands of mussels from each exposure condition and compared these data to profiles from control animals. Microarray analysis revealed distinct patterns for 115 differentially expressed genes (DEGs) under

at least one condition (Fig. 2; Table S3, Table S4). The n-TiO_2 exposure group displayed 48 DEGs of which 68.75% (33 DEGs) were up-regulated. In TCDD-treated mussels, microarray analysis displayed 49 DEGs with 19 up-regulations (38.77%), while the n-TiO_2/TCDD group showed 62 DEGs, mostly down-regulated (33 genes, 54.83%). About 27.53% of DEGs (n= 19) found in the n-TiO_2/TCDD group overlapped with those modulated by n-TiO_2, whereas 31.88% (22 genes) was shared with TCDD. The expression of another set of 32 DEGs was modulated only in n-TiO_2/TCDD samples (Fig. 2, Table S4). Of the 115 DEGs, only 11 genes were shared among the three experimental conditions datasets (Table 1; S4).

To gain a further insight into the major patterns of gene expression, we identified significantly enriched GO terms to reveal the biological processes contributing to the responses to n-TiO_2, TCDD and their mixture (Table 2; Table S4). Analysis of the 48 DEGs in animals exposed to n-TiO_2 alone (versus control) highlighted the following contributing biological processes: "microtubule-based movement", "cellular catabolic processes" and "component organization at cellular level" (Table S3). In addition, the 49 DEGs obtained from animals exposed to TCDD were significantly associated with 4 biological processes, largely composed of "Intracellular signal transduction" and "microtubule-based movement" processes (6 DEGs). "Response to unfolded proteins" as well as "endocrine disruption- related" processes were also depicted (Table S3). GO analysis of the 62 DEGs in samples from the mixture exposure group highlighted 4 biological processes. Among them, "intracellular signal transduction and endocrine disruption" , as reported in TCDD exposed mussels, and "microtubule-based movement", as reported in n-TiO_2 exposed animals. "Response to chemical stimulus" was the process newly generated in animals exposed to the mixture. Expression of genes involved in the most relevant processes depicted in the transcriptomic response to each individual compound and their mixture (microtubule-based movement and endocrine disruption-related processes) are reported in Fig. 3. The results clearly indicate distinct patterns of transcription of target genes in the three experimental conditions.

We also carried out qRT-PCR to confirm and refine the relative expression levels of 6 homologue genes belonging to the most important depicted biological processes: dynein light chain 2, beta tubulin, beta-n-acetylhexosaminidase, matrilin 2, heat shock protein 90, fk506-binding protein. Microarray and qPCR data showed a positive relationship in all cases (Fig. S1).

3.3 Immunofluorescence analysis of tubulin in digestive gland tissue sections

Since transcriptomic data showed DEGs related to microtubule-based movement in all exposure conditions, with differences among treatments, tubulin expression at the protein level was evaluated in mussel tissue sections by immunofluorescence microscopy using an anti-tubulin antibody. As shown in Fig. 4 (A-D), exposure to different treatments increased tubulin expression compared to controls. These observations were supported by quantification of the fluorescence signal by digital imaging, indicating a significant increase in tubulin expression in all exposure groups with respect to controls, with the strongest effect induced by n-TiO_2, followed by the mixture and TCDD alone (Fig. 4E). Interestingly, the observed increase in tubulin fluorescence was partly due to the presence in the digestive gland cells of exposed mussels of highly stained protein containing granules.

3.4 Immunofluorescence detection of TCDD

In order to highlight the presence and distribution of TCDD in mussel tissues, an immunohistochemical method recently developed by Sforzini et al. (2014), which involves the use of an antibody against dioxins, was employed. The results obtained in the different experimental conditions are reported in Fig. 5 (A-D). No anti-dioxins immunoreactivity was detected in control (Fig. 5A) nor in n-TiO_2 -exposed samples (Fig. 5 B), whereas a clear fluorescence signal (green) was observed in the digestive gland tubules from mussels exposed to TCDD, alone (Fig. 1 C) and in combination with n-TiO_2 (Fig. 5 D). However, quantification of the fluorescence signal by digital

imaging showed that TCDD accumulation was lower in samples exposed to the mixture than to TCDD alone (Fig. 5 E).

4. Discussion

Previous data obtained in *M. galloprovincialis* showed combined exposure to n-TiO_2 and TCDD induced interactive effects on biomarkers measured at different levels of biological organization, related to immune, gill and digestive gland function (Canesi et al., 2014). However, the relationship between exposure to the mixture and biological responses observed at the tissue level, as well as the molecular targets involved, were not elucidated. In this work, these aspects were investigated in the digestive gland of mussels exposed to the same experimental conditions using a transcriptomic/immunohistochemical approach.

Histological observations revealed strong effects of TCDD, but not of n-TiO_2, on the cells of digestive tubules, as well as hemocytic infiltration; less severe alterations were induced by the mixture. These data indicate that exposure to the mixture decreased, rather than increased stressful conditions induced by TCDD at the tissue level. It should be noted that in all exposure conditions several small brown granules were observed: these granules, resulting insoluble in organic solvents (Pearse, 1985) and mostly positively stained with Fontana-Masson (data not shown), could probably be considered melanin-reach bodies. Melanin deposition as well as hemocytic infiltration are important components of the invertebrate inflammatory stress response (Palmer et al., 2008).

The capacity of a stressed organism to regulate its cellular processes through transcriptional control can allow it to cope with the alteration of cellular functions and to avoid non-reversible cellular alterations. In order to investigate how the transcriptome of mussel digestive gland was affected by exposure to n-TiO_2, TCDD and their mixture, we employed a 1.7k microarray that has been successfully utilized for identifying differential transcriptional responses to chemical mixtures

in mussel tissues (Canesi et al., 2011; Dondero et al, 2011), as well as for characterizing physiological process during an annual cycle (Banni et al., 2011). In the present study, the microarray data identified 48 DEGs, 49 DEGs and 62 DEGs in response to n-TiO_2, TCDD and their mixture, respectively. GO analyses revealed that distinct biological processes were involved in the response to each individual compound and to their mixture.

