Structurally stable but functionally disrupted marine microbial communities under a future climate change scenario: Potential importance for nitrous oxide emissions

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Running title Marine microbes' sensitivity to global change

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Abstract

The blue mussel *Mytilus edulis* is a widespread and abundant bivalve species along the North Sea with high economic and ecological importance as an engineer species. The shell of mussels is intensively colonized by microbial organisms that can produced significant quantities of nitrous oxide (N_2O), a potent greenhouse gas. To characterize the impacts of climate change on the composition, structure and functioning of microbial biofilms on the shell surface of *M. edulis*, we experimentally exposed them to orthogonal combinations of increased seawater temperature (20 vs. 23 °C) and decreased pH (8.0 vs. 7.7) for six weeks. We used amplicon sequencing of the 16S rRNA gene to characterize the alpha and beta diversity of microbial communities on the mussel shell. The functioning of microbial biofilms was assessed by measuring aerobic respiration and nitrogen emission rates. We did not report any significant impacts of climate change treatments on the diversity of mussel microbiomes nor on the structure of these communities. Lowered pH and increased temperature had antagonistic effects on the functioning of microbial communities with decreased aerobic respiration and N_2O emission rates of microbial biofilms in acidified seawater compared to increased rates in warmer conditions. An overriding impact of acidification over warming was finally observed on N_2O emissions when the two factors were combined. Although acidification and warming in combination significantly reduced N_2O biofilm emissions, the promotion of aquaculture activities in coastal waters where shellfish do not normally occur at high biomass and density could nonetheless result in unwanted emissions of this greenhouse gas in a near future.

Key words: ocean acidification; ocean warming; microbial biofilms; bivalves; nitrogen fluxes, greenhouse gas emission.

1. Introduction

Since the beginning of the Industrial Revolution, anthropogenic carbon dioxide (CO_2) emissions have constantly increased and contributed to a raise in the global mean sea surface temperature (SST) of 0.68-1.01°C and a decrease of ocean surface pH of 0.1 units (IPCC, 2022). These shifts are expected to continue with a further mean pH decrease of 0.08-0.37 units and a mean temperature increase of 0.86-2.89°C by the end of this century relative to the 1986-2005 period under the shared socio-economic pathway (SSP) 1-2.6 and 5-8.5. Changes in seawater pH and temperature have direct physical and chemical consequences such as alteration of gas solubility, enhanced evaporation rates, modified seawater carbonate equilibria or changes in salinity (Bindoff et al., 2019; Hoegh-Guldberg et al., 2014; Pörtner et al., 2014). They also have knock-on impacts on marine biogeochemistry and ecology (Bindoff et al., 2019; Harvey et al., 2013; Pörtner et al., 2014).

The nitrogen cycle consists of a series of microbially mediated oxidation-reduction transformations which are sensitive to changes in pH and temperature (Beman et al., 2011; Hicks et al., 2018; Kitidis et al., 2011), hence ocean acidification (OA) and warming (OW) may critically influence the roles of microbes in the nitrogen cycle (Cavicchioli et al., 2019; Currie et al., 2017; Hutchins & Fu, 2017). For instance, projected future seawater pCO_2 is expected to lower ammonia oxidation rates of oceanic microbial communities (Beman et al., 2011) while enhancing their nitrogen fixation rate (Hutchins et al., 2007; Lomas et al., 2012). Changes in oceanic pH can indeed affect the availability of substrates for nitrogen transformations such as the NH_3-NH_4^+ equilibrium (Bange, 2008). Additionally, OA and OW have the potential to (1) directly impact the activity and performance of pelagic (e.g., Endres et al., 2014; Grossart et al., 2006; Hutchins et al., 2007; Lindh et al., 2013; Piontek et al., 2009) and benthic (Braeckman et al., 2014; Laverock et al., 2013; Rastelli et al., 2015) microbiota and (2) drive changes in structure and diversity of microbial communities (Currie et al., 2017; Krause et al., 2012; Vezzulli et al., 2012). Impacts of climate change on the structure and functioning of microbial communities have mainly been studied with a focus on either OA or OW as single stressors. However, their combined effects can differ from their individual ones (Lindh et

al., 2013; Minich et al., 2018), and interactive effects of OA and OW on marine microbes, as well as their implications for nitrogen cycling, should therefore be investigated.

In addition to being affected by global climate change, the nitrogen cycle can also contribute to radiative forcing in the atmosphere as some microbially-mediated redox conversions produce nitrous oxide (N_2O), a potent greenhouse gas (GHG) (Bange et al., 2010; Devol, 2008). While water bodies and (sub)oxic sediments are broadly recognized as important sources of microbial N_2O production (Voss et al., 2013), there are also other biogenic emissions of N_2O. Microbes in the gut and on the gills of marine invertebrates, for instance, can release N_2O during nitrification and respiratory denitrification processes (Heisterkamp et al., 2016, 2010; Welsh and Castadelli, 2004). These two processes also occur in microbial biofilms on the shells of mollusks (Caffrey et al., 2016; Heisterkamp et al., 2013). This biological matrix is an important site of N_2O emissions with up to 94 % of the total mollusk-associated emissions of the GHG due to microbial assemblages on bivalve shells (Heisterkamp et al., 2013).

Along European coasts, an increasing number of offshore wind farms (OWF) are being installed to meet the requirements for green renewable energy (Degraer et al., 2020; Wind Europe, 2021). The submerged foundation of wind turbines constitutes an artificial hard substrate (AHS) that is rapidly and intensely colonized by fouling organisms, of which the blue mussel *M. edulis* is commonly one of the most dominant, both in terms of abundance and biomass (De Mesel et al., 2015; Degraer et al., 2020; Lindeboom et al., 2011). This species is of high economic interest, with a production of ca. 170-200 10^3 tons per year in Europe (FAO Fisheries Division, Statistics and Information Branch, 2020). Over the next decade, mussel aquaculture will be promoted within OWF concession areas in the North Sea to optimize the use of the available space (Buck et al., 2008, 2004). Blue mussels can emit N_2O *via* the activity of microbial organisms on their outer shell (Heisterkamp et al., 2013). As a result, human actions promoting availability of substrates (bivalve shells) where N_2O is produced might partly counteract the objective of OWF to limit anthropogenic GHG emissions. It is therefore critical to better understand how changes in the marine environment related to OA and OW would affect the biogenic production of N_2O (Hutchins & Fu, 2017; Voss et al., 2013; Wannicke et al., 2018).

Here, we conducted laboratory experiments aiming to (1) characterize microbial communities on shells of the blue mussel *M. edulis* in the North Sea; (2) investigate the effects of OA and OW (separately and in combination) on the composition, α-diversity and structure of biofilm microbial communities; and assess the effect of OA and/or OW on (3) the contribution of shell biofilms to nitrogen compounds' production/consumption and (4) the relative abundance of different nitrogen pathways.

2. Materials and methods

2.1. Experimental design

A fully crossed factorial experiment was conducted in July-August 2018 to test the separate and combined influence of seawater temperature increase and pH decrease according to the IPCC "business as usual" scenario representative concentration pathway (RCP) 8.5 (IPCC, 2014) on compositional (i.e., the microbial "species"), structural (i.e., the diversity of the microbial "species") and functional aspects of microbial communities on the shells of blue mussels, *Mytilus edulis*. The warming and acidification factors encompassed two levels each: ambient summer SST in the Belgian part of the North Sea (BPNS) vs. a temperature increase of 3°C, and ambient pH of the BPNS water in summer vs a pH decrease of 0.3 units. The experiment thus consisted of four treatments: (1) "Control", i.e. ambient SST and pH, (2) "Acidification", i.e. ambient SST and a 0.3 units decrease in ambient pH, (3) "Warming", i.e. a 3°C increase in the SST at ambient pH, and (4) "Climate change", i.e. a 3°C increase in the SST and a decrease in ambient pH of 0.3 units. The experiment lasted six weeks to assess the relatively long-term effects of the investigated stress factors.

2.2. Experimental setup

2.2.1. Sampling and acclimation of *Mytilus edulis*

Adult blue mussels (mean shell length ± SE = 45.0 ± 0.3 mm) were sampled in July 2018 off the coast of Nieuwpoort, Belgium (51°11.024'N, 002°39.883'E), at an aquaculture pilot project site where mussels are farmed using longlines. Mussels were maintained in 80-L barrels filled with oxygenated *in situ* seawater and transported within 4 h to the laboratory. The SST and pH at time of sampling were 19.9°C and 7.9, respectively.

Back at the laboratory, approximately 200 mussels were added to each of four mesocosms (one per experimental treatment; length = 100 cm, height = 45 cm, width = 70 cm) filled with unfiltered natural seawater (height of the water column = ca. 30 cm). Mussels were left to acclimatize to mesocosm conditions

for 24 h before manipulation began (see 2.2.2). Seawater temperature during the acclimation period was 20.0°C, salinity was 32.4 and pH was 7.96. Oxygen pumps were placed in each mesocosm to keep the water fully oxygenated throughout acclimation and incubation (see 2.2.2).

