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Exposure to nanoplastics and nanomaterials either single and combined affects the gill-associated microbiome of the Antarctic soft-shelled clam *Laternula elliptica*

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HIGHLIGHTS

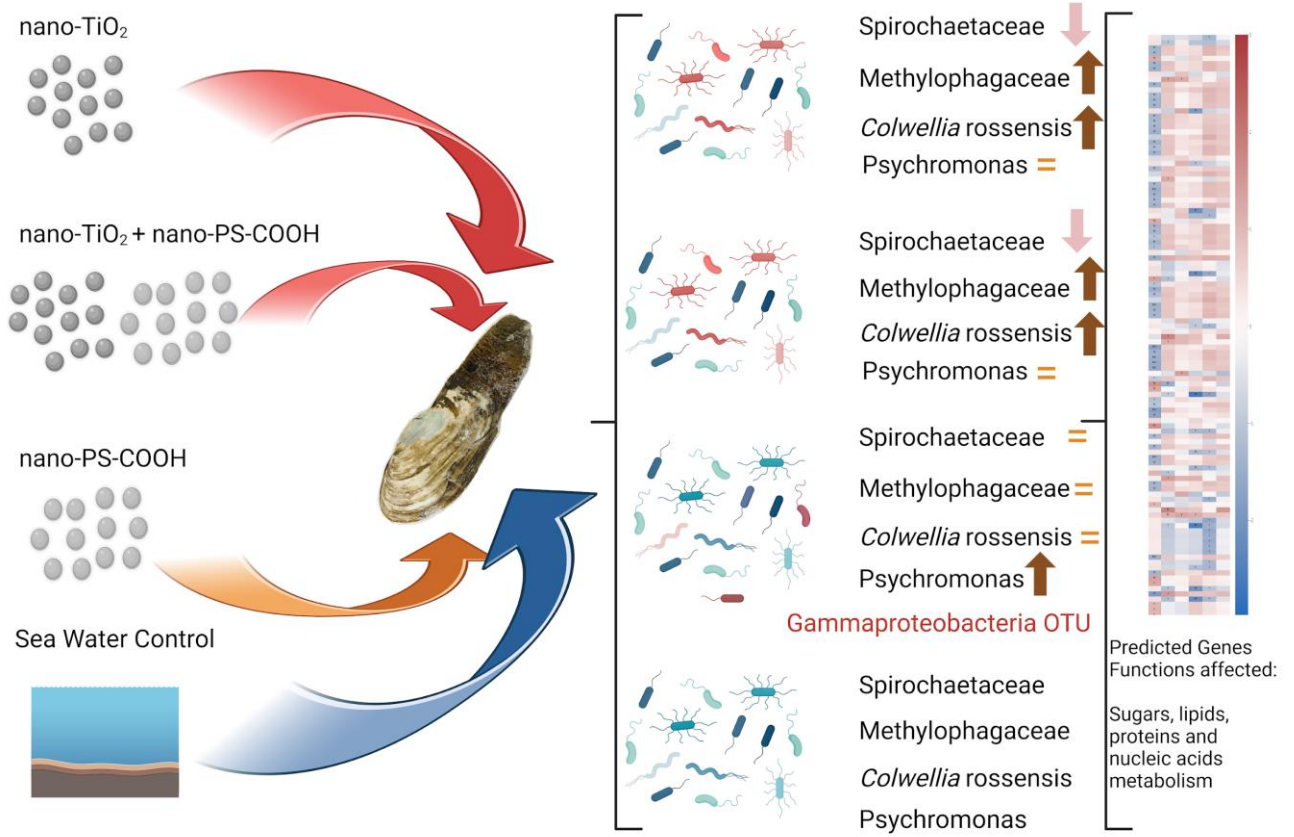
- Antarctic soft-shell clam microbiome affected by nanoplastic and nanoscale titanium dioxide exposure.
- The main predicted gene functions affected were implicated in gill's metabolism
- Single and combined exposure to nano-TiO₂ affect prokaryotic symbiosis
- An increase of two low abundance OTUs in PS-COOH NPs exposed clams was observed
- A decrease in relative abundances of *Spirochaetia* occurred upon exposure to nano-TiO₂

ABSTRACT

Nanoplastics and engineering nanomaterials (ENMs) are contaminants of emerging concern (CECs), increasingly being detected in the marine environment and recognized as a potential threat for marine biota at the global level including in polar areas. Few studies have assessed the impact of these anthropogenic nanoparticles in the microbiome of marine invertebrates, however combined exposure resembling natural scenarios has been overlooked. The present study aimed to evaluate the single and combined effects of polystyrene nanoparticles (PS NP) as proxy for nanoplastics and nanoscale titanium dioxide (nano-TiO₂) on the prokaryotic communities associated with the gill tissue of the Antarctic soft-shell clam *Laternula elliptica*, a keystone species of marine benthos. Wild-caught specimens were exposed to two environmentally relevant concentrations of carboxylated PS NP (PS-COOH NP, ~ 62 nm size) and nano-TiO₂ (Aeroxide P25, ~ 25 nm) as 5 and 50 µg/L either single and combined for 96h in a semi-static condition. Our findings show a shift in microbiome composition in gills of soft-shell clams exposed to PS NP and nano-TiO₂ either alone and in combination with a decrease in the relative abundance of OTU1 (Spirochaetaceae). In addition, an increase of gammaproteobacterial OTUs affiliated to MBAE14 and Methylophagaceae (involved in ammonia denitrification and associated with low-quality water), and the OTU *Colwellia rossensis* (previously recorded in polluted waters) was observed. Our results suggest that nanoplastics and nano-TiO₂ alone and in combination induce alterations in microbiome composition by promoting the increase of negative taxa over beneficial ones in the gills of the Antarctic soft-shell clam. An increase of two low abundance OTUs in PS-COOH NPs exposed clams was also observed. A predicted gene function analysis revealed that sugar, lipid, protein and DNA metabolism were the main functions affected by either PS-COOH NP and nano-TiO₂ exposure. The molecular functions involved in the altered affiliated OTUs are novel for nano-CEC exposures.

Key Words: CECs, bivalve, prokaryotic, nanoplastic, titanium dioxide, microbiome, Antarctica

GRAPHICAL ABSTRACT



INTRODUCTION

Contaminants of Emerging Concern (CECs) constitute a group of compounds with a wide amount of chemicals such as Flame-retardants, pharmaceutical chemicals, as well as micro- and nanoparticles. Although the Southern Ocean is considered a highly remote and isolated region, it is not exempt from CECs, which have been found in Antarctic Peninsula Waters, Weddel and Ross Seas (Chen et al., 2015; Perfetti-Bolaño et al., 2022; Szopińska et al., 2022; Wolschke et al., 2015). For example, macro- and meso-plastic have been widely reported in Southern Ocean waters (as reviewed in Rota et al., 2022), with even records of high concentrations of microplastics in Antarctic coastal areas (Isobe et al. 2017). According to Waller et al. (2017) microplastic concentrations in the Southern Ocean are similar to those found in populated regions, raising the concern that these compounds may have an impact on Antarctic wildlife. A relevant source of pollution is vessels operating in Antarctic waters, with recent studies showing that 45% of microplastic collected can derive from vessel-induced contamination (Leistenschneider et al. 2021). Some examples of microplastics in Antarctica include marine sediments in the Ross Sea (Munari et al., 2017), and sea ice cores (Kelly et al., 2020). Considering biotic matrix with the presence of micro-plastics and micro-fibers, there is growing evidence of bioavailability and presence in marine benthos, especially filter-feeders and grazers (Sfriso et al., 2020), and where the internalization of natural- and synthetic microfibers in the Antarctic whelk *Neobuccinum eatoni* (Bergami et al., 2022). In this regard, CECs can potentially affect all levels of the food web. Recently, planktonic species such as Antarctic krill and salps have been shown to ingest microplastic fragments and fibers (Wilkie Johnston et al. 2023; Zhu et al. 2023a), potentially transferring these pollutants to predators such as fishes (Ergas 2023; Zhu 2023b) and penguins, where microplastics have been observed in excreta (Bessa et al., 2019; Le Guen et al., 2020; Fragão et al., 2021).