Cytoskeletal protection was previously suggested as a general potential mechanism for increased tolerance to environmental stressors in mussels (Negri et al., 2013; Lockwood et al., 2010). Transcriptomics data in exposed mussels showed changes in transcription of genes associated with cytoskeletal structures, revealing an up-regulation of microtubule-based movement related genes in samples from n-TiO_2 (8 genes) and mixture (5 genes) exposure groups. Among genes involved in this process, tubulin up-regulation at transcriptional level was confirmed by qRT-PCR, indicating that the mRNA levels for tubulin were increased in response to n-TiO_2 and the mixture, whereas no effects were observed with TCDD. These data apparently indicate a specific response to n-TiO_2 exposure.

This aspect was further investigated by an immunohistochemical approach using an anti-tubulin antibody. Digestive gland cells of control animals showed a well-developed microtubular cytoskeleton. In the tissues of treated organisms a significant increase in tubulin immunolabelling was observed; interestingly, in mussels exposed to n-TiO_2 and, to a lesser extent, in those exposed to the mixture, part of the fluorescence was detected in form of granules. Microtubules are dynamic structures that undergo continual assembly and disassembly within the cell (Cooper, 2000); moreover, the synthesis of tubulin is autoregulated depending on the level of unpolymerized tubulin (Cleveland, 1989). Depolymerization of the microtubule network and formation of granules containing tubulin has been previously described in mammalian cells as a consequence of different stress/pathological conditions (Clark and Shay, 1981; Martin et al., 2010). Our data seem to support the hypothesis that in animals exposed to n-TiO_2 and the mixture the cytoskeleton disassembly

could involve a loss of tubulin that is sequestrated in granules; such an effect may be compensated by an increase in tubulin biosynthesis at the transcriptomic level. In TCDD-exposed animals, a small but significant increase in tubulin immunoreactivity was observed. Such variation is not apparently dependent on changes in gene transcription and could reflect a limited variation in the balance between synthesis and degradation of this protein.

It is generally thought that TCDD exposure results in little adverse effects in invertebrates, although significant effects on bivalve reproduction have been previously reported (Wintermyer et al., 2007). Our data indicated that TCDD up-regulated transcription of vitelline envelope-related genes, alone and in combination with n-TiO_2, whereas no effects were induced by n-TiO_2 alone. TCDD also affected processes related to signal transduction, and the effects where reduced in the presence of n-TiO_2. Up-regulation of *Mytilus* Estrogen Receptors (MER1 and MER2) by the mixture n-TiO_2/TCDD was previously observed (Canesi et al., 2014). These results represent the first data on the effects of TCDD in marine invertebrates at the molecular level, and support the hypothesis that TCDD could exert some endocrine-related effects in mussels.

Transcriptomics data also underlined contrasting effects of n-TiO_2 and TCDD on lysosomal function. n-TiO_2 alone induced up-regulation of B-hexosoaminidase, the major lysosomal hydrolytic enzyme, and of Atg5, one of those "autophagy essential genes" required for autophagosome formation, as well as for biogenesis of lysosomes in an autophagy-independent manner (Peng et al., 2014). These effects, related to stimulation of the lysosomal function, were not observed in samples exposed to TCDD alone and to the mixture. These data are in line with previous results obtained in the same experimental conditions on lysosomal biomarkers, indicating that n-TiO_2 alone induced a modest decrease in lysosomal membrane stability, suggesting activation of the lysosomal function, whereas strong lysosomal damage was induced by TCDD alone and the mixture (Canesi et al., 2014).

Finally, tissue TCDD accumulation was evaluated in digestive gland tissue sections utilizing the immunohistochemical method recently developed in earthworm tissues by Sforzini et al. (2014). This method proved as a sensitive tool for demonstrating dioxin accumulation in different tissues of animals exposed to low concentrations of TCDD (0.01-0.1 ppb), in accordance with chemical data. The results here presented show that the anti-dioxins antibody can be suitably applied to detect TCDD accumulation in the tissues of mussels exposed to TCDD in the same nominal concentration range. Interestingly, our data show that in mussel digestive gland, co-exposure with n-TiO_2 resulted in a decreased TCDD accumulation. This observation may partly explain why in the digestive gland certain responses induced by the mixture were reduced with respect to those elicited by each contaminant alone. However, in whole soft tissues of mussels exposed to the mixture higher TCDD concentrations were detected with respect to the TCDD exposure group, this probably due to the contribution of the lipid rich mantle fraction (Canesi et al., 2014). In aquatic organisms, co-exposure to different types of NPs, including n-TiO_2, with organic contaminants can result in increased, decreased or no change in xenobiotic accumulation and toxicity, depending on the chemical, model organism and target tissue, and experimental settings; however, the mechanisms involved are far to be elucidated (Canesi et al., 2015).

Conclusions

Few studies on gene expression patterns in response to exposure to different types of NPs in aquatic organisms are so far available, and restricted to freshwater species (Gagnè et al., 2010; Jovanović and Palić, 2011; Adam et al., 2015). The results obtained in this work represent the first data on transcriptional responses of marine invertebrates to exposure not only to n-TiO_2 as a model NPs, but also to a legacy organic contaminant like TCDD. The results confirm both distinct and interactive effects of n-TiO_2 and TCDD in mussel digestive gland at the molecular level, identify

the main molecular targets involved in the response to each chemical, and underline how combined exposure to n-TiO_2 and TCDD does not result in increased toxicity or harmful effects in mussels.