2.2.2. Incubation procedure

A large tank supplied each mesocosm with seawater in a closed circuit of ca. 400 L each. Following acclimation (see 2.2.1), seawater temperature and/or pH was increased in steps of 1°C and decreased in steps of 0.1 unit per day over three days, respectively. The seawater temperature was manipulated in the supply tanks using a heater/cooler (TK-200H, TECO®). A sensor (IKS) in each of the four mesocosms continuously recorded seawater temperature in the circuits. Water pH was manipulated by bubbling seawater with pure CO_2. To this end, an electrode (IKS) was placed in each mesocosm and connected to a modular system (aquaristic computer IKS aquastar) to continuously record water pH. Electrodes were calibrated weekly using buffer solutions (MetrOhm, pH = 4 and pH = 7). The CO_2 bubbling method alters dissolved inorganic carbon at constant total alkalinity and is considered efficient to maintain constant conditions over a long period of time (Riebesell et al., 2011). Approximately one third of the total seawater volume for each treatment was renewed weekly. Mesocosms were kept under a 15h30:8h30 light-dark regime throughout the experiment, mimicking the natural day-night regime at time of sampling (summer 2018, see 2.1). Air-bubbling systems were added to the mesocosms to keep the water fully oxygenated during the six weeks of the incubation period. A summary of the physico-chemical water parameters for the four treatments throughout the experiment is available in Supplement S1.

Throughout the experiment, mussels were fed twice a week by adding 3 mL of a mix of marine microalgae (Shellfish Diet 1800®, Reed Mariculture) to each mesocosm. Mortality of mussels was checked on a daily basis and dead organisms were removed but not replaced. After six weeks, three mussels were randomly sampled in each mesocosm, placed individually in an incubation chamber (diameter = 10 cm, height = 20 cm) and their respiration rates and those of their microbial biofilm were assessed (see 2.2.3).

2.2.3. Aerobic respiration of microbial biofilms

144 The oxygen consumption rate of microbial biofilms on the shells of three randomly sampled blue mussels
145 per mesocosm was measured using a direct flux approach. To assess the respiration rate of the "pure"
146 biofilms, the shells were opened by cutting the posterior adductor muscle and all soft tissues were removed.
147 Empty mussel shells and their microbiome (N = 3 per treatment) were then individually placed in incubation
148 chambers (diameter = 10 cm, height = 20 cm) along with water from their respective mesocosms. Control
149 incubation chambers without empty mussel shell were also set up to. Each incubation chamber was then
150 placed in a water bath in which the temperature corresponded to that in the associated treatment. Chambers
151 were then sealed and an oxygen probe (OXROB10, PyroScience) was introduced in the water column
152 through the lid. A magnetic stirrer homogenized the water column. Incubations lasted for 3 h. The 0% and
153 100% oxygen calibration were performed in a sodium ascorbate solution and in the saturated overlying
154 water of the incubation core, respectively, before the beginning of the incubation. Aerobic respiration rate
155 was calculated as the difference in oxygen concentration in the water column between the beginning and
156 end of the incubation divided by its duration. The latter were corrected for bacterioplankton respiration rates
157 using control incubations.

158 **2.2.4. Influence of microbial communities on nitrogen fluxes**

159 During respiration measurements of biofilms on mussel empty shells (see 2.2.3), we assessed the
160 contribution of microbial biofilms to dissolved ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and nitrous
161 oxide (N_2O) concentrations in the surrounding water. To this end, we sampled 30 and 25 mL of water at the
162 start and end, respectively, of the three-hour incubation to quantify dissolved N_2O and NH_4^+, NO_3^- and NO_2^-
163 concentrations, respectively. Water samples for N_2O analysis were fixed with 100 µL of a saturated $HgCl_2$
164 solution and N_2O concentration in the water was determined by column gas chromatography (8610C Gas
165 Chromatograph, SRI Instruments). A continuous flow analyser (SAN++, Skalar) was used to quantify
166 dissolved concentrations of NH_4^+, NO_3^- and NO_2^-. Concentrations were corrected for background nitrogen.
167 Nitrogen fluxes (net emission or consumption rates) were calculated as the difference between initial and

final concentrations relative to the duration of the incubation. At the end of the incubation, mussel shells were individually placed in plastic bags and frozen at -20°C pending DNA extraction (see 2.3).

2.3. DNA extraction and PCR amplification

Microbial biofilms were scraped from the shell surface of frozen mussels using sterile tweezers and razors. Total DNA was obtained using phenol-chloroform extraction. In detail, scraped biofilms were individually placed in 1.5 mL Eppendorf® tubes with 500 µL of Tris/EDTA (TE buffer, 10mM Tris – 1 mM EDTA), 500 µL of phenol (ROTI®Phenol, Roth) and 0.5 g zirconia beads. Extracts were mechanically homogenized (Mixer Mill MM400, Retsch; 1 min 25 sec at 30 Hz, 3 times) and centrifuged for 5 min (11,292 rcf at 4°C). The supernatant of each sample was transferred into a new sterile tube with 500 µL of chloroform:isoamylalcohol (25:24:1 v:v:v). Tubes were shaken by hand and then centrifuged for 5 min (11,292 rcf at 4°C). This step was repeated twice before the supernatant was harvested and transferred into a new tube with 50 µL of sodium acetate (3 M), 1 mL of ethanol (96%) and 2 µL of glycogen (20 mg mL^{-1}). Samples were manually mixed and incubated overnight at -20°C. They were then centrifuged for 30 min (18,213 rcf at 4°C); the supernatant was discarded, 1 mL of ethanol (70%) was added and tubes were centrifuged for 5 min (18,213 rcf at 4°C). The content of the collection tube was discarded and the centrifugation step repeated to ensure there was no remaining ethanol. Pellets were air-dried for 20 min before addition of 100 µL of TE buffer. Finally, tubes were incubated for 20 min at 55°C for a complete dissolution of the DNA pellets. DNA extracts were then stored at -20°C pending PCR amplifications.

Bacterial and Archaeal V4 regions of the 16S small-subunit rRNA gene were amplified by PCR using the primer pair 515F–806R (Caporaso et al., 2011). Each sample was amplified in three technical replicates. The PCR was carried out in a total volume of 20 µL comprising 4 µL of PCR reaction buffer (Thermo Fisher Scientific), 0.4 µL of dNTP mix (10 mM each), 0.2 µL of DNA polymerase (Phusion™ Hot Start II High-Fidelity DNA polymerase, Thermo Fisher Scientific; 2 U μL^{-1}), 2 µL of extracted DNA, 11.4 µL of nuclease-free water and 1 µL of each primer (10 µM). Next to the target-specific region, the reverse primers contained an identifier index for each sample and an Illumina adapter (Caporaso et al., 2011). The cycling

conditions were: 1 cycle of initial activation at 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, 65°C for 30 s and 72°C for 15 s; then a final step at 72°C for 10 min. PCR products were then analyzed by electrophoresis in a 1% w/v agarose gel stained with ethidium bromide. The technical replicates of amplified PCR products for each sample were pooled before purification using the Agencourt AMPure XP PCR Purification kit (Beckman Coulter). The quantity of DNA for each purified amplicon was measured using a Qubit 3.0 fluorometer (Thermo Fisher Scientific). The samples (N = 12 in total) were finally pooled using 10 ng of amplicon DNA for each, and the library was sent to Edinburgh Genomics, University of Edinburgh, for Illumina MiSeq sequencing using a 2 x 300-bp paired-end protocol.

2.4. Bioinformatics analyses

Sequencing results were analyzed using QIIME 2 (Bolyen et al., 2019). Forward and reverse primers were first removed from the reads. The DADA2 QIIME 2 plugin (Callahan et al., 2016) was then used to detect and remove low-quality regions of the reads (quality scores < 25) and filter chimeric sequences. An amplicon sequence variant (ASV, Callahan et al., 2017) table was produced, with the number of times each ASV was observed in a sample. Taxonomy was assigned to each of the ASVs using the classify-sklearn qiime feature classifier against the SILVA v132 database (Quast et al., 2012). The classified ASV table was filtered by removing all ASVs with a frequency < 0.1 % of the minimum sample depth (minimum number of reads = 32,170 sequences) as well as non-bacterial or archaeal sequences (i.e., mitochondrial and chloroplast 16S sequences). Rarefaction curves indicated that biofilm microbiomes were sequenced at a depth sufficient to capture the diversity of communities under investigation (Supplement S2). A rooted phylogenetic tree was finally generated using the align-to-tree-mafft-fasttree qiime phylogeny command.

QIIME 2 output files were analyzed using R v.3.6.2 (2019-12-12) (R Core Team, 2019). For all analyses, data were rarefied down to 32,170 sequences corresponding to the minimum sample depth. The rarefied number of ASVs per sample was used as a measure of species richness. To evaluate the alpha diversity of mussel-associated bacterial communities within each sample, the Shannon-Wiener and Pielou evenness

indices were calculated. We also defined the core microbiomes of mussel biofilm samples by identifying a list of ASVs that were shared by at least 90% of the samples (Shade and Handelsman, 2012).