The Antarctic krill *Euphausia superba* could further turn ingested microplastic and release it as nanoplastics ($< 1 \mu\text{m}$), possibly making them more available for filter-feeder species (Dawson et al., 2018). Effects of nanoplastics can be detrimental also for the krill molting and decrease sinking velocity of fecal pellets with implications for the carbon pump of the Southern Ocean (Bergami et al., 2020). Precisely nanoplastics have been detected in sea ice, with an average concentration of 52.3 ng/ml, therefore their presence is no longer just hypothetical in Antarctica (Materić et al., 2022). Furthermore, given their high potential toxicity compared to larger microplastic particles, there is an urgent need for more ecotoxicological data about their effects on Antarctic biota (Corsi et al., 2023).

Other relevant nanoscale CECs are the nano-metal-oxides, likely present in Antarctic waters as a result of the increasing activities of scientific stations, tourism, and fisheries. An important one is

nano-titanium-dioxide (nano-TiO₂), largely employed in water treatments but also in commercial products such as sunscreen and cosmetics, which thus act as the main source of metal-oxide nanomaterials in marine coastal waters (Haynes et al., 2017). Since the production and use of synthetic nanoparticles as nano metal-oxides are growing rapidly (Labille et al., 2020), their occurrence in the environment including polar areas seems inevitable (Clemente et al., 2012).

In invertebrates, the microbiome associated with key tissues and organs plays a critical role in organism homeostasis (Lesperance and Broderick, 2020; Ferguson and Foley, 2022), being crucial in organisms' health and resilience (Bourne et al., 2016), especially for metabolic performance in extreme environments (Bang et al., 2018; Yang et al., 2022). Microbiome functionality can be disturbed by environmental changes and pollutants such as pesticides, heavy metals, and synthesized micro- and nanoparticles (Zhu et al., 2020; Cao et al., 2022; Ye et al., 2022). In some cases, tissue-associated microbiota disruption can elicit a starvation-like condition in invertebrates (Vera-Ponce de León et al., 2021) and in marine bivalves, the perturbation of microbiota by pollutants could allow the spread of potential opportunistic pathogens (Iori et al., 2020).

Considering anthropogenic global changes, it is crucial to assess if prokaryotic symbionts could help benthic invertebrates face current and projected threats such as those linked to human activities (Li et al., 2023). The microbiome associated with metazoan tissues and organs could be perturbed by anthropogenic pollution as CECs such as micro- and nanoplastic, as well as nanoparticles of metal-oxides (reviewed in Santos et al. 2022). Both impact in a single and combined way. Recent studies have reported microbiome shifts in moribund freshwater mussels compared with healthy ones, suggesting a possible bacterial invasion during mass mortality (Richard et al., 2021). These outcomes indicate the importance of monitoring tissue-associated microbiomes in aquatic metazoan as filter-feeder benthic bivalves.

Micro- and nano-pollutants as plastic particles could impact the microbiota associated with bivalve species, with potential human pathogens found among operational taxonomic units (OTUs) with increased abundance induced by micro-plastic exposure in the digestive tract. (Li et al., 2020). Equally, metal-oxide nanoparticles have been reported impacting microbiota in bivalves, as in the case of nano-TiO₂ affecting the abundance of different genera, with decreases in some (e.g. *Shewanella*, *Kistimonas*, *Vibrio*) and increases in others (e.g. *Stenotrophomonas*) (Auguste et al., 2019). In other taxa as crustaceans, changes in microbial composition have been recorded after microplastic exposure, with the relative abundance of Firmicutes and Bacteroidetes decreased following exposure to 40 mg/L microplastics, whereas that of Fusobacteria and Proteobacteria

increased (Liu *et al.* 2019). Studies on the effect of nanoplastics on microbial communities associated with fishes, specifically Zebrafish, have shown significant changes in the composition after 14-day exposure to 1000 mg/L of both sizes of polystyrene MP, while other studies have reported also changes in terms of species richness (Jin *et al.*, 2018; Zhang *et al.*, 2021b).

Among benthic invertebrates, the Antarctic soft-shell clam *Laternula elliptica* is a key species in the benthic-pelagic carbon flux in coastal areas, filtering sedimentary particles and planktonic organisms (Momo *et al.*, 2002). According to Peck *et al.* (2018) filter filter-feeding mollusks are among the highest sensitive species to environmental and anthropogenic perturbations in Antarctica, In fact, several cellulosic, plastic micro-fragments and micro-fibers have been recorded in this species (González-Aravena *et al.* 2024). The microbiome of *L. elliptica* was recently described by González-Aravena *et al.* (2023) revealing that it is a sensitive indicator of water quality, including the presence of potential pathogen Prokaryotic OTUs. In this regard, the gill is a key tissue, directly exposed to the environment, which can display important microbiome structural composition alterations, where principal prokaryotic groups are Gammaproteobacteria, Bacteroidia, Fusobacteriia, Campylobacteria and Spirochaetia (González-Aravena *et al.*, 2023). Considering filter-feeder behaviour and the environmentally exposed gill tissue, we aim to evaluate the structural composition of the microbiome associated with *L. elliptica* gills by comparing individuals exposed to single and combined nano-sized CECs.

METHODS

Experimental design

A total of 21 Antarctic soft-shell clams *Laternula elliptica* (average shell length of 71.66 cm \pm 11.75 SD) were collected by SCUBA diving from one population in Fildes Bay, King George Island (South Shetland Islands) in January 2020 and transferred in the aquarium facility of the Chilean scientific base Julio Escudero.

