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The effect of ultrasonic antifouling control on the growth and microbiota of farmed European sea bass (*Dicentrarchus labrax*)

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ABSTRACT

Biofouling is a serious threat to marine renewable energy structures and marine aquaculture operations alike. As an alternative to toxic surface coatings, ultrasonic antifouling control has been proposed as an environmentally friendly means to reduce biofouling. However, the impact of ultrasound on fish farmed in offshore structures or in marine multi-purpose platforms, combining renewable energy production and aquaculture, has not yet been assessed. Here we study the impact of ultrasound on the growth and microbiota of farmed European sea bass (*Dicentrarchus labrax*) under laboratory conditions. Whereas growth and survival were not reduced by ultrasound exposure, microbiological analysis using plate counts and 16S rRNA gene based metataxonomics showed a perturbation of the gill and skin microbiota, including an increase in putative pathogenic bacteria. This warrants further research into the long-term effects of ultrasonic antifouling control on the health and wellbeing of farmed fish.

1. Introduction

Human activities developed within maritime areas are constantly increasing. Among these activities, renewable energies and aquaculture are the most emergent and will grow significantly in the future. The marine environment offers multiple sources to produce renewable energy (wind, wave, tides, currents, temperature and pressure gradients) (Abhinav et al., 2020) and, by 2030, the demand for seafood will exceed the current supply by 40 million metric tonnes (FAO, 2018). Consequently, to optimize marine spatial planning, co-location opportunities for renewable energies and aquaculture facilities must be exploited to develop multi-purpose platforms (offshore platforms serving the needs of multiple offshore industries; Abhinav et al., 2020) in future decades. Case studies are essential to explore co-location options and several recent projects were carried out to study the feasibility to combine offshore energy production and aquaculture (Abhinav et al., 2020; Benassai et al., 2014; Dalton et al., 2019; Papandroulakis et al., 2017; Schütz and Slater, 2019; Weiss et al., 2018). These studies have demonstrated that co-location reduces operating costs (Weiss et al., 2018) and that offshore aquaculture is a viable alternative for increasing the global seafood production while offering several advantages such as ample space for expansion, low exposure to pollution and optimal environmental conditions for a wide variety of marine species (Holm et al., 2017).

Nevertheless, offshore activities are associated with high technical risks (e.g., mechanical forces, corrosion, extreme conditions, unreliable moorings and biofouling) and the measures put in place to manage these risks must not harm any of the activities developed around the multi-purpose platforms. Biofouling, which refers to the attachment of undesirable molecules and fouling organisms to the submerged surface, is a major problem facing the marine industry. In the marine renewable energy industry, it initiates or accelerates the corrosion process of metal and concrete structures, compromises device performance and requires additional maintenance activities (Blackwood et al., 2017; Loxton et al., 2017; Want et al., 2017). In the aquaculture industry, biofouling hinders nutrient exchange and deforms cages due to extra weight which can lower productivity (Fitridge et al., 2012). Therefore, the use of anti-biofouling agents is of high economic importance. In general, to limit

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biofouling and help to decrease device maintenance requirements and costs associated with the removal of biofouling, the submerged structures are protected with antifouling solutions, such as paints and coatings (Finnie and Williams, 2010; Gu et al., 2019). However, these paints and coatings contain toxic substances, such as trace metals and booster co-biocides (zinc and copper pyrithione, chlorothalonil, ziram, zineb, dichlofluanid, diuron) (Turner, 2010), which can be released into the marine environment and impact non-target marine organisms (Chen and Lam, 2017; Soon et al., 2019; Soroldoni et al., 2020). Thus, it is necessary to develop new environmental-friendly antifouling systems.

To date, several environmental-friendly antifouling approaches have been considered to combat marine biofouling, including coatings with dynamic surfaces (Xie et al., 2019), foul release coatings (Tian et al., 2020), bioinspired coatings (Li and Guo, 2019), electrolyzing seawater (Wake et al., 2006) and acoustic methods (Legg et al., 2015). Among acoustic treatments studied, ultrasound has been reported to be effective at inhibiting the formation of biofouling on surfaces suspended in freshwater and seawater (Legg et al., 2015; Park and Lee, 2018). The most promising results have been obtained for frequencies between 20 and 50 kHz. At these frequencies, ultrasound is, for example, capable of causing mortality of numerous crustacean and bivalve larvae (Haque and Kwon, 2018; Legg et al., 2015), removing fouling organisms (Mazue et al., 2011), inhibiting settlement (Guo et al., 2012), inactivating zooplankton (Holm et al., 2008) and reducing biofilm thickness (Bott, 2000). Consequently, ultrasonic treatment could be an interesting alternative to the use of toxic antifouling molecules in order to protect offshore platforms combining energy production and aquaculture. Nevertheless, ultrasound can be considered as an anthropogenic noise, which could have a potential negative impact on marine life and farmed fish. Currently, only one study has been carried out to evaluate the impact of a long-term ultrasound exposure on a commonly farmed fish species (common carp exposed to a dual-frequency of 23 and 46 kHz from an antibiofilm ultrasound device over a 30-day period; Techer et al., 2017). The results of this study did not provide an indication that ultrasound negatively affects the welfare and physiological homeostasis of carp. However, it is essential to expand our knowledge on the impact that ultrasound could have on different farmed or wild fish species. In addition, recent advances in microbiology show that animal health and welfare are closely linked to its host-associated microbiota (Douglas, 2019; Merrifield and Rodiles, 2015). Since ultrasound is known to affect microorganisms, for instance, by impacting their growth, inducing metabolite production or inactivating cells through membrane disruption (Piyasena et al., 2003), it is important to investigate whether ultrasound can impact the microbiome of farmed fish and thereby their growth performance, health or welfare. Indeed, a recent study has demonstrated a correlation between the growth performance of European eel and its associated microbiota (Shi et al., 2020).