The "phylogenetic investigation of communities by reconstruction of unobserved states" approach was applied to predict the functional potential of the microbial communities on mussel shells from ASV taxonomic affiliations (PICRUSt2; Douglas et al., 2019; Langille et al., 2013). The unrarefied ASV table was used as input. We primarily used PICRUSt2 instead of PICRUSt because it does not require input sequences to be operational taxonomic units (OTUs) (Douglas et al., 2019). From the Kyoto encyclopedia of genes and genomes (KEGG) prediction table, we then identified genes predicted to be involved in the nitrogen cycle. According to the KEGG nitrogen metabolism reference pathway (https://www.genome.jp/kegg/pathway/map/map00910.html), the following pathways were identified: assimilatory nitrate reduction (K00360, K00366, K00367, K00372), denitrification (K00368, K15864, K04561, K02305, K00376), dissimilatory nitrate reduction to ammonium (DNRA; K00367, K00372, K00360, K00366, K00362, K00363, K03385, K15876), nitrate reduction (first step of denitrification and DNRA; K00370, K00371, K00374, K02567, K02568), nitrification (K10944, K10945, K10946, K10535) and nitrogen fixation (K02588, K02586, K02591, K00531).

2.5. Statistical analyses

2.5.1. Microbial community composition and structure

One-way analysis of variance (ANOVA) was used to assess the influence of the experimental treatments (four levels: "Control", "Acidification", "Warming" and "Climate change") on alpha diversity metrics and on the proportion of the total microbiome belonging to the core microbiome. Normality of the data and homogeneity of variances were checked using Shapiro-Wilk and Levene tests, respectively. Data were log-transformed when assumptions were not met. Finally, posthoc Tukey tests were conducted to assess significant differences between entities.

Principal coordinate analyses (PCoA) based on generalized UniFrac distances (Chen et al., 2012) were used to visualize the effect of the factor "Treatment" on the structure of microbial communities (i.e., relative

abundance of the ASVs) associated with the mussel shells. Permutational multivariate analyses of variance (PERMANOVA) (Anderson, 2001) were run to assess the significance of the separation of groups on the PCoA plot (number of permutations = 999). The PERMDISP procedure was used to test the effects of the experimental treatments on among-replicate variability (i.e. dispersion) (Anderson, 2006). We conducted posthoc pairwise comparison tests to assess significant differences between entities when a significant effect of the "Treatment" factor was reported. All analyses were performed with R (R Core Team, 2019).

2.5.2. Functioning of microbial communities

One-way ANOVA was used to assess the effect of experimental treatment on respiration rates and N_2O, NH_4^+, NO_3^- and NO_2^- net production/consumption rates of microbial communities on the shell of blue mussels after six weeks of incubation. Posthoc Tukey tests were conducted to assess significant differences between entities. Normality of the data and homogeneity of variances were checked using Shapiro-Wilk and Levene tests, respectively. PERMANOVA was run to evaluate the effect of experimental treatments on the relative abundance of the different nitrogen pathways All analyses were performed with R (R Core Team, 2019).

3. Results

3.1. Survival of mussels

After six weeks of incubation, mussel mortality in the "Control" mesocosm was < 3 % (97.3% survival; $N = 75$) (Supplement S3). A few mussels died after three weeks in the "Warming" treatment and a cumulated mortality of 10.7% was observed after six weeks. Mussel mortality in "Acidification" and "Climate change" mesocosms was observed as soon as three and one days after initiation of the experiment, respectively, and reached 26 and 33.4%, respectively, after six weeks (Supplement S3).

3.2. Microbial communities: alpha diversity

A total of 615,224 amplicon reads were sequenced with a mean of 51,269 reads per sample (range = 32,857–67,837 reads). After filtering and removal of rare ASVs and chimeras, 608,737 reads remained, i.e., the filtering steps reduced the dataset by ca 1.1 %. A total of 226 to 438 ASVs per sample was identified (Supplement S4).

There was no significant effect of the experimental treatments on species richness (Fig. 1A; One-way ANOVA, $F = 0.76$, $p = 0.55$), Shannon-Wiener index (Fig. 1B; One-way ANOVA, $F = 0.11$, $p = 0.95$) and Pielou evenness (Fig. 1C; One-way ANOVA, $F = 0.68$, $p = 0.59$).

3.3. Microbial community structure and taxonomic composition

3.3.1. Community structure

Permutational multivariate analysis of variance (PERMANOVA) revealed a significant effect of the experimental treatment on the microbial community structure (PERMANOVA, pseudo-$F = 1.68$, p-perm = 0.004; Permdisp, pseudo-$F = 2.63$, p-perm =0.11). There was no clear discrimination of the biofilm communities according to treatment along the first two axis of the PCoA ordination plot (Fig. 2A). In contrast, a distinct separation of "Control" and "Acidification" microbial communities along the third axis of the PCoA ordination plot was observed (Fig. 2B). However, this was not supported by PERMANOVA pairwise tests.

3.3.2. Taxonomic composition

Microbial communities on the shell of mussels were dominated by Bacteroidia, Alphaproteobacteria, Gammaproteobacteria and Planctomycetacia, irrespectively of the experimental treatment (Fig. 3). Together, these four bacterial classes represented 79.2 ± 11.4 to 90.9 ± 0.6 % of the total microbial community (mean ± SE) (Table 1). At the order level, Flavobacteriales (Bacteroidia), Rhodobacterales (Alphaproteobacteria), Planctomycetales (Planctomycetacia) and, to a lesser extent, Oceanospirillales (Gammaproteobacteria), Verrucomicrobiales (Verrucomicrobiae), Pirellulales (Planctomycetacia), and Rhizobiales (Alphaproteobacteria) dominated the biofilm assemblages (Fig. 3B, Table 1). As suggested by the PCoA ordination plot (Fig. 2A), the relative abundance of the different bacterial classes and orders were similar between the four experimental treatment (Fig. 3, Table 1).

3.3.3. Core microbiome

Fifteen ASVs were shared between 90% of the mussel biofilm samples and represented 4.7-20.4% of the total number of reads (mean ± SE = 12.0 ± 1.3%) (Supplement S5) with no significant differences between treatments (one-way ANOVA, $F_{1,3}$ = 0.36, p = 0.78). The core ASVs belonged to Bacteroidia (Flavobacteriales: Flavobacteriaceae: *Aquimarina*; N = 9 ASVs), Planctomycetacia (Planctomycetales: Rubinisphaeraceae: *Planctomicrobium* and *Fuerstia*; N = 2 and 3 ASVs, respectively) and Gammaproteobacteria (Oceanospirillales: Nitrincolaceae: *Neptuniibacter*; N = 1 ASV). There was no effect of "Treatment" on the structure of the core microbiome (PERMANOVA, pseudo-F = 1.068, p-perm = 0.40; Permdisp, pseudo-F = 0.86, p-perm =0.52).

3.4. Functional analyses

3.4.1. Biofilm respiration rates

Respiration rates of microbial communities on the shell of blue mussels ranged between 1.2 and 8.1 µmol O_2 ind^{-1} $hour^{-1}$ (Fig. 4). There was a main effect of the "Treatment" factor (one-way ANOVA, F_3 = 10.9 and p <0.05) on respiration rates. Posthoc tests showed that acidification caused a decrease in oxygen

consumption of biofilms as compared to the control samples. In contrast, the "Warming" (Tukey HSD, p = 0.45) and "Climate change" (Tukey HSD, p = 0.16) treatments did not significantly affect the biofilm respiration rates as compared to the "Control" (Fig. 4).

3.4.2. Emission and consumption of nitrogen compounds

There was a net production of nitrous oxide N_2O by mussel biofilms that ranged between 0.59 and 10.7 nmol N_2O ind.$^{-1}$ h^{-1} after six weeks of incubation (Fig. 5A). Treatments significantly affected net N_2O production rates (one-way ANOVA, F_3 = 1066.4, $p < 0.001$). In acidified treatment there was a significant reduction in N_2O production compared to "Control" (Fig. 5A; Tukey posthoc test, $p < 0.05$). In contrast, we noted a 3.0-fold increase in the emission rate of N_2O in "Warming" treatment compared to the "Control" (Fig. 5A; Tukey posthoc test, $p < 0.05$). There was also a net production of ammonium NH_4^+ by the shell biofilms (Fig. 5B), but NH_4^+ net emission rates did not significantly vary between treatments (one-way ANOVA, F_3 = 1.4, p = 0.30). Finally, there was no significant effect of treatments on NO_3^- (Fig. 5C; one-way ANOVA, F_3 = 0.44, p = 0.73) and NO_2^- (Fig. 5D; one-way ANOVA, F_3 = 1.3, p = 0.35) fluxes due to a large inter-individual variability.

3.4.3. Predicted nitrogen cycling functional genes

We identified 15-25 nitrogen metabolism KEGG ortholog (KO) genes in the predicted metagenome of each sample. These genes represented 0.13-0.23 % of the total number of predicted KO genes in mussel biofilm samples (Supplement S6). The nitrogen metabolism KO genes belonged to a diverse range of functional pathways, including assimilatory and dissimilatory nitrate reduction to ammonium (DNRA), denitrification, nitrification and nitrogen fixation (Fig. 6). The most common functional pathways were "denitrification" and "dissimilatory nitrate reduction to ammonium", irrespective of treatments. Together, they accounted for 70.4 to 76.6% of the predicted nitrogen metabolism pathways (Fig. 6). There was no significant effect of experimental treatments on the relative abundance of different nitrogen pathways (PERMANOVA, pseudo-F = 1.21, p-perm = 0.35; Permdisp, pseudo-F = 2.1, p-perm =0.16).