Preparation of nanoparticle suspensions

Nano-PS-COOH were purchased from Bangs Laboratories Inc. and provided as nanospheres with a nominal size of 62 nm and density of 1.06 g/cm³ (see Bergami *et al.*, 2020 for further details on the stock). Nano-TiO₂ Aeroxide® P25 stock was composed of nanoparticles with a semi-spherical shape, a nominal size of 25 nm and density of 4.1 g/cm³ (Della Torre *et al.*, 2015). Briefly, nano-PS-COOH and nano-TiO₂ stock suspensions were prepared in MilliQ water at the concentration of 10 mg/mL, sonicating the suspensions using a bath sonicator DENTSPLY Ultrasonik 57H for 2 min (Murano *et al.*, 2021), and vortexed before use, according to Bergami *et al.*, 2020. The natural sea water (NSW)

used for the experiments (temperature of $0\pm 1^\circ\text{C}$, pH of 8.42 and salinity of 32.8‰) was collected from Fildes Bay at the same sampling site of the specimens and filtered (1 μm) and using 40 watt UV lamp, before filling the 5 L aquariums. Then, two working suspensions at 250 and 25 $\mu\text{g}/\text{mL}$ were prepared, and 1 mL of each suspension was added to 5 L tanks of NSW to achieve final concentrations of 50 and 5 $\mu\text{g}/\text{L}$, respectively. Tested suspensions were replaced every 24 h for a semi-static *in vivo* exposure. *L. elliptica* individuals were maintained for 2 days in NSW, and then two independent experiments (96 h) were carried out placing five individuals in 5 L tanks with 3 aquarium replicates by the following conditions: NSW (control), nano-PS-COOH at 5 and 50 $\mu\text{g}/\text{L}$, nano-TiO₂ at 5 and 50 $\mu\text{g}/\text{L}$ and combined exposure (nano-PS-COOH and nano-TiO₂) at 5 and 50 $\mu\text{g}/\text{L}$. These concentrations were comparable to those reported by Materić et al (2022) in Antarctic sea ice, and corresponded to $3.78\cdot 10^{10}$ particles/L and $3.78\cdot 10^{11}$ particles/L of nano-PS-COOH at 5 and 50 $\mu\text{g}/\text{L}$, respectively (see Bangs Laboratories Inc. TechNote 206 reported in Bergami et al., 2020 for calculation). For the experiments, $1.89\cdot 10^{11}$ PS particles (at 5 $\mu\text{g}/\text{L}$) and $1.89\cdot 10^{12}$ PS particles (at 50 $\mu\text{g}/\text{L}$) were thus added to 5 L tanks.

For nano-TiO₂, by approximating to nanospheres, 5 and 50 $\mu\text{g}/\text{L}$ corresponded to estimated concentrations of $1.49\cdot 10^{11}$ particles/L and $1.49\cdot 10^{12}$ particles/L at 5 and 50 $\mu\text{g}/\text{L}$, respectively, thus with $7.46\cdot 10^{11}$ TiO₂ particles (at 5 $\mu\text{g}/\text{L}$) and $7.46\cdot 10^{12}$ TiO₂ particles (at 50 $\mu\text{g}/\text{L}$) added to 5 L tanks. Considering that anthropogenic UV-filters have been found in Antarctica (D'Amico et al, 2022) and in temperate waters such as the Mediterranean Sea, the concentrations of TiO₂ can reach from 20 to 900 $\mu\text{g}/\text{L}$ in water column and surface water respectively (Labille et al., 2020), the concentration of the present study are environmentally relevant.

Tanks were kept well oxygenated and specimens were maintained with a photoperiod of 18/6 light/dark intervals. After 96 h, clams were dissected for tissue extractions (gills), frozen with liquid nitrogen, and kept in 1ml of RNAlater at -80°C until DNA isolation.

DNA Isolation

Genomic DNA from *L. elliptica* gill tissues for 16S rRNA gene amplicon sequencing was isolated using the Precellys© Evolution homogenizer with a Cryolys cooling unit (Bertin Technologies, Montigny-Le-Brettonneux, France) and the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA concentrations were measured using a Nanoquant spectrophotometer (Tecan, Switzerland). Ten microliters of DNA samples were sent to Dalhousie University CGEB-IMR for V6–V8 rRNA gene library preparation and sequencing. Samples were sequenced using an Illumina Miseq with paired-end 300 C 300 bp reads. All PCR procedures,

primers, and Illumina sequencing details were as described in Comeau et al. (2017). The cDNA sequences of 21 libraries, corresponding to 2 controls, 2 nano-PS-COOH at 5 µg/L, 2 nano-PS-COOH at 50 µg/L, 3 nano-TiO₂ at 5 µg/L, 3 nano-TiO₂ at 50 µg/L, 3 combined-NP at 5 µg/L and combined-NP at 50 µg, were deposited at NCBI as a BioProject with accession ID PRJNA1043728.

Data Processing and Analysis

Microbiome data was analyzed with the QIIME2 (version 2021.4) pipeline (Bolyen et al., 2018). Briefly, 21 paired-end libraries in fastq compressed files (.gz) were imported to perform merge reads from R1 and R2 libraries per sample, trimming primers and low-quality nucleotides at the end of the sequences, denoising, removing chimaeras, and clustering sequences. Only samples with more than 2000 reads passing quality filters were used for posterior analysis. Clustered and filtered sequences were then affiliated using the SILVA132 database with a 99% identity for seven levels of taxonomy. Before statistical analyses, clusters affiliated with chloroplasts and mitochondria were removed. A core metric plugin of Qiime2 was applied to the operational taxonomic unit (OTU) abundance data for each sample to obtain values for observed OTUs, Chao1, Shannon, and Simpson diversity indexes. Prokaryotic community compositions at Class and OTU levels were plotted with the most frequent prokaryotic Classes and OTUs. In order to determine the existence of OTUs present in all treatments, we examined the core OTUs in control and exposed individuals, selecting only OTUs that were present in all 21 samples from the whole OTU list.

Differences in the beta diversity of the prokaryotic community between treatments at the order and OTU levels were tested with PERMANOVA analyses based on Bray-Curtis distance matrices, after square root transformation relative abundance data using Primer v7 (Anderson et al., 2008). Univariate statistics were performed using Shapiro test and Bartlett test to evaluate normal distribution and homogeneity of variance, respectively. Depending on these results, then we applied Kruskal-Wallis or ANOVA to evaluate differences between experimental conditions, when significant results were revealed a Dunn post hoc pairwise analyses were performed. The predicted functional pathways abundances by experimental conditions were analyzed with Qiime2 outputs using PICRUSt2 program (Douglas et al., 2020) from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. All samples were randomly resampled, selecting 1500 reads without replacement, and replicating based on the minimum number of sequences to eliminate the effect of the sequencing depth (Zhang et al., 2021a). To perform pathway differential expression analysis between treatments, ALDEx2 (Fernandes et al., 2013) was used. Significant pathways were determined by an absolute significant value greater than 1 and a p-value below 0.05, as assessed using the Wilcoxon signed-rank test.