Acknowledgments

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References

Adam, N., Vergauwen, L., Blust, R., Knapen, D., 2015. Gene transcription patterns and energy reserves in Daphnia magna show no nanoparticle specific toxicity when exposed to ZnO and CuO nanoparticles. Environ. Res. 138, 82-92.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Altschul, S.F., Wootton, J.C., Gertz, E.M., Agarwala, R., Morgulis, A., Schäffer, A.A., Yu, Y.K., 2005. Protein database searches using compositionally adjusted substitution matrices. FEBS J. 272(20), 5101-5109.

ASTM, 2004. International Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Salt Water Bivalve Molluscs. E 724-798.

Banni, M., Negri, A., Mignone, F., Boussetta, H., Viarengo, A., Dondero, F., 2011. Gene expression rhythms in the mussel *Mytilus galloprovincialis* (Lam.) across an annual cycle. PLoS ONE 6(5), e18904 doi:10.1371/journal.pone.0018904.

Barmo, C., Ciacci, C., Canonico, B., Fabbri, R., Cortese, K., Balbi, T., Marcomini, A., Pojana, G., Gallo, G., Canesi, L., 2013. *In vivo* effects of n-TiO_2 on digestive gland and immune function of the marine bivalve *Mytilus galloprovincialis*. Aquat. Toxicol. 132-133, 9-18.

Baun, A., Hartmann, N.B., Grieger, K., Kusk, K.O., 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. Ecotoxicology 17, 387-395.

Brunelli, A., Pojana, G., Callegaro, S., Marcomini, A., 2013. Agglomeration and sedimentation of titanium dioxide nanoparticles (n-TiO_2) in synthetic and real waters. J. Nanopart. Res. 15, 1-10.

Canesi, L., Negri, A., Barmo, C., Banni, M., Gallo, G., Viarengo, A., Dondero, F., 2011. The organophosphate chlorpyrifos interferes with the responses to 17β-Estradiol in the digestive gland of the marine mussel *Mytilus galloprovincialis*. PLoS One. 6(5), e19803.

Canesi, L., Ciacci, C., Fabbri, R., Marcomini, A., Pojana, G., Gallo, G., 2012. Bivalve molluscs as an unique target group for nanoparticle toxicity. Mar. Environ. Res. 76, 16-21.

Canesi, L., Frenzilli, G., Balbi, T., Bernardeschi, M., Ciacci, C., Corsolini, S., Della Torre, C., Fabbri, R., Faleri, C., Focardi, S., Guidi, P., Kočan, A., Marcomini, A., Mariottini, M., Nigro, M., Pozo-Gallardo, K., Rocco, L., Scarcelli, V., Smerilli, A., Corsi, I., 2014. Interactive effects of n-TiO_2 and 2,3,7,8-TCDD on the marine bivalve *Mytilus galloprovincialis*. Aquat. Toxicol. 153, 53-65.

Canesi, L., Ciacci, C., Balbi, T., 2015. Interactive effects of nanoparticles with other contaminants in aquatic organisms: Friend or foe? Mar. Environ. Res. doi: 10.1016/j.marenvres.2015.03.010.

Cano-Sancho, G., Sioen, I., Vandermeersch, G., Jacobs, S., Robbens, J., Nadal, M., Domingo, J.L., 2015. Integrated risk index for seafood contaminants (IRISC): Pilot study in five European countries. Environ. Res. doi: 10.1016/j.envres.2015.03.006.

Chan, J.K., 2014. The wonderful colors of the hematoxylin-eosin stain in diagnostic surgical pathology. Int. J. Surg. Pathol. 22, 12-32.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-169.

Clark, M.A., Shay, J.W., 1981. The role of tubulin in the steroidogenic response of murine adrenal and rat Leydig cells. Endocrinology 109, 2261-2263.

Cleveland, D.W., 1989. Autoregulated control of tubulin synthesis in animal cells. Curr. Opin. Cell. Biol. 1, 10-14.

Cold Spring Harb Protoc, 2006. doi: 10.1101/pdb.rec10255.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M., 2005. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674-3686.

Cooper, G.M., 2000. The Cell, 2nd edition. A Molecular Approach. Boston University Sunderland (MA): Sinauer Associates.

Corsi, I., Cherr, G.N., Lenihan, H.S., Labille, J., Hassellov, M., Canesi, L., Dondero, F., Frenzilli, G., Hristozov, D., Puntes, V., Della Torre, C., Pinsino, A., Libralato, G., Marcomini, A., Sabbioni, E., Matranga, V., 2014. Common strategies and technologies for the ecosafety assessment and design of nanomaterials entering the marine environment. ACS Nano 8, 9694-9709.

Della Torre, C., Buonocore, F., Frenzilli, G., Corsolini, S., Brunelli, A., Guidi, P., Kočan, A., Mariottini, M., Mottola, F., Nigro, M., Pozo, K., Randelli, E., Vannuccini, M.L., Picchietti, S., Santonastaso, M., Scarcelli, V., Focardi, S., Marcomini, A., Rocco, L., Scapigliati, G., Corsi, I., 2015. Influence of titanium dioxide nanoparticles on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin bioconcentration and toxicity in the marine fish European seabass (*Dicentrarchus labrax*). Environ. Pollut. 196, 185-193.

D'haeseleer, P., 2005. How does gene expression clustering work? Nat. Biotechnol. 23(12), 1499-1501.

Domingo, J.L., Bocio, A., 2007. Levels of PCDD/PCDFs and PCBs in edible marine species and human intake: A literature review. Environ. Int. 33, 397-405.