4. Discussion

Microbial biofilms on the shell surface of mollusks are important sites of nitrous oxide N_2O production (Heisterkamp et al., 2013, 2010), a potent greenhouse gas. Shellfish constitute more than 45 % of aquaculture production in Europe (*FAO yearbook. Fishery and Aquaculture Statistics 2017*, 2019) and their production could be promoted through the development of large aquaculture areas in European coastal waters (Buck et al., 2008, 2004). This raises concern because enhanced shellfish farming may lead to unwanted emissions of GHG. At the same time, coastal waters are strongly impacted by global climate change (Bindoff et al., 2019; IPCC, 2022) and the importance for N_2O emissions of microbial biofilms on the shells of aquaculture species can therefore not be accurately understood without taking into account anthropogenic impacts on seawater characteristics. Here we demonstrate that biofilm communities on the shells of blue mussels, *Mytilus edulis*, are compositionally (identity of ASVs) and structurally (relative importance of the different ASVs) robust to future climate change, but also that they were functionally affected by both OA and OW. Notably, ocean warming significantly enhanced the biofilm production of N_2O whereas acidification decreased it.

4.1. Compositional and structural responses of microbial communities to climate change

Ocean acidification (OA) and warming (OW), alone or in combination, did not significantly affect alpha diversity nor composition of microbial communities on the shell surface of blue mussels. Such a low compositional and structural susceptibility of biofilm communities to OA, OW, and climate change (CC) is surprising since several studies have reported that bacterioplankton communities (Lindh et al., 2013; Wang et al., 2021), bacterial consortia on living (Mensch et al., 2020, 2016) and inert surfaces (Ferguson et al., 2021) are sensitive to these stressors, notably OW. The blue mussel inhabits coastal areas and microbes on their shell might thus be able to withstand environmental changes projected for the end of the 21st century. Indeed, biofilms on the shells of blue mussels are naturally exposed to naturally fluctuating pH and temperature in their environment since variability in coastal environments encompasses a range of, among others, daily and seasonal variations expected in the global climate change context (Duarte et al., 2013; Joint

et al., 2011; Newbold et al., 2012; Wang et al., 2021). Blue mussels may also have influenced the compositional and structural stability of the microbial communities they host on their shell, explaining the biofilms low susceptibility OA and/or OW. Hosts can produce metabolites that can either inhibit growth of some bacteria (Harder et al., 2003; Persson et al., 2011) or promote microbial settlement (Wahl et al., 1994) and thus modulate the colonization of their surface by microbes. Finally, through its own activity, the mussel biofilm itself might have also provided microbial cells with stable micro-environmental conditions. Microbes synthetize and secrete extracellular polymeric substances that can affect the physical and biogeochemical environment in their proximity (Decho, 1990) and could notably help to buffer changing environmental conditions (de Carvalho, 2018; Decho, 1990).

4.2. Functional changes in microbial communities exposed to climate change

While OA and/or OW did not significantly affect the composition of microbial communities nor in the relative abundance of the different groups of microbes, direct measurements of the activity of bacterial consortia reveal significant and contrasting effects of OA and OW on their functioning. In acidified waters, we noted a 2.2 and a 2.6-fold decrease in the respiration rate and N_2O production rate of microbial biofilms, respectively, as compared to the control. In sharp contrast, there was a 3-fold increase in the N_2O production rate but no significant change in the aerobic respiration rate of microbial organisms in the "Warming" treatment. Opposite effects of OA and OW on the functioning of microbial communities are thus observed, as the pH effects counteract the effects of warming, resulting in similar respiration and N_2O production rates in the "Acidification" and "Climate change" treatments. Nonetheless, it remains unclear how OA, OW and CC affect the relative importance of the diverse N_2O emissions pathways, that is nitrification and denitrification (Heisterkamp et al., 2013). Indeed, the predicting analysis (PICRUSt2) did not suggest any significant effect of OA, OW nor CC on the relative contribution of the nitrogen transformation pathways and only assumptions can then be made.

4.2.1. Influence of ocean acidification on microbial communities

In mussel shell biofilms, nitrification and denitrification contribute equally to N_2O production (Heisterkamp et al., 2013). Several studies have reported a significant detrimental effect of OA on

nitrification activities of marine microbes which could lead to a concomitant lower production of N_2O (Beman et al., 2011; Kitidis et al., 2011; Wannicke et al., 2018). In contrast, the modulating role of OA on denitrification activities of marine microbes is less clear. Some studies have highlighted that OA could enhance microbes' N_2O production by stimulating the activity of denitrifiers (Su et al., 2021; Wu et al., 2022) whereas others did not reveal any significant mean effect of this stressor (Wannicke et al., 2018). In this study, we did not observe any significant accumulation of NH_4^+ in the "Acidification" treatment but an increase in the aerobic respiration activity of microbial biofilms. Moreover, the oxygen availability was high (ca. 210 µmol O_2 L^{-1}) in the first 1500 µm above the shell surface of blue mussels in acidified water (Voet et al., 2023). Together, it may suggest a low negative effect of OA on the level of activity of ammonia-oxidising bacteria whereas the activity of anoxic denitrifiers, and concomitant emissions of N_2O, might have been limited. Finally, Voet et al. (2023) have also reported that OA decreases the thickness of marine biofilms on the shell of mussels. Therefore, lower production rate of N_2O may results from a combined negative effect of OA on nitrogen transformation pathways (nitrification and/or denitrification) and microbial abundances.

4.2.2. Influence of ocean warming on microbial communities

Warmer temperatures usually enhance microbial activity through stimulatory effects on biochemical reaction rates (Brown et al., 2004; Gillooly et al., 2001; Pomeroy and Wiebe, 2001), and a similar trend was observed here with a substantial increase in the microbial production of N_2O compared to the "Control" treatment. The processes underlying this pattern are expected to involve higher denitrification rates promoted by more favourable conditions for the activity of denitrifiers, notably lower oxygen availability (Battaglia and Joos, 2018; Codispoti, 2010). Indeed, OW tended to enhance the aerobic respiration rates of microbes which lead to reduced dissolved oxygen concentrations in the close vicinity of the shell surface of mussels (ca. 170-190 µmol O_2 L^{-1} in the first 500 µm above the shell) (Voet et al., 2023).

Although there is still little information about the effects of OW on the nitrogen cycle (but see Voet et al., 2023), such indirect stimulatory effects of OW on N_2O emissions are widely expected (Hutchins and Fu, 2017; Voss et al., 2013; Wannicke et al., 2018). In particular, the net production of N_2O during

405 denitrification happens at the interface between suboxic and anoxic waters (Bange et al., 2010; Devol, 2008).
406 Global warming could increase ocean stratification, negatively affect O_2 diffusion and lower subsurface
407 oxygen concentrations, and ultimately promote oxygen minimum zones (Gruber, 2011). In this way, it
408 would thus expand favourable denitrifying conditions for microbial organisms and subsequently N_2O
409 production (Battaglia and Joos, 2018; Codispoti, 2010).

410 **4.2.3. Influence of climate change on microbial communities**

411 In this study, the "Acidification" and "Climate change" treatments had similar restricting impacts on the
412 N_2O production by microbial communities on the shell of blue mussels, whereas OW stimulated the
413 production of this GHG. Therefore, OA has an overriding impact over OW. Nonetheless, it remains unclear
414 whether the magnitudes of the OA and CC effects on the different N_2O production pathways are similar.
415 For instance, we observed a net consumption of NO_2^- in some replicates of the "Acidification" treatment
416 and a net production in others, whereas there consistently was a clear net production under "Climate
417 change". Although the high among-replicate variability led to non-significant test results, these tendencies
418 may suggest a lower denitrification rate of microbes in the "Climate change" treatment as compared to the
419 "Acidification" one. Since nitrous oxide production rates are highly similar in the "Acidification" and
420 "Climate change" treatments, it follows that the relative importance of denitrifiers in N_2O production in the
421 climate change scenario may have been reduced while the activity of ammonia-oxidizing microbes may
422 have gained importance. Although the overall singular effects of OA, or OA and OW in combination, on
423 N_2O production could be the same, the nitrogen pathways involved in the production of the GHG may thus
424 be differently affected in the two contexts. Labelling experiments with stable isotopes remain necessary for
425 an accurate characterisation of the underlying mechanisms involved in the nitrous oxide emission under
426 future climate change conditions (Heisterkamp et al., 2013).

427 **4.3. Implications for the nitrogen cycle at the ecosystem level**

428 Our study suggest that climate change might interfere with nitrogen transformations driven by bacterial
429 biofilms on the shell surface of blue mussels, notably denitrification. However, the net and relative

importance of this functional shift at the ecosystem level is uncertain. Knowledge of the roles of microbial associations with macrobiota for nitrogen cycling is still limited (Moulton et al., 2016). A few studies have reported that macrobiota can enhance microbially-mediated N transformations (Diaz and Ward, 1997; Moulton et al., 2016) and that the latter can also be quantitatively important. For instance, ammonium oxidation rates of microbes in association with the Californian mussel *M. californianus* exceeded those of seawater alone by at least two orders of magnitude (Pfister and Altabet, 2019). Here, we observed a 2-fold reduction in the GHG emission in the CC treatment as compared to the control. As such, it could be argued that aquaculture installations in the North Sea would not result in unwanted emissions of GHG under future climate change scenarios. Nevertheless, the promotion of the presence of high densities of bivalves and microbial organisms on their shell through offshore wind farms and/or aquaculture installations in areas that are not naturally colonized by mussels will still result in net unwanted emissions of N_2O irrespective of the climate scenario under consideration.