RESULTS

A total of 157 OTUs were recorded with highthrough V6-V8 hypervariant region amplicons sequencing (Supplementary File 1), all of them bacterial groups belonging to 30 Class taxa (Supplementary File 2). The prokaryotic composition showed alterations for some taxa at the Class level with a decrease of most abundant classes in control clams such as the Spirochaetia. In contrast, an increase in other classes such as Bacteroidia, gammaproteobacterial and Campylobacteria, and particularly the Actinobacteria and Fusobacteria were recorded in samples that were not present in controls but in exposed clams. The co-occurrence network analysis revealed these alterations caused by nano-TiO₂ at single and combined exposures with PS-COOH NPs, with the decrease of Spirochaetia Class and the increase of Bacteroidia, Gammaproteobacteria and others (Fig. 1). Intriguingly, at OTUs level, two of them belonging to Spirochaetaceae decreased their relative abundances in exposed clams, with an increase of OTUs belonging to Bacteroidia, Gammaproteobacteria (including the Alteromonadales *Colwellia rossensis*), and Campylobacter. The co-occurrence network analysis at OTU level showed a decrease of OTU1 and increases of OTU3, OTU4, OTU5 and OTU7 (Fig. 2). Three OTUs were significantly affected by both nanoscale particles. These OTUs included OTU8 which is only present in PS-COOH NP at 50 µg/L, OTU9 which decreases upon combined exposures at 5 µg/L and OTU12 which is not present in control and increases in PS-COOH NP at 5 µg/L. To summarize, the biggest differences were observed implicated in nano-TiO₂ and combined exposures with the most abundant OTUs, and differences in less abundant OTUs were found involved in PS-COOH NP treatments.

The core community consisted only of three OTUs (OTU1, OTU6 and OTU9) that were presented in all individuals (Fig. 3). On the other hand, for the Chao1 index an increase was observed for PS-COOH NP and nano-TiO₂ at 50 µg/L but without statistical significance (Fig. 4A; SF1). Indeed, an increase in observed OTUs and Shannon index was found for clams exposed to nano-TiO₂ at 50 µg/mL (Fig. 4B-C) although not statistically significant. Regarding the Simpson index, an increase was obtained for nano-TiO₂ at single and co-exposure at both concentrations tested (Fig. 4D), although not significant. Despite the alteration mentioned above for some classes and OTUs, the global microbiome structure did not show significant differences between control and treatments (PERMANOVA, p-value = 0.0512).

The predicted functional analyses revealed 19, 65 and 3 pathways with corrected p-value statistic significant differences (< 0.05) for PS-COOH NP, nano-TiO₂ and co-exposures at 50 µg/L with respect to the control group. At the lower concentration tested (5 µg/L), zero, 16 and 14 functional

pathways displayed significant differences, with corrected p-value (< 0.05) at PS-COOH NP, nano-TiO₂ and co-exposures at 5 µg/L in comparison to control. These functional predicted analyses showed that the pathways with significant differences between treatments and control were mainly implicated in sugar, aminoacidic and lipid metabolisms in gill-associated prokaryotes identified with V6-V8 hyper-variant 16S gene region (Fig. 5).

DISCUSSION

Gills are key organs that are directly exposed to seawater and implicated in various physiological processes such as food filtration, respiration, immunity and osmoregulation (Shao et al., 2021; Zhou et al., 2022). By analyzing gills microbiome of the Antarctic soft-shell clam upon single and combined exposure to PS-COOH NP and nano-TiO₂ at environmentally realistic concentrations (5 and 50 µg/L), we were able, for the first time, to assess the impact of single and combined effects. This is also the first microbiome assessment of nanoscale CECs in an Antarctic species, considering predictive molecular functions and pathways.

Diversity

In the present study, the diversity indexes of microbiome composition did not change after gill's soft shell clam exposure to single and combined PS-COOH and nano-TiO₂, indicating that effects were only in relative abundance shifts and the presence of some new opportunist OTUs. Previous studies reported limited effects of nano-TiO₂ alone in the gut microbiota of the marine bivalve *Mytilus coruscus* from Gouqi Island in Shengsi (Zhejiang Province), where an impact in microbial richness and diversity was only observed in combination with other stressors such as pentachlorophenol and the presence of a predator (Chen et al., 2023).

Community changes and their implications

The taxa composition at Class and OTU levels suggests that the microbiome of *L. elliptica* gills remains relatively stable to nano-PS-COOH exposure regardless of the tested concentration, with changes only in the relative abundance change of low presence OTUs, such as OTU12 affiliated as *Psychromonas* and OTU8 belonging to Gammaproteobacteria Class. Meanwhile, the biggest alteration occurred in clams exposed to nano-TiO₂ and in combination at both concentrations. This observed shift in the microbiome was characterized by a decrease of known bivalve gill bacterial OTUs belonging to Spirochaetia, and the increase of other Classes such as Bacteroidia and Campylobacteria. On the other hand, the Class Fusobacteria was only present in nano-PS-COOH, nano-TiO₂ and in co-exposed specimens at the highest concentration tested (50 µg/L); similarly, Lentisphaeria Class was only present in the nano-TiO₂ and upon combined exposure at 50 µg/L.

Some Gammaproteobacterial OTUs were highly abundant in nano-TiO₂ treatments (OTU3, OTU4, OTU7 and OTU8). It is worth mentioning that some of these OTUs are indicators of water quality for gill tissue and ill conditions (Callac et al., 2022; Krolicka et al., 2019; Mori et al., 2019).

At OTUs level, OTU1 (Spirochaetaceae) showed a decrease in its relative abundance for nano-TiO₂ and in combination. In contrast, under the same conditions, an increase in the abundance of OTU3 (MBAE14 family), OTU4 (Methylophagaceae), OTU5 (an *uncultured* genus of Spirochaetaceae) and OTU7 (*Colwellia rossensis*) was observed. To a minor degree but consistently, OTU8 (Gammaproteobacteria) was only found in specimens exposed to PS-COOH NP at 50 µg/L and OTU12 was not present in control and increased in specimens exposed at 5 µg/L. The co-occurrence networks also showed dysregulation of this important bacterial group for bivalve gills. Regarding Spirochaetaceae OTUs, they are common members of core OTU among bivalve tissues, normally overrepresented in gills (King et al., 2020). In this regard, the effects of nano-TiO₂ and in combination with PS-COOH NPs are concerning as it has been found in oysters resistant to viruses such as OsHV-1 (King et al., 2019). Regarding bacteria with increases in relative abundance such as OTU3 and OTU4 belonging to MBAE14 and Methylophagaceae family, both are implicated in ammonia-rich environments and deteriorated conditions (Mori et al., 2019; Callac et al., 2022), suggesting that detrimental conditions of nano-TiO₂ in gills allow the development of these Bacteria. Several *Colwellia* OTUs, including *Colwellia rossensis* which was also recorded in this study, have been described as abundant in oil treatments in comparison to control water experiments from Norwegian and Arctic regions, suggesting their role as indicators of pollution (Krolicka et al., 2019). In addition, *Colwellia* has been reported as abundant in other invertebrate diseases such as sponges and corals (Simister et al., 2012; Fan et al., 2013; Gajigan et al., 2017), with other studies showing an increase in injured tissues (Rondon et al., 2020) and in individuals exposed to thermal stress (De Castro-Fernández et al., 2023). Altogether these OTUs alterations induced by nano-TiO₂ suggest their key function for health and common bivalve-associated bacterial taxa Spirochaetaceae decrease, signal of detriment condition for gill tissue. Further, considering the increase in taxa indicators of adverse conditions, such as pollution, diseases and injuries, these results raise concern about the effect of metal-oxide NP at realistic concentrations of 5 and 50 µg/L.