Dondero, F., Placentini, L., Banni, M., Rebelo, M., Burlando, B., Viarengo, A., 2006. Quantitive PCR analysis of two molluscan metallothionein genes unveils differential expression and regulation. Gene 345, 259-270.

Dondero, F., Negri, A., Boatti, L., Marsano, F., Mignone, F., Viarengo, A., 2010. Transcriptomic and proteomic effects of a neonicotinoid insecticide mixture in the marine mussel (*Mytilus galloprovincialis*, Lam.). Sci. Total Environ. 408, 3775-3786.

Dondero, F., Banni, M., Negri, A., Boatti, L., Dagnino, A., Viarengo, A., 2011. Interactions of a pesticide/heavy metal mixture in marine bivalves: a transcriptomic assessment. BMC Genomics 12, 195.

Gagné, F., Fortier, M., Yu, L., Osachoff, H.L., Skirrow, R.C., van Aggelen, G., Gagnon, C., Fournier, M., 2010. Immunocompetence and alterations in hepatic gene expression in rainbow trout exposed to CdS/CdTe quantum dots. J. Environ. Monit. 12(8), 1556-1565.

Hartmann, N.B., Baun, A., 2010. The nano cocktail: ecotoxicological effects of engineered nanoparticles in chemical mixtures. Integr. Environ. Assess. Manag. 6, 311-314.

Jovanović, B., Ji, T., Palić, D., 2011. Gene expression of zebrafish embryos exposed to titanium dioxide nanoparticles and hydroxylated fullerenes. Ecotoxicol. Environ. Saf. 74(6), 1518-1525.

Lockwood, B.L., Sanders, J.G., Somero, G.N., 2010. Transcriptomic responses to heat stress in invasive and native blue mussels (genus *Mytilus*): molecular correlates of invasive success. J. Exp. Biol. 213(Pt 20), 3548-3558.

Martin, D., Duarte, M., Lepault, J., Poncet, D., 2010. Sequestration of free tubulin molecules by the viral protein NSP2 induces microtubule depolymerization during rotavirus infection. J. Virol. 84, 2522-2532.

Matranga, V., Corsi, I., 2012. Toxic effects of engineered nanoparticles in the marine environment: model organisms and molecular approaches. Mar. Environ. Res. 76, 32-40.

Menard, A., Drobne, D., Jemec, A., 2011. Ecotoxicity of nanosized TiO_2. Review of *in vivo* data. Environ. Pollut. 159, 677-684.

Negri, A., Oliveri, C., Sforzini, S., Mignione, F., Viarengo, A., Banni, M., 2013. Transcriptional response of the mussel Mytilus galloprovincialis (Lam.) following exposure to heat stress and copper. PLoS ONE 8(6), e66802.

Palmer, C.V., Mydlarz, L.D., Willis, B.L., 2008. Evidence of an inflammatory-like response in non-normally pigmented tissues of two scleractinian corals. Proc. Biol. Sci. 275, 2687-2693.

Pearse, A.G.E., 1985. Histochemistry: theoretical and applied. Vol. 2. Edinburgh: Churchill Livingstone, pp. 441-1055.

Peng, J., Zhang, R., Cui, Y., Liu, H., Zhao, X., Huang, L., Hu, M., Yuan, X., Ma, B., Ma, X., Takashi, U., Masaaki, K., Liang, X., Yu, L., 2014. Atg5 regulates late endosome and lysosome biogenesis. Sci. China Life Sci. 57(1), 59-68.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Robichaud, C.O., Uyar, A.M., Darby, M.R., Zucher, L.G., Wiesner, M.R., 2009. Estimates of upper bounds and trends in nano-TiO_2 production as a basis for exposure assessment. Environ. Sci. Technol. 43, 4227-4233.

Schecter, A., Birnbaum, L., Ryan, J.J., Constable, J.D. 2006. Dioxins: An overview. Environ. Res. 101, 419-428.

Sforzini, S., Moore, M.N., Boeri, M., Benfenati, E., Colombo, A., Viarengo, A., 2014. Immunofluorescence detection and localization of B[*a*]P and TCDD in earthworm tissues. Chemosphere 107, 282-289.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol Biol. 3, Article 3 doi: 10.2202/1544-6115.1027.

Sturn, A., Quackenbush, J., Trajanoski, Z., 2002. Genesis: cluster analysis of microarray data. Bioinformatics 18(1), 207-208.

Sun, T.Y., Gottschalk, F., Hungerbühler, K., Nowack, B., 2014. Comprehensive probabilistic modelling of environmental emissions of engineered nanomaterials. Environ. Pollut. 185, 69-76.

US EPA, 2010. EPA's Reanalysis of Key Issues Related to Dioxin Toxicity and Response to NAS Comments (External Review Draft). US Environmental Protection Agency, Washington, DC, EPA/600/R-10/038A.

Venier, P., De Pittà, C., Pallavicini, A., Marsano, F., Varotto, L., Romualdi, C., Dondero, F., Viarengo, A., Lanfranchi, G., 2006. Development of mussel mRNA profiling: can gene expression trends reveal coastal water pollution? Mutat. Res. 602, 121-134.

Wade, T.L., Sweet, S.T., Sericano, J.L., Defreitas, D.A., Lauenstein, G.G., 2014. Polychlorinated dibenzo-p-dioxins and dibenzofurans detected in bivalve samples from the NOAA National Status and Trends Program. Mar. Pollut. Bull. 81(2), 317-324.

Wintermyer, M., Cooper, K., 2007. The development of an aquatic bivalve model: evaluating the toxic effects on gametogenesis following 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) exposure in the eastern oyster (*Crassostrea virginica*). Aquat. Toxicol. 81, 10-26.