5. Conclusion

In this study, we used an experimental *ex situ* approach to assess the effects of climate change (ocean acidification OA and warming OW, in isolation and in combination) on the composition, diversity and structure of microbial communities associated with the shell of the blue mussel *Mytilus edulis*. We also evaluated the effects of climate change on mussel shell biofilm-associated nitrogen transformations. Predicted changes in seawater pH and/or temperature had negligible effects on the composition, diversity, and structure of microbial biofilms on the outer shell surface of blue mussels. By contrast, substantial functional shifts occurred with respect to the influence of the microorganisms on the nitrogen cycle. In particular, OW increased the nitrous oxide production rate of bacteria on mussel shells, whereas ocean acidification, in isolation and in combination with ocean warming, reduced it. Nonetheless, we cannot conclude about a positive or a negative effect of climate change on net N_2O emissions. Indeed, the artificial expansion of substrates where N_2O is produced through aquaculture could still result in net emissions of the GHG. At the ecosystem level, the importance of these emissions for large-scale processes remains uncertain,

but considering that the marine nitrogen cycle is tightly coupled to other major cycles, complementary studies appear critical to better understand interactive effects between marine biogeochemical cycles under future climate change (Battaglia and Joos, 2018).

Compliance with ethical standards

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Declarations of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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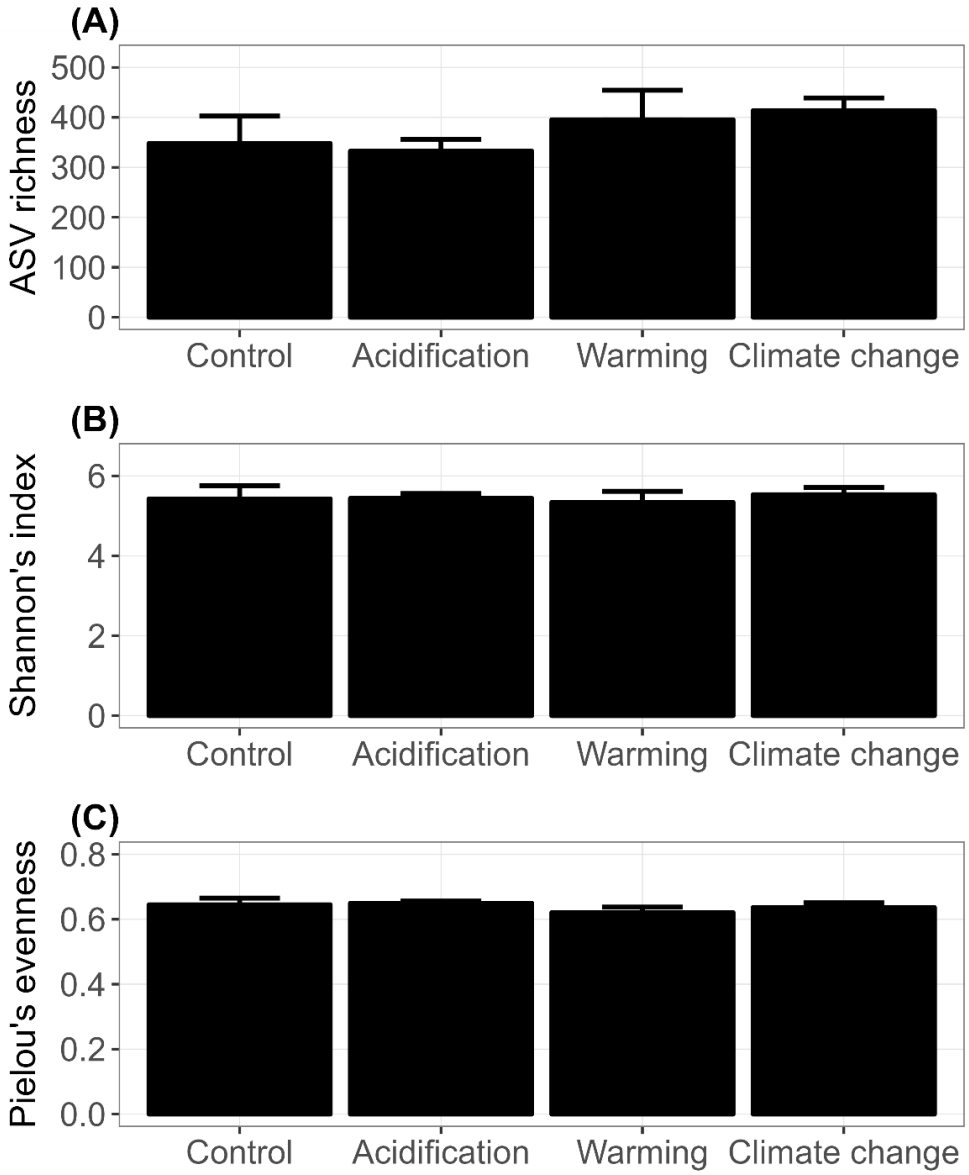

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766 **Figure 1** Influence of experimental treatments ("Treatment" factor, four levels: "Control", "Acidification,"
767 Warming" and "Climate change") on (A) species richness (amplicon sequence variants ASV), (B) Shannon-
768 Wiener's index and (C) Pielou's evenness for microbial communities sampled on the shell of mussels after
769 six weeks of incubation. Results are given as mean + SE. *N* = 3 per experimental treatment.

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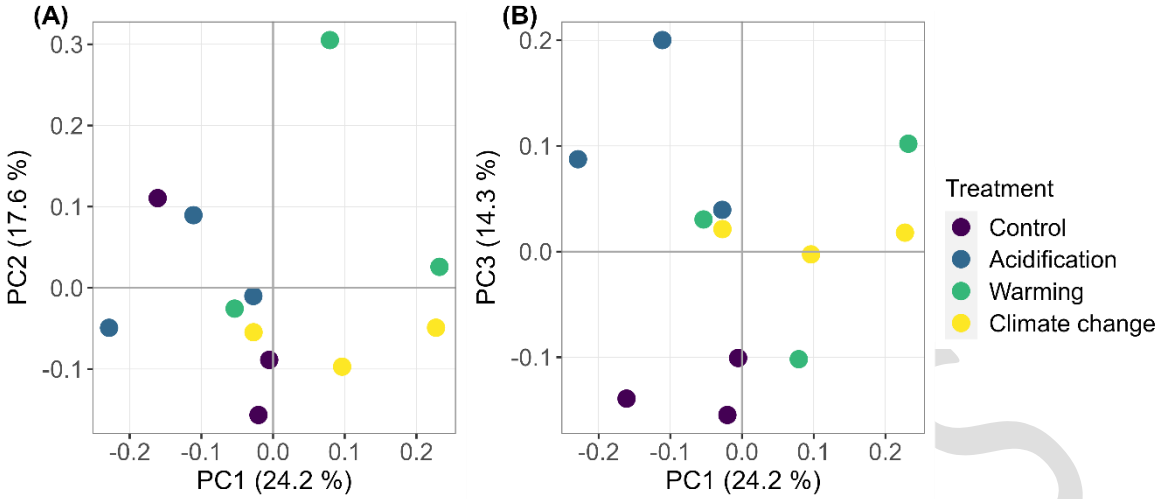

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774 **Figure 2** Principal coordinate analysis (PCoA) ordination based on GUniFrac distance matrix evaluating
775 the influence of simulated seawater acidification and/or warming on microbial communities sampled on
776 the shell of mussels after six weeks of incubation. *N* = 3 per experimental treatment.