Microbiome alterations produced by micro- and nanoparticles exposures have been mainly reported for soil worms (Zhu et al., 2018; Ding et al., 2020; Ma et al., 2020; Xu and Yu, 2021), and few marine invertebrates, mainly in the gut of bivalves and decapods (Liu et al., 2019; Li et al., 2020), but also in bivalve haematocytes (Auguste et al., 2019). Differences in the abundance of 25 genus were recorded after exposures to microplastics in juvenile crab intestines (Liu *et al.*, 2019), while 126

OTUs suffered abundance alterations in the gut of *Mytilus edulis* (Li *et al.*, 2020), including the genus *Colwellia* similar to the present study. Spirochaetes OTUs are commonly present in bivalve gill microbiomes, but also Gammaproteobacteria, Bacteroidia and Campylobacter OTUs (Roterman *et al.*, 2015; Lim *et al.*, 2021; González *et al.*, 2022). This suggests that the increase of Campylobacter and Bacteroidia after exposure to nano-TiO₂ at single and combined exposures (both concentrations) is yet established microbiota, but the increase in their abundances could be considered detrimental especially by those implicated in poor water quality and gill indicators, with the appearance of Fusobacteria and Lentisphaeria probably as a consequence of these CECs.

Predicted functional alterations

The Class Spirochaetia is known for playing different molecular functions such as the assimilation of nitrate and nitrite, glutamine and glutamate synthesis and others (Lim *et al.*, 2019). Interestingly, in most treatments, there was an effect in the predicted microbiome functions mainly implicated in nucleotides, sugar, lipids and amino acids metabolisms. This is a novel insight into nano-CEC impacting a marine invertebrate and its associated microbiota.

Among the nucleotide synthesis pathways, “5-aminoimidazole ribonucleotide biosynthesis I” and “5-aminoimidazole ribonucleotide biosynthesis II” were under-represented in nano-TiO₂ at 50 µg/L in comparison to control. Differently, nucleotide degradation pathways, such as “purine ribonucleosides degradation” were under-represented in nano-TiO₂ at 5 µg/L, PS-COOH NP at 50 µg/L and their combined co-exposure at 5 µg/L.

Regarding lipids and phospholipids synthesis pathways, these were under-represented in nano-TiO₂ at 50 µg/L, PS-COOH NP at 50 µg/L and in combination at 5 µg/L. These were “CDP-diacylglycerol biosynthesis I” and “II”, “phosphatidylglycerol biosynthesis I (plastidic)” and “II (non-plastidic)”, and “superpathway of phospholipid biosynthesis I”. On the other hand, fatty acid degradation pathways, such as “superpathway of fatty acid biosynthesis initiation”, were over-represented in nano-TiO₂ at 50 µg/L. Prokaryotic amino acid metabolism was also affected by single exposure to both nanoparticles, as the synthesis pathways (i.e., L-arginine, L-isoleucine, L-lysine, L-ornithine, L-valine and L-phenylalanine biosynthesis) were under-represented in nano-TiO₂ at 50 µg/L and PS-COOH NP at 50 µg/L. As far as the amino acid degradation pathways, e.g., “L-histidine degradation II”, “L-tyrosine degradation I”, these were over-represented in nano-TiO₂ at 5 µg/L and in combination at 50 µg/L, and only under-represented in combined co-exposure at 5 µg/L. Finally, considering sugar degradation such as “Glycolysis”, “Galactose degradation I” and “Sucrose degradation III” were over-represented in nano-TiO₂ both at 50 and 5 µg/L, PS-COOH NP at 50 µg/L

and in combination at 5 µg/L. “Glycogen biosynthesis I” was under-represented in nano-TiO₂ at 50 µg/L. Predicted metabolism pathways in gut-associated microbiome can be affected by nanoplastic alone and when combined with norfloxacin, as observed in *Mytilus coruscus* (Xu et al. 2023). These findings suggested that these anthropogenic nanoparticles impair energy balance in bivalve-associated microbiota. The predicted functional alterations reported by our study revealed a clear trend to degradations and energy needs, contrary to the under-representation of biosynthesis. These could implicate an impact on gill tissue microbiota physiology and, as a consequence, on the whole organism, even at short-term exposure (96 h) and realistic concentrations (5 and 50 µg/L). In this sense, in future ecophysiological metabolism assessments, it is important to include oxygen consumption to determine if the global metabolism is affected in *L. elliptica* clams.

Conclusions

The present study highlights the value of bioindicator gill-associated bacteria for OTUs, considering the important functions and signal of Spirochaetia in bivalves following short and realistic exposures to nano-TiO₂, leaving the place to opportunistic OTUs bacteria, such as *Colwellia*. It was also recorded an increase of two OTUs upon PS-COOH NP exposure, and one of them only present in this treatment, being this relevant information of the nanoplastic effect in associated bacteria in the gills of an aquatic metazoan. The increase in opportunistic bacteria can be an indicator of water deterioration as found in polluted regions, as well as other ones possibly indicating tissue diseases and damage. Altogether our findings suggest that single and combined exposures to nano-CECs affect prokaryotic symbiosis, altering composition and important bacterial metabolic functions. Finally, the molecular functions involved in the altered affiliated OTUs are novel for nano-CECs impact evaluations, showing important considerations to consider in future ecotoxicology studies.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTIONS

Rondon: Designed experimentations, carried out experimental exposure experiments in Antarctica, performed bioinformatic analyses and wrote the manuscript. **Cosseau:** Design experimentations and participated in manuscript drafting. **Bergami:** Designed experimentations, carried out characterization of nanoparticles suspensions and participated in manuscript drafting. **Cárdenas:** participated in manuscript drafting. **Perez-Toledo:** Carried out DNA-extractions and sampling

preparations. **Alvarez:** Performed Picrust2 bioinformatic analyses. **Aldridge:** Performed Picrust2 bioinformatic analyses. **Font:** Participated in Picrust2 bioinformatic analyses. **Garrido:** Performed diving sampling in Antarctica and participated in experimental analyses. **Santa Cruz:** Participated in statistical analyses, figures creation and participated in manuscript drafting. **Perrois:** carried out experimental exposure experiments in Antarctica. **Balbi:** Participated in manuscript drafting. **Corsi:** Designed experimentations, provided nanoparticles and participated in manuscript drafting. **González-Aravena:** Participated in experimentation design and drafting manuscript.