Figure Captions

Figure 1 – Hematoxylin-eosin staining of digestive gland tissue sections from Control (A) and treated (B-D) mussels (B = n-TiO_2; C = TCDD; D = Mixture), these latter showing intracellular vacuolation (V), infiltrating haemocytes (H) and pigmented granules (arrows and inset to Fig. 1B).

Figure 2 - Gene expression profiles in the digestive gland of mussels exposed to n-TiO_2, TCDD and their mixture. The heat map (A) (Pearson correlation, complete linkage algorithm) reports the log2

relative expression level with respect to the reference condition. 115 differentially expressed genes were generated in at least one condition. Microarray data were analyzed using the Linear Mode for Microarray Analysis (LIMMA) software as described in Dondero et al., (2006). B statistics with adjusted p value 0.05 and B.0 were used as threshold for rejection of the null hypothesis (no variation). Supporting information to Fig. 2 is present in Table S3 and Table S4. The Venn diagram representation of gene expression patterns (B) clearly depicted that only 11 DEGs shared between the tree experimental conditions, 19 DEGs between n-TiO2 and mixture and 22 DEGs between TCDD and mixture. All DEGs are obtained respect to the control condition. Data used to generate the Venn-diagram were obtained from microarray analysis (Table S4).

Figure 3 - Expression pattern of genes related to microtubule-based movement processes (A) and endocrine disruption processes (B) in the digestive gland of mussels exposed to n-TiO_2, TCDD and their mixture. Expression level was calculated with respect to the reference condition. Supporting information to Fig. 3 is present in Table 2 and Table S3.

Figure 4 - Anti-tubulin immunohistochemical staining (green: Chromeo™ 488 conjugated secondary antibody; blue: DAPI nuclear staining) of digestive gland tissue sections from mussels exposed to different experimental conditions (A = Control; B = n-TiO_2; C = TCDD; D = Mixture). (E) Quantitative fluorescence analysis of anti-tubulin immunoreaction. Data are mean ± SD of at least five replicates; * = $p < 0.05$ (Mann-Whitney *U*-test).

Figure 5 - Anti-dioxins immunohistochemical staining (green: FITC conjugated secondary antibody; blue: DAPI nuclear staining) of digestive gland tissue sections from mussels exposed to different experimental conditions (A = Control; B = n-TiO_2; C = TCDD; D = Mixture). (E)

Quantitative fluorescence analysis of anti-dioxin immunoreaction. Data are mean ± SD of at least five replicates; * = $p < 0.05$ (Mann-Whitney U-test).

Table 1 - Number of DEGs in all experimental conditions with respect to the control. Data used to generate the table were obtained from microarray analysis (Table S3).

Condition	n-TiO_2	TCDD	Mixture
DEGs	48	49	62
Up-regulated	33 (68.75%)	19 (38.77%)	28 (45.16%)
Down-regulated	15 (31.25%)	30 (61.22%)	34 (54.83 %)

Table 2: GO term over-representation analysis of DEGs in the digestive gland tissue of mussels exposed to n-TiO_2, TCDD and their mixture.

Condition	GO Term	N	Up	Gene ID
n-TiO_2	Microtubule-based movement	8	8	AJ516886,AJ625595,AJ625032,AJ625091, AJ516796, AJ623456, AJ623456, AJ625824
	Cellular component organization at cellular level	6	5	AJ624768,AJ625083,AJ623349,AJ625834, AJ625105, AJ623463
	cellular catabolic process	3	0	AJ624144, AJ624260, AJ624360
TCDD	intracellular signal transduction	4	2	AJ625058, AJ624360, AJ623546, AJ626333
	microtubule-based movement	4	2	AJ623456, AJ626006, AJ625824, AJ625799
	Folding proteins	2	1	AJ624969, AJ625621
	Endocrine disruption	2	2	AJ625803, AJ516818
Mixture	microtubule-based movement	6	6	AJ516886,AJ625595,AJ625032,AJ625091, AJ516796, AJ623456
	response to chemical stimulus	3	3	AJ624848, AJ624144, AJ625621
	Endocrine disruption	2	2	AJ625803, AJ516818
	intracellular signal transduction	2	0	AJ625058, AJ624260

Gene Ontology terms enrichment analysis was carried out comparing the GO term frequency distribution in each cluster against that in the whole microarray set (hypergeometric statistics, $p<0.05$). Only the lowest node per branch of the hierarchical structure of the Gene Ontology that fulfills the filter condition - cut off 2 sequences- was reported. Showed are: Experimental conditions; GO Term, over-represented feature; N, number of mussel sequences associated to each GO term; Gene ID, EMBL accession number of each sequence found. The over-represented GO terms in exposed mussels versus control (hypergeometric stats, $p < 0.05$).