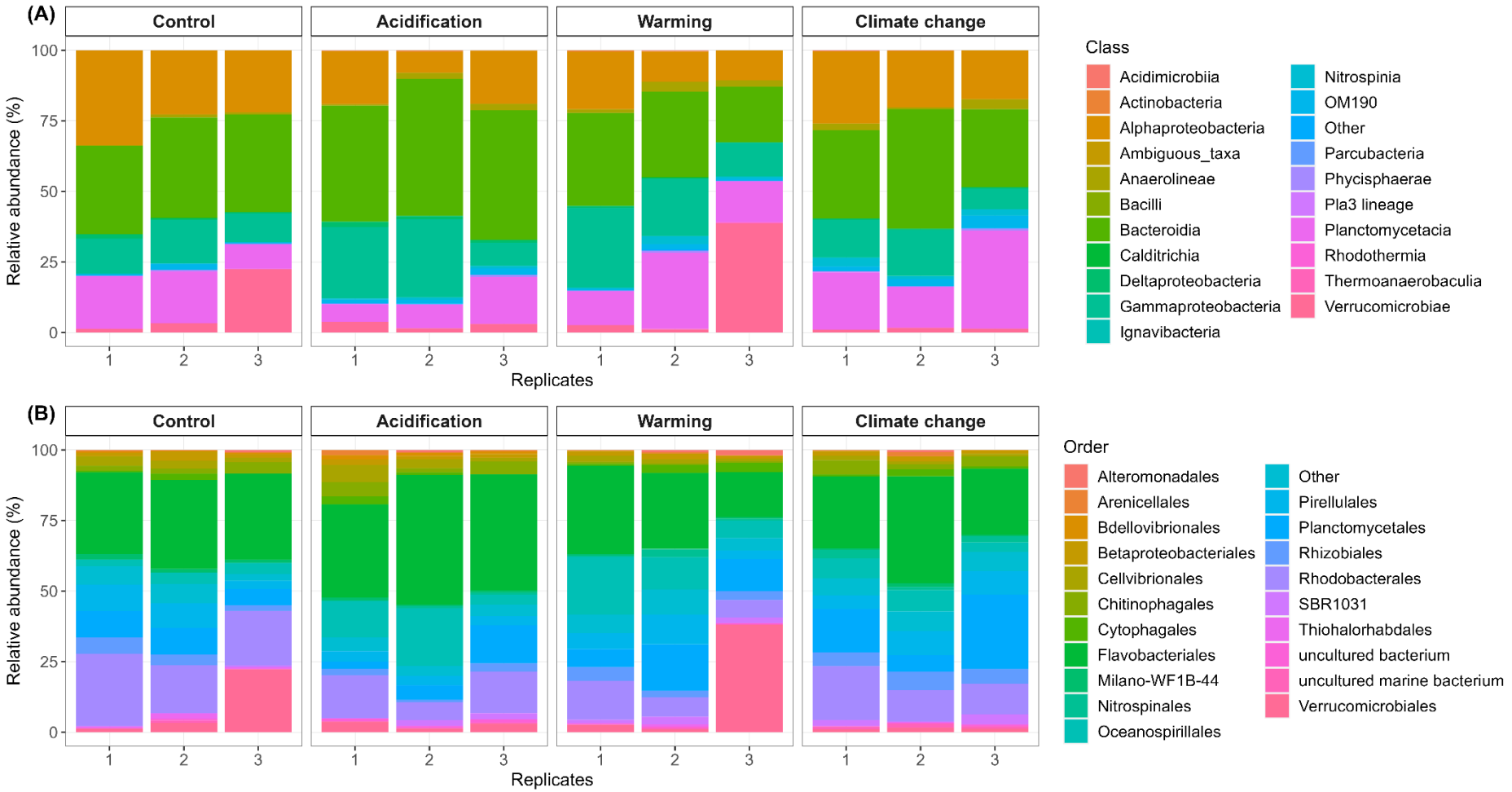
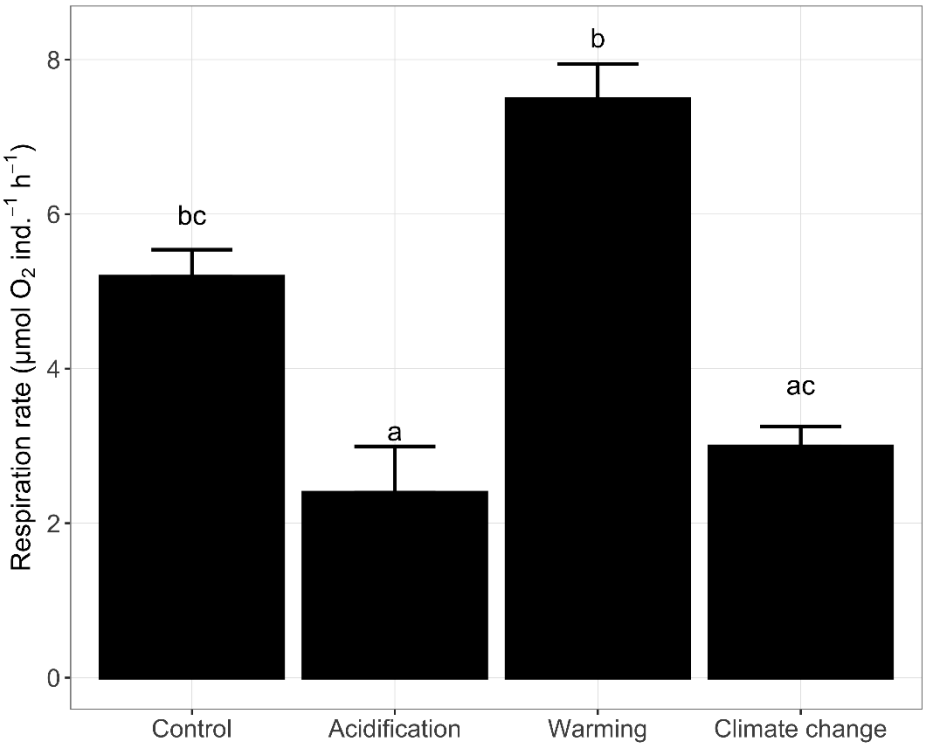


Figure 3 Influence of experimental treatments ("Control", "Acidification", "Warming" and "Climate change") on the relative abundance of the dominant amplicon sequence variants (ASV) at the class (A) and order (B) levels on the shell of mussels *Mytilus edulis* after six weeks of incubation. The twenty most abundant microbial classes and orders are shown. $N = 3$ per experimental treatment.



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782 **Figure 4** Influence of experimental treatments ("Control", "Acidification", "Warming" and "Climate
783 change") on the mean aerobic respiration rates (+ SE) of microbial biofilm developing on the shell
784 surface of blue mussels *Mytilus edulis* after six weeks of incubation. Results of Tukey posthoc tests on
785 the significant effects of experimental treatment are shown (different groups of letters indicate
786 significant differences, $p < 0.05$). Results are given as mean + SE. $N = 3$ per experimental treatment.

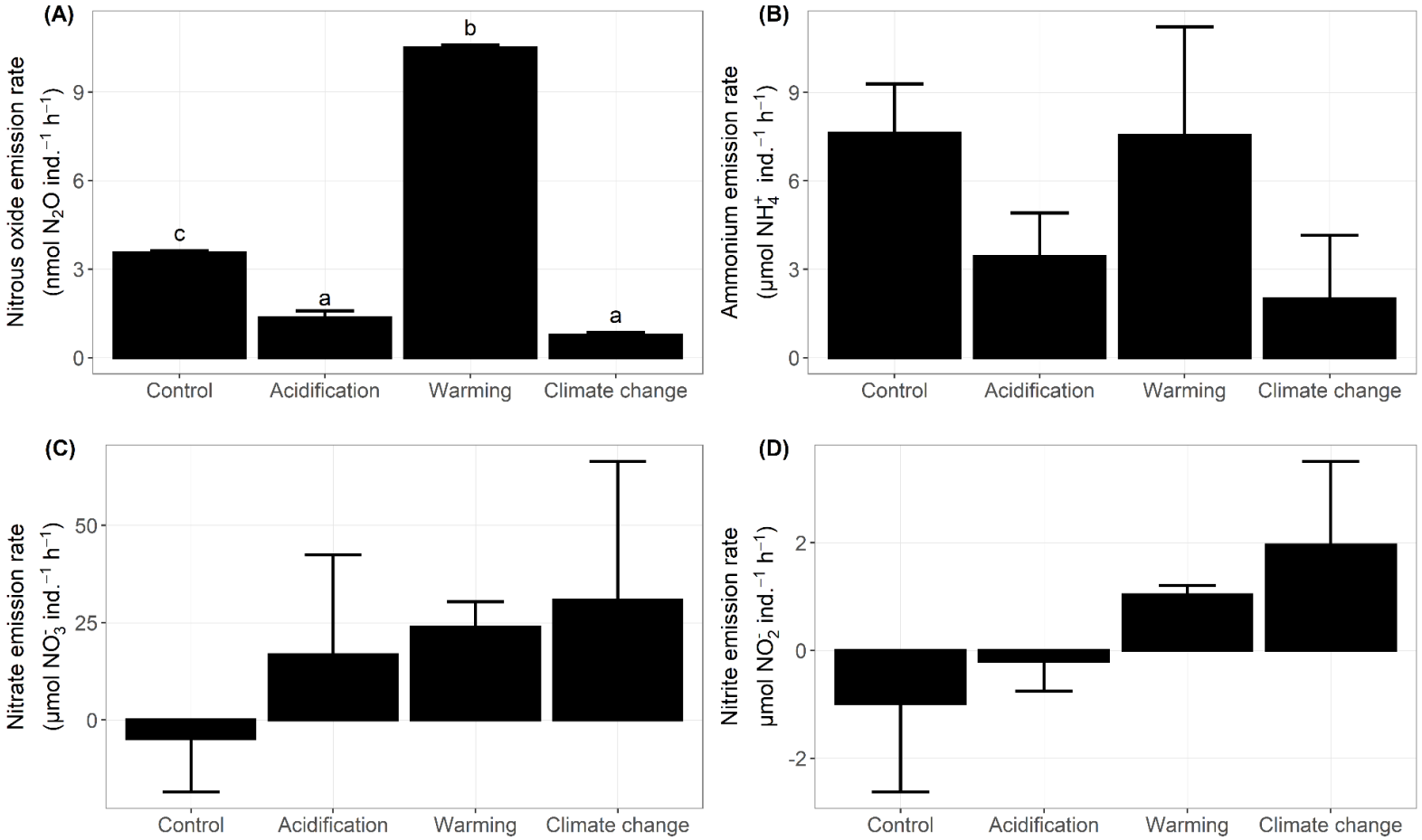
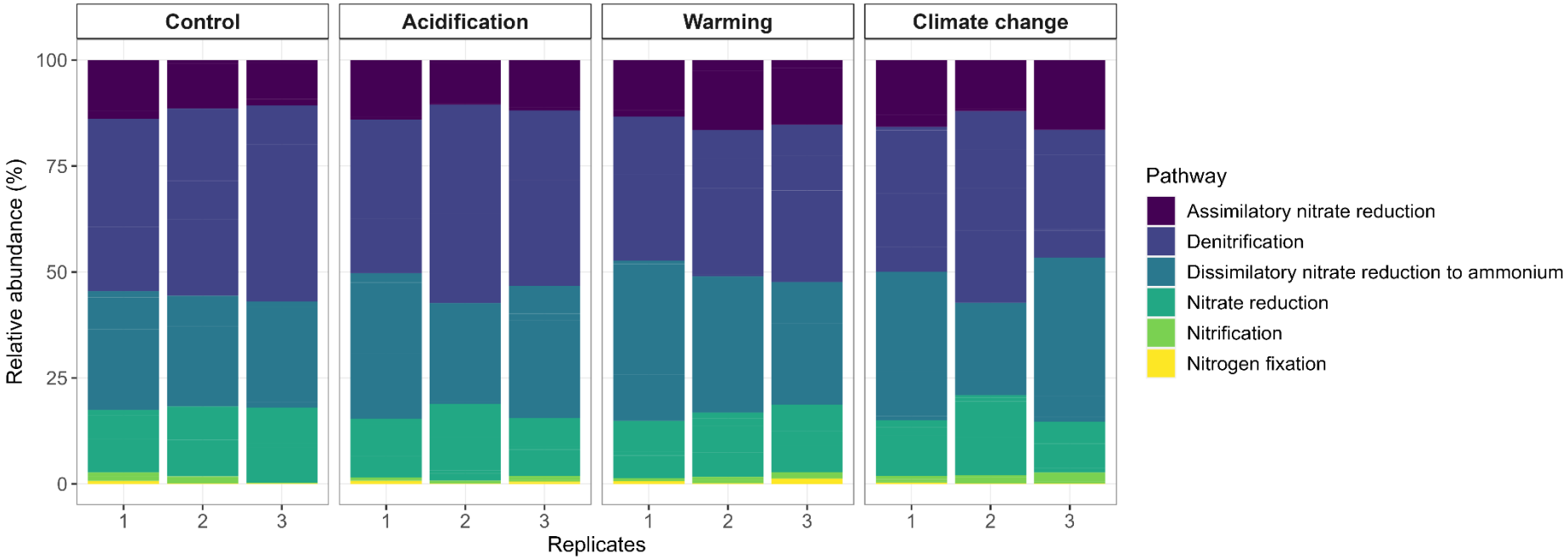