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FIGURES AND TABLES

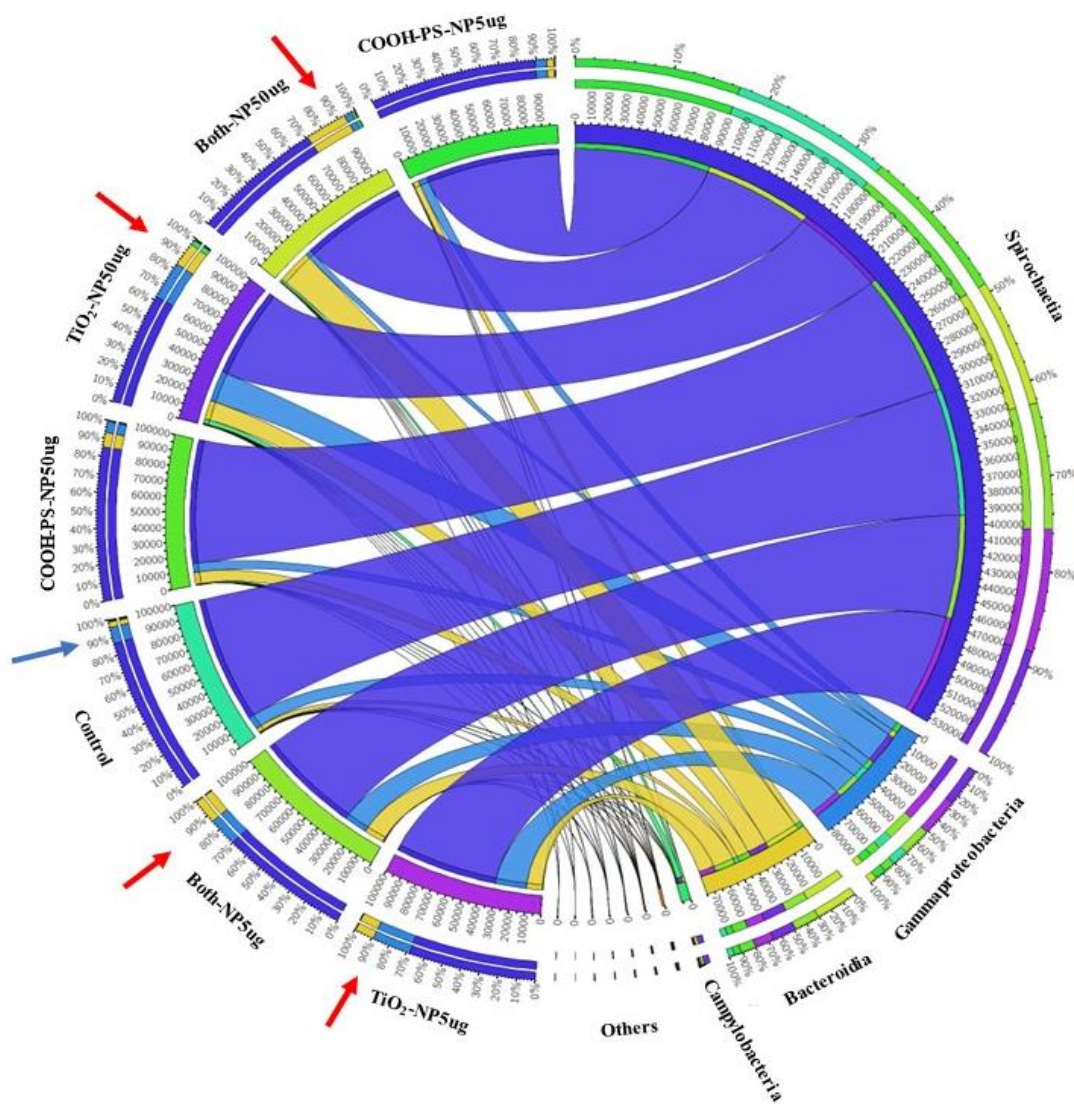


Fig. 1. Co-occurrences networks analysis performed with Circos web program at class level (B). Colours indicate each taxon and the horizontal bar of each condition is the relative abundance (%). The conditions are nanopolystyrene at 5 $\mu\text{g/L}$ (COOH-PS-NP 5 μg), nano-titanium-dioxide at 5 $\mu\text{g/L}$ (TiO_2 -NP 5 μg), combined nanoparticle exposure at 5 $\mu\text{g/L}$ (Both-NP 5 μg), nanopolystyrene at 50 $\mu\text{g/L}$ (COOH-PS-NP 50 μg), nano-titanium-dioxide at 50 $\mu\text{g/L}$ (TiO_2 -NP 50 μg) and combined nanoparticle exposure at 50 $\mu\text{g/L}$ (Both-NP 50 μg). The blue arrow indicates the abundances in the control condition, instead red arrows indicate abundances alterations in some n- TiO_2 -NP and combined co-exposures in both concentrations.