Figure 1

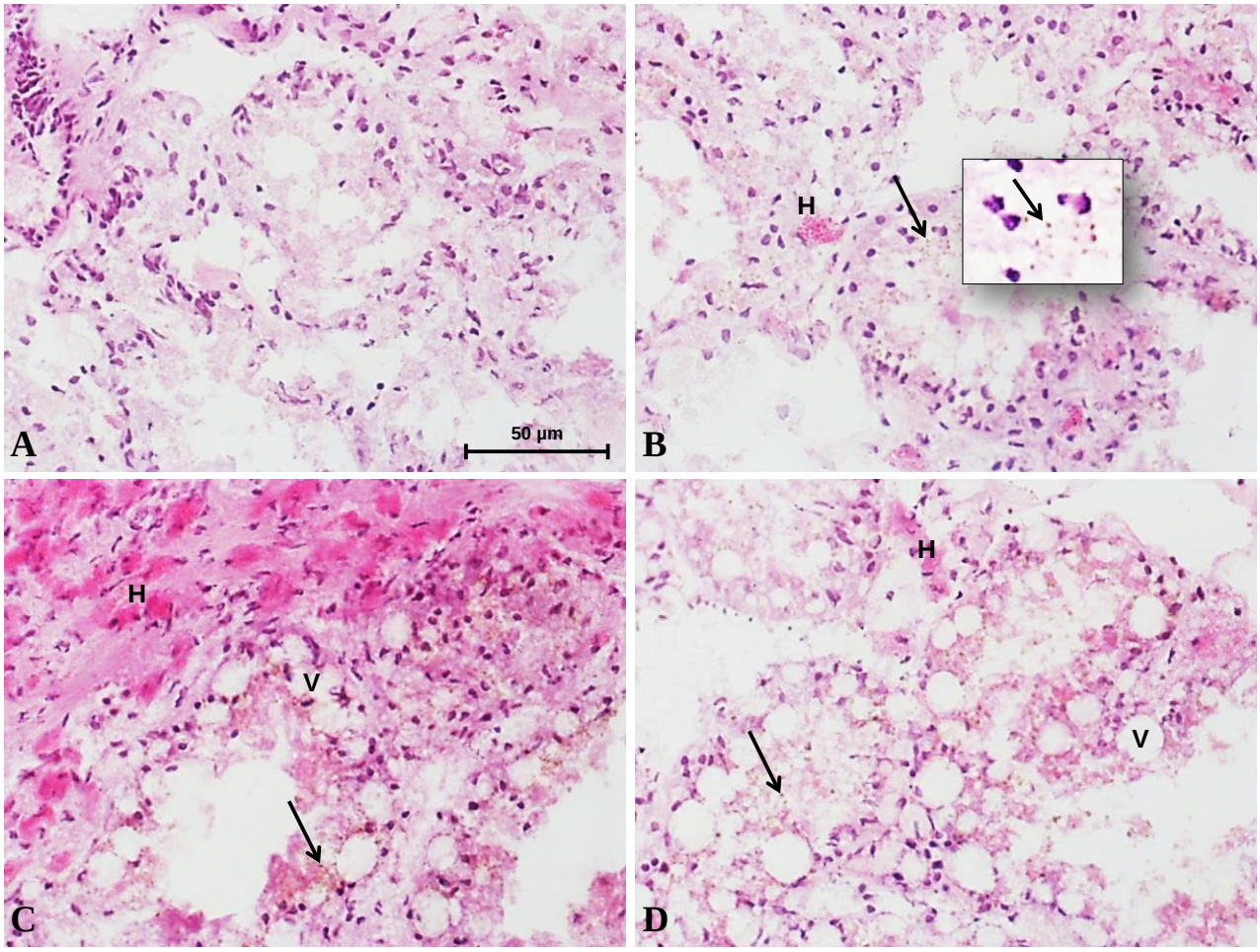

Fig. 1.

Figure 2

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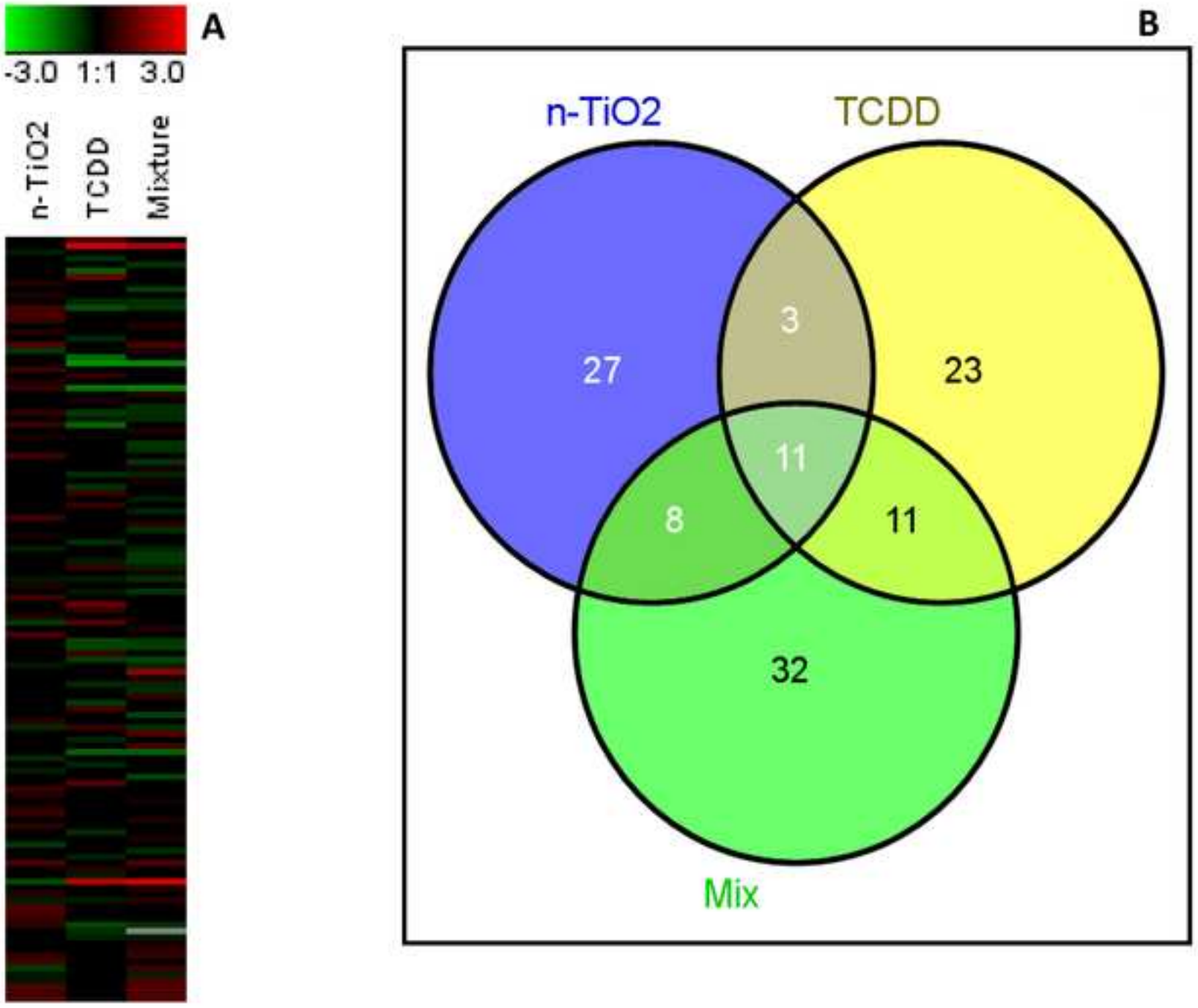