Figure 5 Influence of experimental treatments ("Control", "Acidification", "Warming" and "Climate change") on the mean production or consumption rates of (A) nitrous oxide N_2O, (B) ammonium NH_4^+; (C) nitrate NO_3^- and (D) nitrite NO_2^-. Results of Tukey posthoc tests on the significant effects of experimental treatment are shown (different groups of letters indicate significant differences, $p < 0.05$). Results are given as mean + SE. $N = 3$ per experimental treatment.



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792 **Figure 6** Influence of experimental treatments ("Control", "Acidification", "Warming" and "Climate change") on the relative abundance (%) of predicted nitrogen

793 transformation pathways (PICRUST analysis) associated to the microbiome on the shell of mussels *Mytilus edulis* after six weeks of incubation. Pathways are of

794 the sum of counts of KEGG ortholog genes. *N* = 3 per experimental treatment.

795 **Table 1** Relative abundance (mean ± SE, %) of the most abundant taxa (class and order levels) in microbial communities on the shell of blue mussels *Mytilus*

796 *edulis* after a six-week incubation period under different experimental treatments ("Control", "Acidification", "Warming" and "Climate change").

CLASS	**Control**	**Acidification**	**Warming**	**Climate change**
Alphaproteobacteria	25.9 ± 3.9	15.1 ± 3.7	13.9 ± 3.3	21.0 ± 2.4
Bacteroidia	34.1 ± 1.4	45.1 ± 2.2	27.6 ± 4.2	33.8 ± 4.3
Gammaproteobacteria	12.5 ± 1.4	20.3 ± 6.2	20.0 ± 4.5	12.2 ± 2.6
Planctomycetacia	15.1 ± 3.2	10.5 ± 3.1	17.7 ± 4.5	23.0 ± 5.9
Total proportion of the total community	87.5 ± 6.3	90.9 ± 0.6	79.2 ± 11.4	90.1 ± 1.8
ORDER				
Alteromonadales				
Cellvibrionales				
Flavobacteriales	30.5 ± 0.9	39.9 ± 3.8	24.7 ± 4.7	29.1 ± 4.4
Oceanospirillales	3.4 ± 0.5	12.1 ± 4.9	12.6 ± 4.0	5.9 ± 1.3
Pirellulales	7.0 ± 2.0	3.5 ± 0.2	6.3 ± 2.1	7.5 ± 1.3
Planctomycetales	8.0 ± 1.2	7.0 ± 3.2	11.3 ± 2.9	15.5 ± 5.9
Rhizobiales	3.8 ± 1.1	2.1 ± 0.6	3.5 ± 0.9	5.5 ± 0.5
Rhodobacterales	20.5 ± 2.7	12.1 ± 2.8	8.9 ± 2.4	13.8 ± 2.5
Verrucomicrobiales	9.0 ± 6.7	2.7 ± 0.7	14.0 ± 12.1	1.4 ± 0.1
Total proportion of the total community	82.2 ± 2.5	79.3 ± 3.1	81.3 ± 3.2	78.7 ± 0.4

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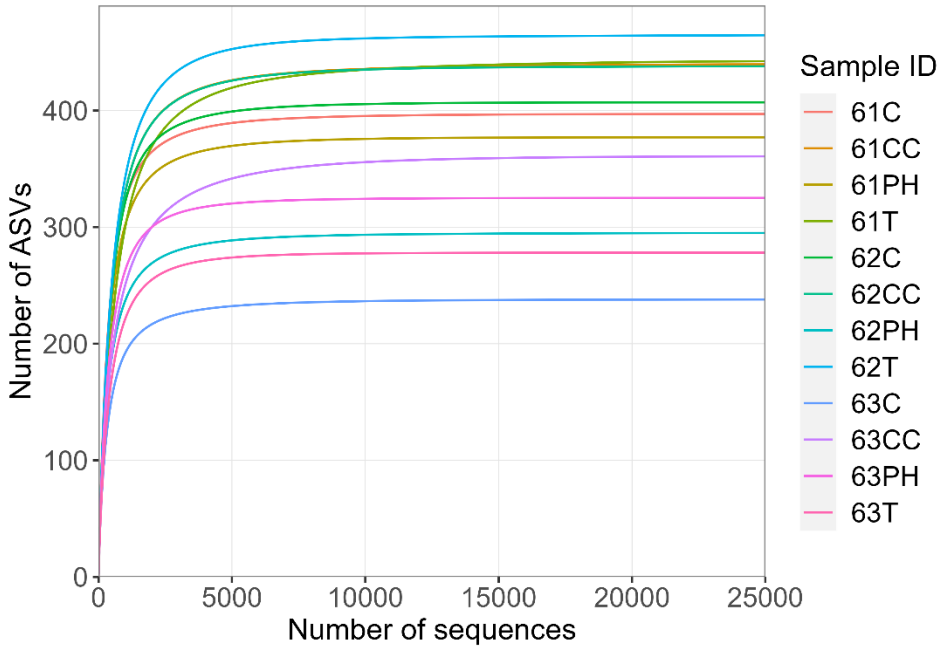
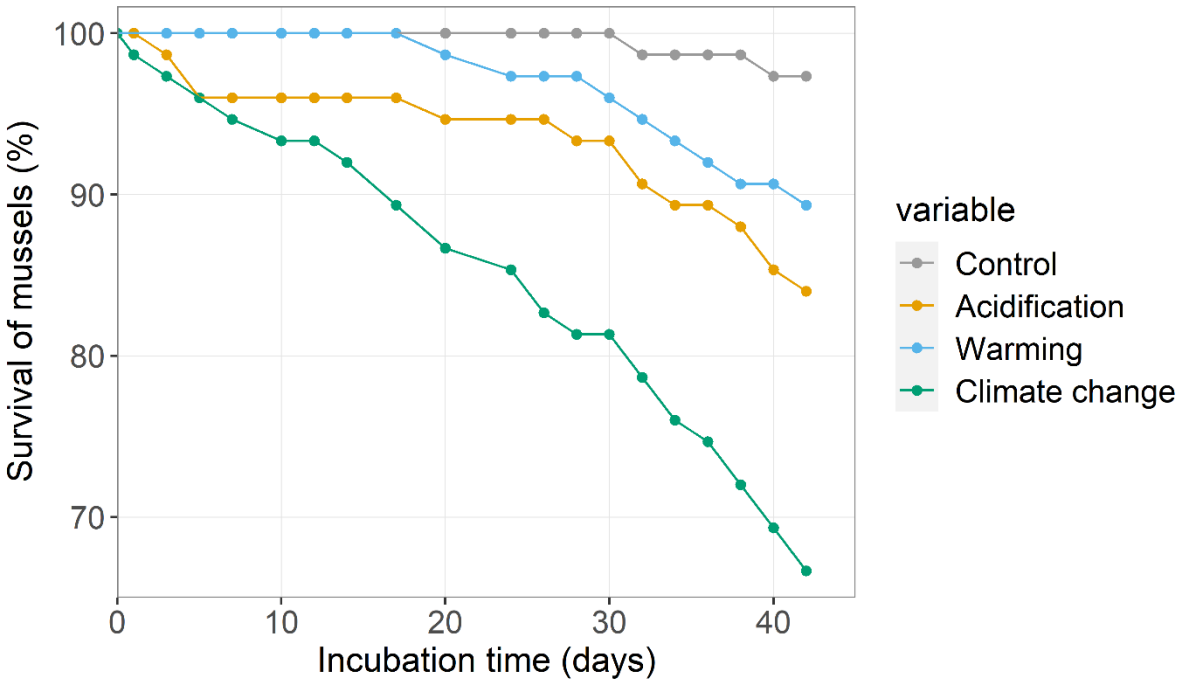
Supplementary data

Supplement S1 Summary of the physico-chemical parameters of the mesocosm waters for the four experimental treatments. Values are given as mean ± standard deviation. pH was measured on the NBS scale.