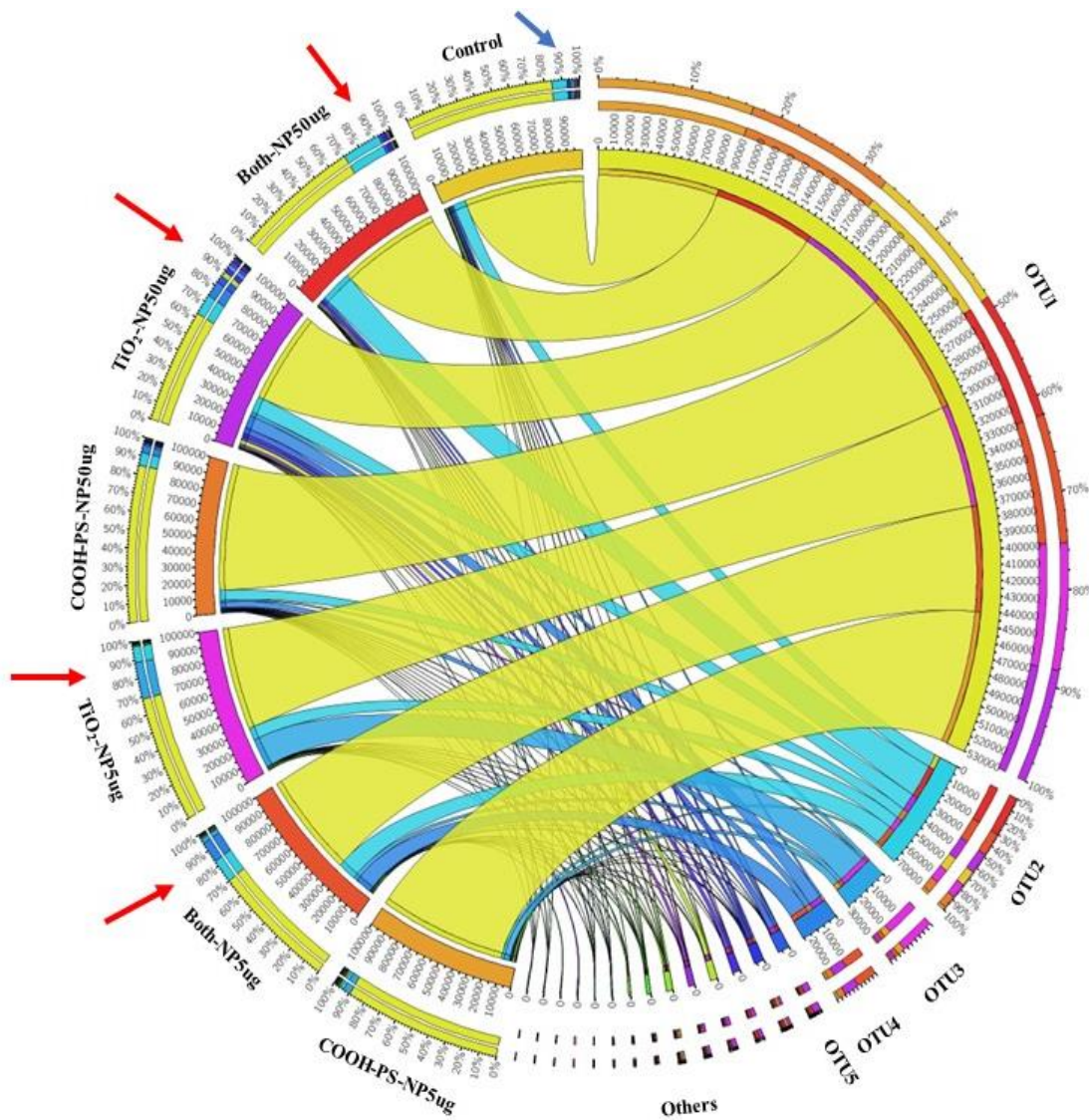


Fig. 2. Co-occurrences networks analysis performed with Circos web program at OTUs levels (B). Colours indicate each taxon and the horizontal bar of each condition is the relative abundance (%). The conditions are nanopolystyrene at 5 $\mu\text{g/L}$ (COOH-PS-NP 5 μg), nano-titanium-dioxide at 5 $\mu\text{g/L}$ (TiO₂-NP 5 μg), combined nanoparticle exposure at 5 $\mu\text{g/L}$ (Both-NP 5 μg), nanopolystyrene at 50 $\mu\text{g/L}$ (COOH-PS-NP 50 μg), nano-titanium-dioxide at 50 $\mu\text{g/L}$ (TiO₂-NP 50 μg) and combined nanoparticle exposure at 50 $\mu\text{g/L}$ (Both-NP 50 μg). The blue arrow indicates the abundances in the control condition, instead the red arrows indicate abundances alterations in some n-TiO₂-NP and combined co-exposures in both concentrations.

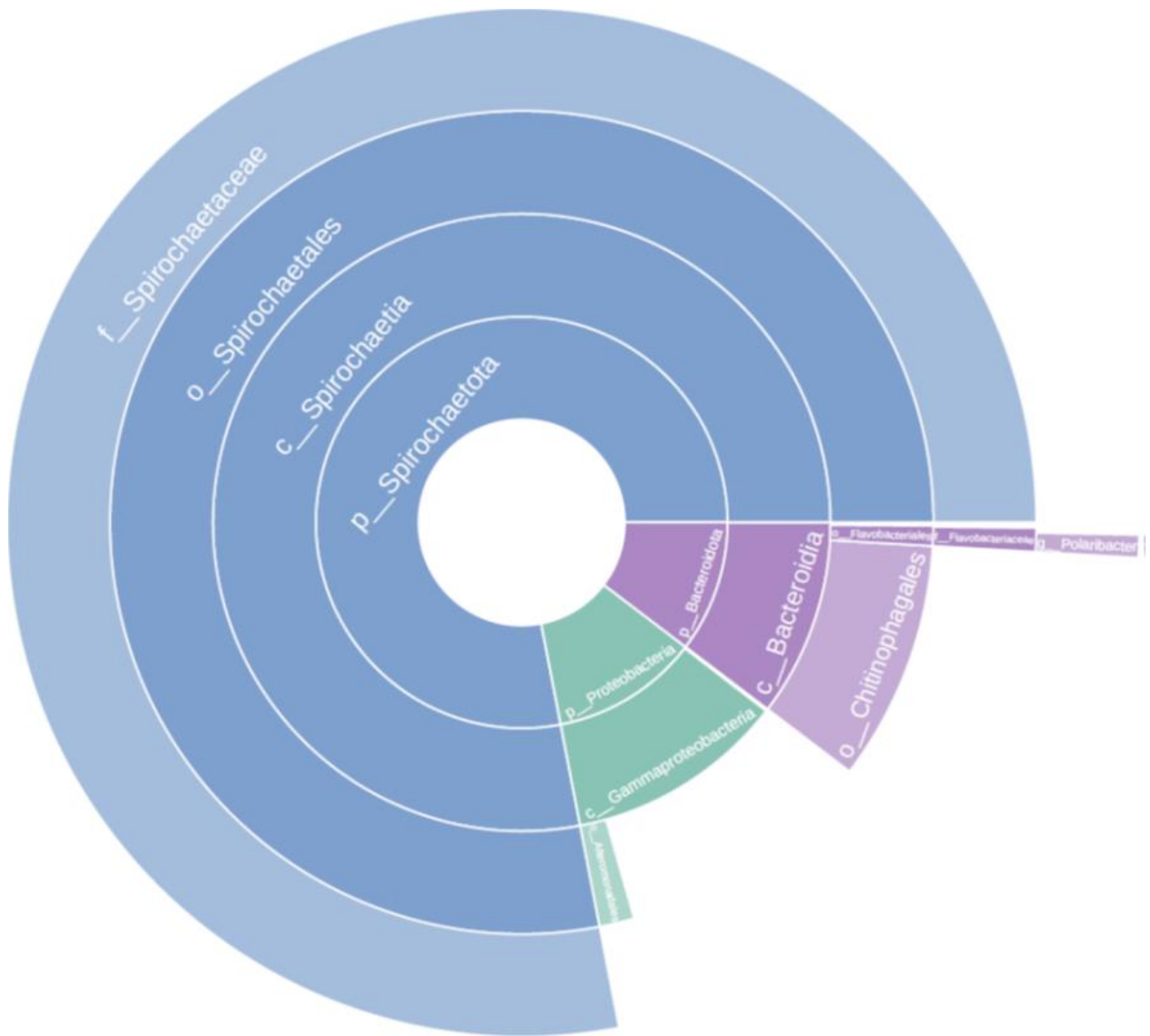


Fig. 3. Core microbiota present in all samples, indicating their different taxonomic levels. Colours represent the different prokaryotic taxa.