Figure 3

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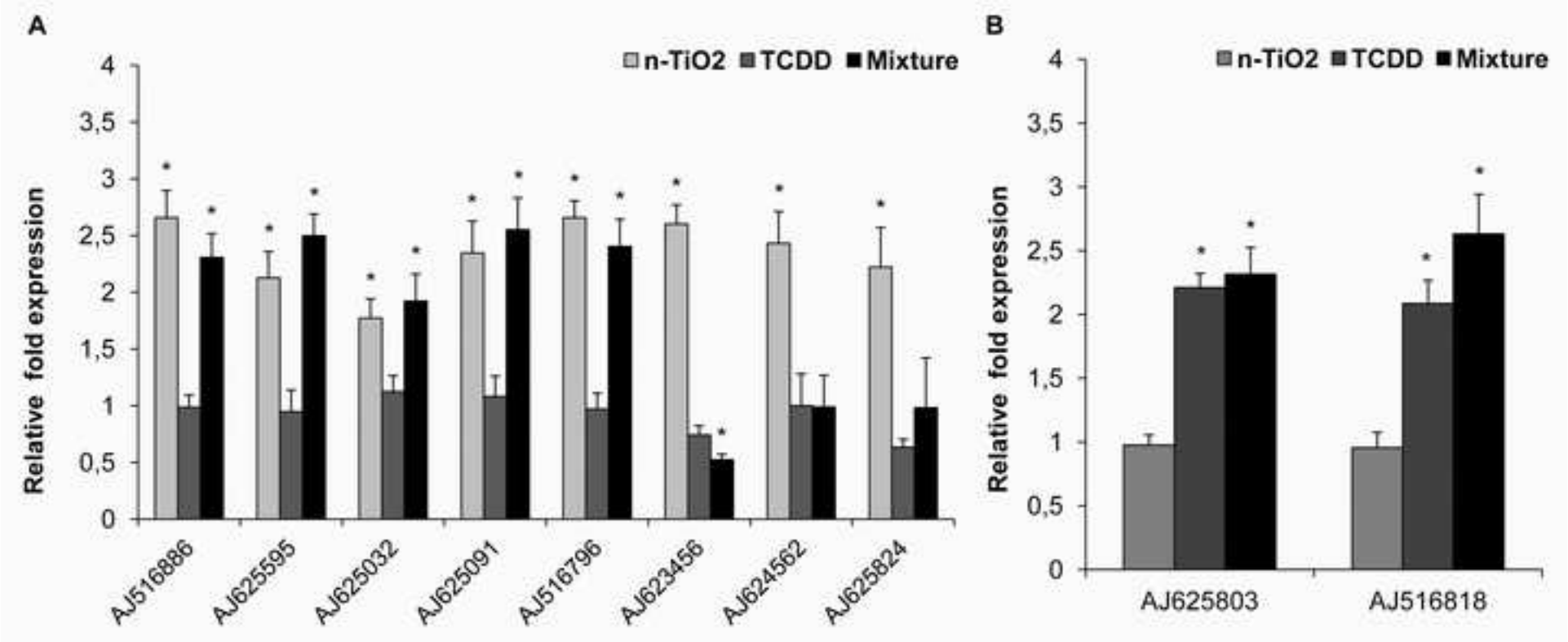