	Temperature (°C)	pH	Salinity	Total alkalinity (µmol kg^{-1})	pCO_2 (µatm)	Dissolved inorganic carbon C_T µmol kg^{-1}	Bicarbo ion HC (µmol
Control	20.0 ± 0.1	8.0 ± 0.0	33.0 ± 0.5	2106.9 ± 247.5	453.3 ± 45.7	1910.6 ± 225.6	1753.9 ±
Acidification	20.0 ± 0.3	7.6 ± 0.0	32.4 ± 0.4	1984.8 ± 280.1	966.6 ± 127.1	1809.0 ± 270.8	70.0 ± 25
Warming	23.2 ± 0.2	8.0 ± 0.0	33.8 ± 0.5	2113.3 ± 268.5	441.9 ± 39.8	1711.4 ± 237.3	161.7 ± 2
Climate change	23.1 ± 0.3	7.7 ± 0.0	33.0 ± 0.5	2037.7 ± 317.3	990.2 ± 145.9	1834.8 ± 306	81.4 ± 28



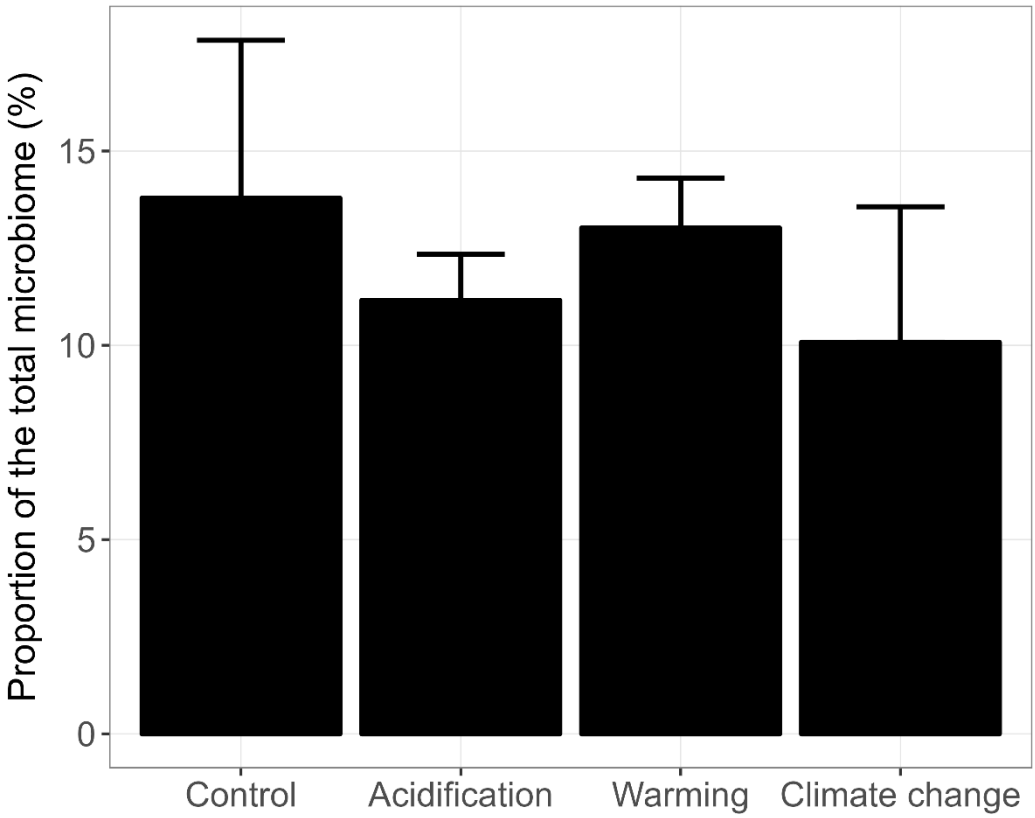
Supplement S2 Rarefaction curves of microbiomes on the shell surface of blue mussels *Mytilus edulis* after six weeks of incubation. All samples are plotted.



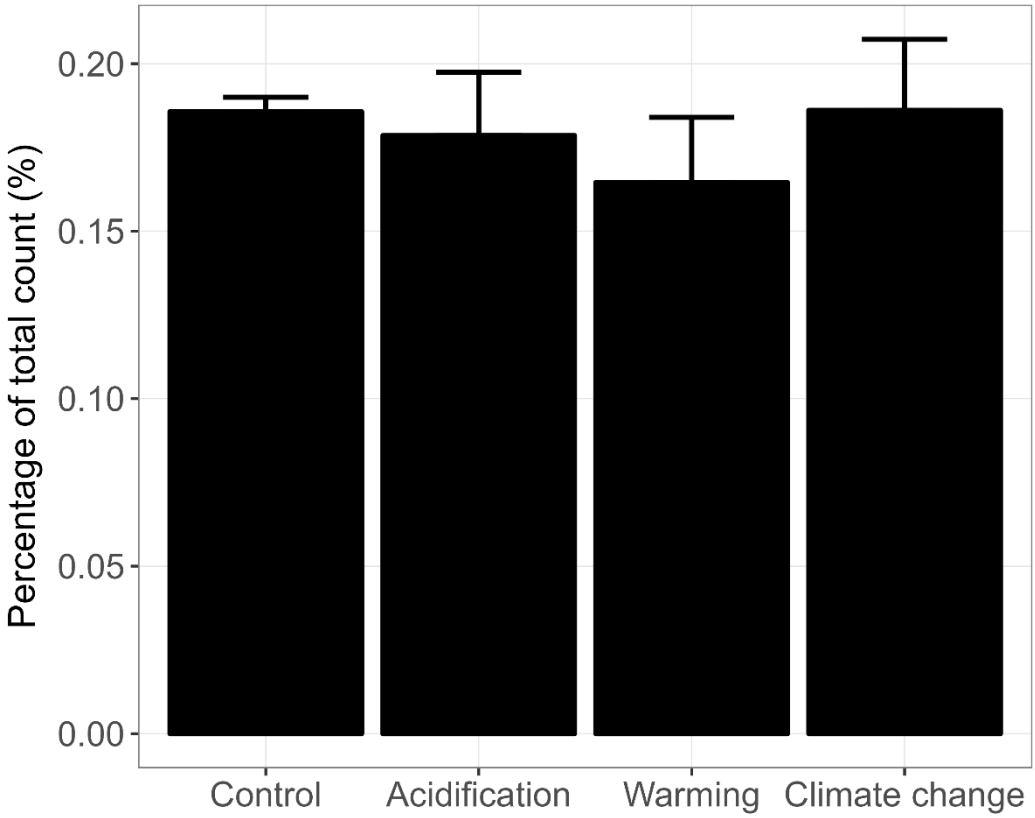
Supplement S3 Survival of blue mussels *Mytilus edulis* over six weeks (42 days) of incubation under four experimental treatments. *N* = 75 individuals per experimental treatment.

Supplement S4 Results of sequence data processing for the different groups of samples according to the experimental treatments.

Experimental treatment	Number of replicates	Number of sequences before the filtering steps (min-max)	Number of sequences after the filtering steps (min-max)	Number of ASV (min-max)
Control	3	36,322-51,163	36,011-50,689	226-386
Acidification	3	44,828-49,524	44,259-48,838	270-351
Warming	3	32,857-67,837	32,170-67,180	261-438
Climate change	3	58,340-64,835	57,830-64,366	332-417



Supplement S5 Percentage of the total bacterial community belonging to the core microbiome for biofilms on the shell of blue mussels *Mytilus edulis* after six weeks of incubation under different experimental treatments. Core microbiomes were identified as a list of amplicon sequence variants (ASV) shared between 90 % of the mussel biofilm samples. *N* = 15 shared ASVs for mussel biofilm samples.



Supplement S6 Percentage of the KEGG ortholog genes involved in the nitrogen metabolism in the microbial biofilms on the shell of blue mussels *Mytilus edulis* after six weeks of incubation under different experimental treatments.