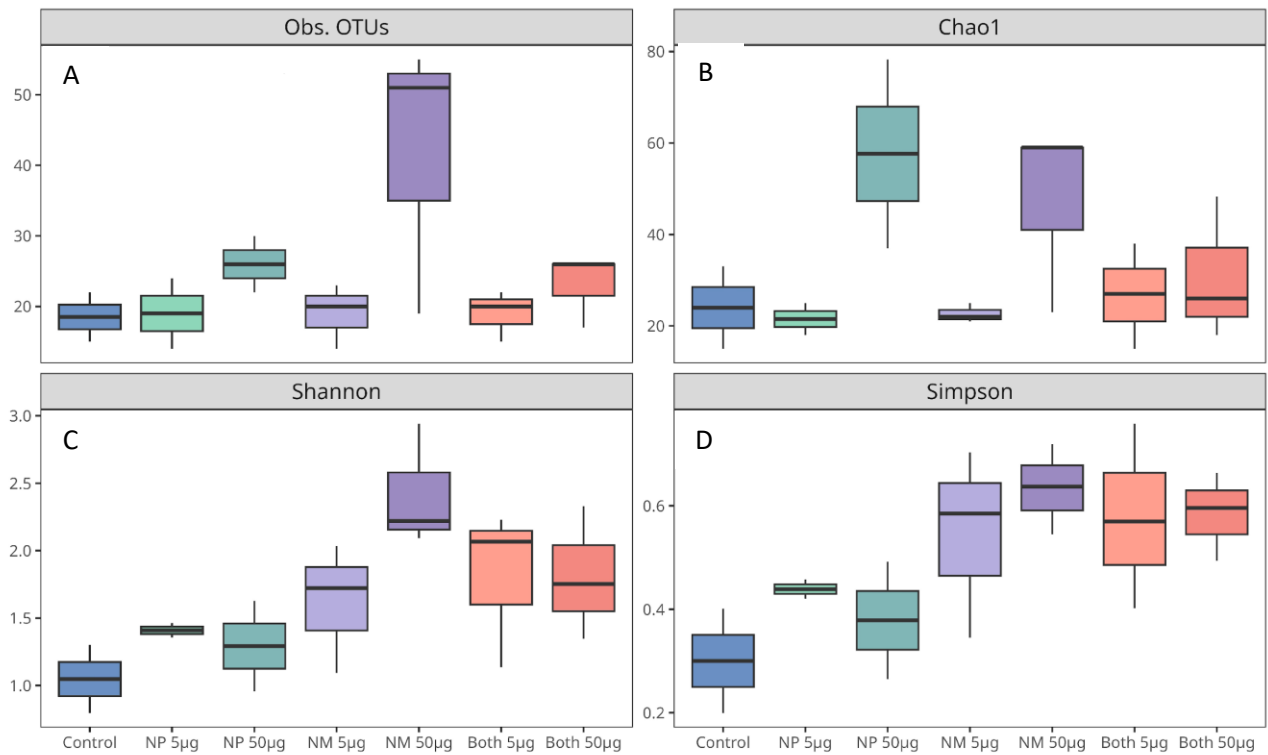


Fig. 4. Alpha diversity indexes, observed OTUs (A), Chao1 (B), Shannon (C) and Simpson (D) for control and treatments with the black lines indicating the Media and inferior and superior values. The conditions are Control at blue, nanopolystyrene at 5 µg/L (NP5µg: COOH-PS-NP 5 µg; light-green), nanopolystyrene at 50 µg/L (NP50µg: COOH-PS-NP 50 µg; dark-green) nano-titanium-dioxide at 5 µg/L (NM5µg: TiO₂-NP 5 µg; light-purple), nano-titanium-dioxide at 50 µg/L (NM50µg: TiO₂-NP 50 µg; dark-purple), combined nanoparticle exposure at 5 µg/L (Both5µg: Both-NP 5 µg; light-orange) and combined nanoparticle exposure at 50 µg/L (Both50µg: Both-NP 50 µg; dark-orange).

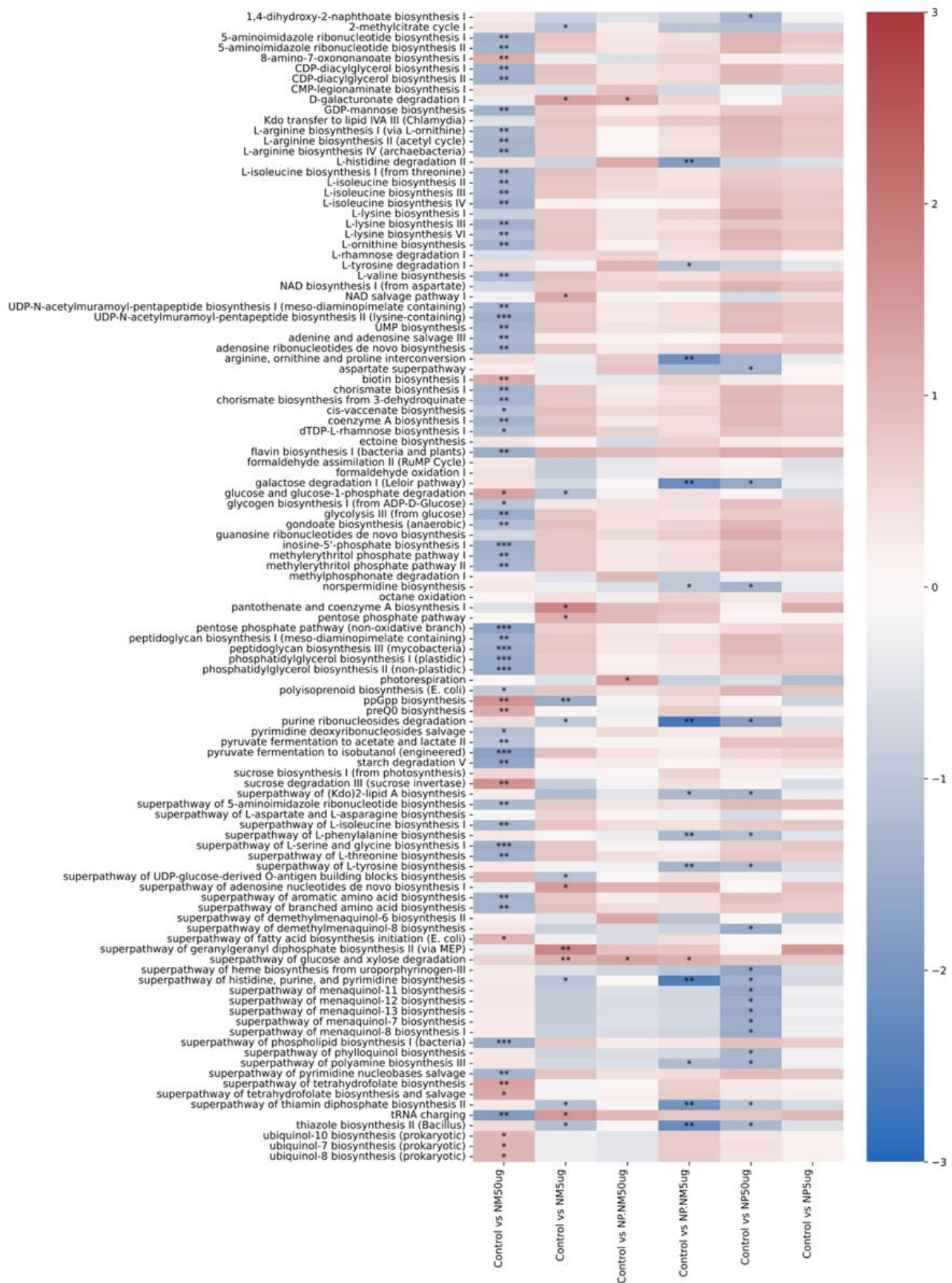


Fig. 5. Difference in predicted molecular pathways, based on abundances, between control and treatment conditions, revealed with Picrust2. The treatments displayed are: nanopolystyrene at 5 $\mu\text{g/L}$

(NP5 μ g), nano-titanium-dioxide at 5 μ g/L (NM5 μ g), combined nanoparticle exposure at 5 μ g/L (NP-NM5 μ g), nanopolystyrene at 50 μ g/L (NP50 μ g), nano-titanium-dioxide at 50 μ g/L (NM50 μ g) and combined nanoparticle exposure at 50 μ g/L (NP-NM50 μ g). Blue colour indicates Kegg term under-representation and red colour over-representation. Significance is indicated by asterisks: * < 0.05; ** < 0.001 and *** < 0.0001.

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