Figure 4

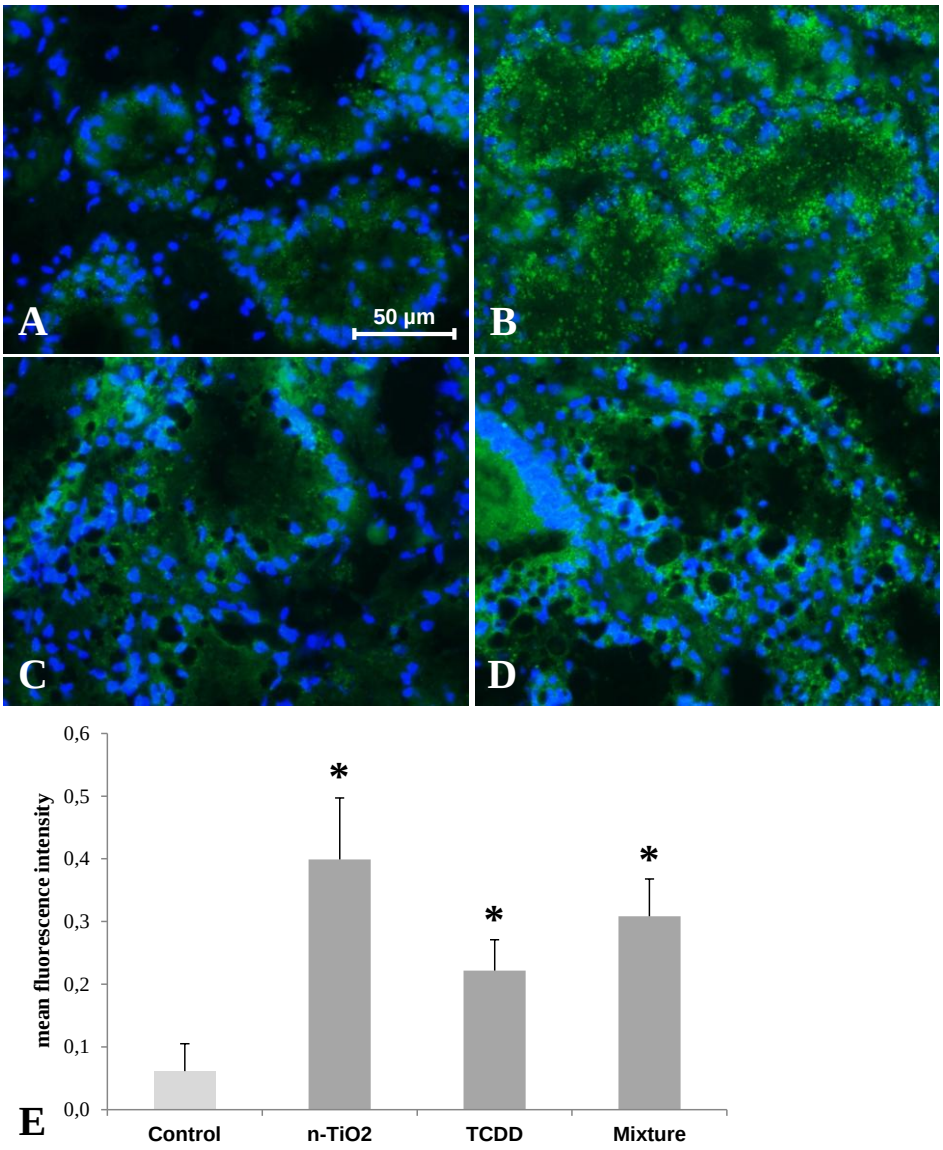

Fig. 4.

Figure 5

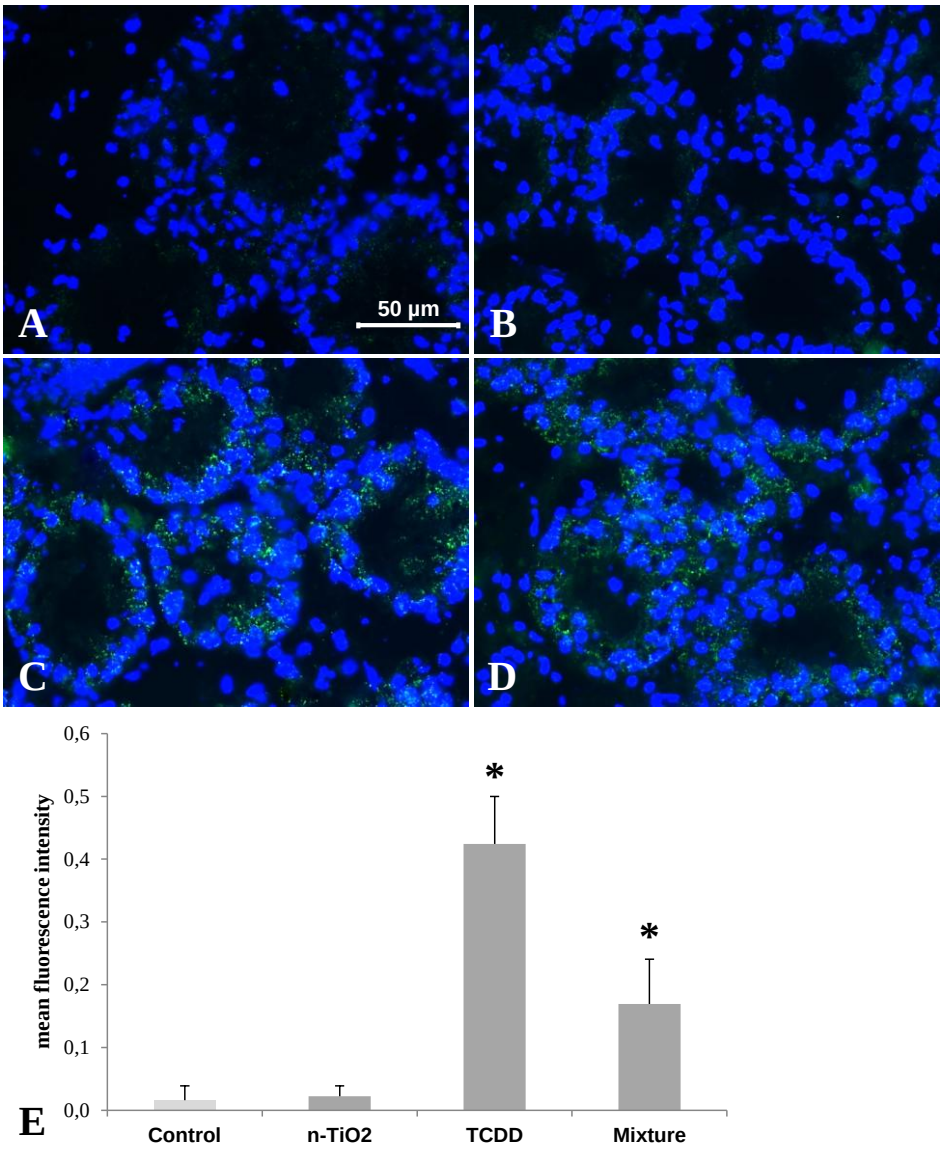

Fig. 5.

Fig.S1

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Table S1

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Table S2

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Table S3

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Table S4

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