The present study was conducted in order to examine the impact of ultrasound on the growth and microbiota of European sea bass (*Dicentrarchus labrax*), a commercially important aquaculture species. This work is essential to assess the feasibility and safety of using ultrasound as an environmental-friendly antifouling agent within offshore platforms combining energy production and fish farming.

2. Material and methods

2.1. Study species and animal maintenance

European sea bass, *Dicentrarchus labrax* L. (Moronidae, Perciformes), used in this experiment were sourced from a seabass aquaculture broodstock at the Ifremer aquaculture station (Palavas-les-Flots, France). A batch of 569 individuals was transferred to the experimental station at the CNAM Intechmer (Tourlaville, France) at 240 days-post-hatching (dph). After a 56-day acclimation period, all individuals were tagged with passive integrated transponder (PIT tag) at 296 dph to track individual fish weight. This operation was performed under benzocaine anaesthesia (200 ppm). A sub-sample of 360 individuals was randomly split into six 600 L tanks ($N = 60$ individuals per tank) supplied with filtered seawater in a recirculating system (flow rate: 0.4 m^3 h^{-1} and 100% water renewal per day). Mean water temperature, oxygen concentration and salinity were measured throughout the experiment with average values being 20.4 ± 1.7 °C, 7.21 ± 2.8 mg L^{-1}, and 35 ± 0 g L^{-1}, for each parameter respectively. The light was switched on for 14 h and off for 10 h of each 24-h cycle (light switched on at 07:00 U.T. + 1), with twilight transition periods of 30 min. The individuals were hand-fed twice a day (8:30 a.m. and 4:00 p.m.), with commercial food (Le Gouessant Aquaculture). The size of the pellets and the rations were adjusted after each weight measurement according to the manufacturer's recommendations.

2.2. Ultrasound equipment and experiment set-up

Ultrasonic frequencies were generated using ultrasound transducers (20–80 kHz, Model 2 Harsonic for Propellers, Harsonic) connected to a frequency generator (Virtual Bench, VB8012, National instruments). The LabVIEW (NXG 2017, National instrument) software was programmed to generate randomly alternated frequency sequence loops between 20 and 80 kHz. At 462 dph, an ultrasound transducer was placed at the centre-bottom of each tank to avoid any biases due to the presence of the device in the tank. However, only the transducers in three tanks were activated (Ultrasound exposure group, US) while the three others were kept inactive (Control group). The ultrasound devices remained active for 71 days and were turned off at 533 dph (see Fig. 1). The frequencies generated by the transducers in the three tanks were measured at 512, 514 and 517 dph using a wideband hydrophone (Hydrophone HTI-99-HF, Scorpions Oceanics). The 999 first frequencies generated by the transducers were recorded (See Supplemental Fig. S1) giving average frequencies of 26.2 ± 49.7 kHz, 26.7 ± 45.0 kHz and 26.0 ± 17.5 kHz in each of the tanks with active devices, respectively.

2.3. Growth performance

Individual fish weight was assessed four times during the experiment (Fig. 1). Before each measurement, the fish were fasted for 24 h then anesthetized with Benzocaine (Sigma Aldrich, 200 ppm). Individual growth performance was assessed by calculating the specific growth rate (SGR, % increase in body weight per day) for the periods before (386–462 dph, 76 days), during (462–533 dph, 71 days) and after (533–589 dph, 56 days) ultrasound exposure. Individual fish weight and SGR are presented as averages with standard deviations.

2.4. Cultivation-based microbiological analyses

2.4.1. Sample preparation

Samples for cultivation-based microbiological analysis were collected before ultrasound exposure (T0), after 55 days of ultrasound exposure (T1) and 55 days after cessation of ultrasound exposure (T2) (see Fig. 1). At each occasion, two sea bass and 100 mL seawater were sampled from each experimental tank. Sea bass were caught one at a time, euthanized using a lethal dose of 300 ppm benzocaine placed individually in sterile stomacher bags (400 mL, 180 × 300 mm, Stomacher, Seward) and immediately transported to the laboratory in a cooler with ice packs. Cooler and ice packs were previously cleaned with 70% ethanol.

For the gill samples, whole gills were weighed and placed in a sterile stomacher bag (80 mL, 100 × 150 mm, Stomacher, Seward) with 20 mL sterile seawater. They were then mashed for 3 min using a stomacher (Stomacher, Lab-Blender 400, Seward), in order to obtain the initial suspension. Each initial suspension was then serially diluted up to 10^2-fold in sterile seawater. At times T0 and T2, the gills of the two sea bass from the same tank were mixed to form a single sample, while at time T1, the gills of the two sea bass from the same tank were analysed

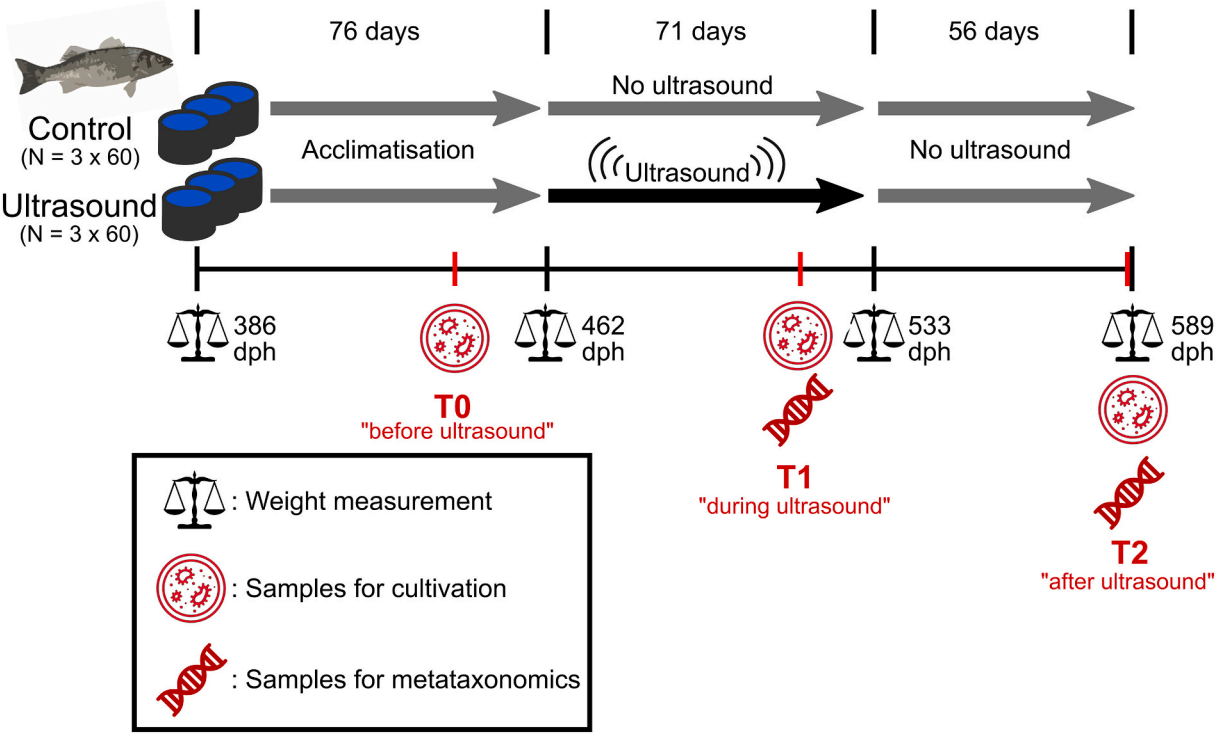

Fig. 1. Overview of experimental timeline, weight measurements and sample collection.

separately. Therefore, in total, six microbiological analyses were performed at T0, twelve microbiological analyses were performed at T1 (six analyses for control condition and six analyses for ultrasound condition) and six microbiological analyses were performed at T2 (three analyses for control condition and three analyses for ultrasound condition).

For the skin mucus samples, 1 g of skin mucus was collected at the lateral line using a sterile Teflon spatula and suspended in a tube containing 9 mL of sterile seawater. This suspension was homogenized using a vortex mixer (initial suspension). Each initial suspension was then serially diluted up to 10^1-fold in sterile seawater. Sample pooling was performed as for the gill samples resulting in the same number of samples at T0 ($n = 6$), T1 ($n = 12$) and T2 ($n = 6$), respectively.

For the tank seawater, water samples from each tank were collected using a 250 mL sterile vial and serially diluted up to 10^2-fold in sterile seawater.

2.4.2. Enumeration of cultivable bacteria by plate count method

100 µL of all initial suspensions and dilutions were plated in triplicates onto marine agar plates (Difco) for heterotrophic plate counts (HPC) (Lyons et al., 2007); onto Pseudomonas agar base supplemented with cephalothin, fucidin, cetrimide supplement (Merck) and modified according to Stanbridge and Board (1994) (CFC agar modified) to enumerate *Pseudomonas* spp.; and onto thiosulfate citrate bile salts sucrose agar plates (TCBS agar; Merck) to enumerate *Vibrio* spp. (Bolinches et al., 1988). All plates were incubated for 48 h to 72 h at 22 °C. After incubation, plates showing 30–300 colonies on marine agar and 15–150 colonies on CFC agar and TCBS agar were used to calculate the Colony Forming Units (CFU) per g of gill, per g of skin mucus and per mL of seawater. Counts of CFU are presented as logarithmic values and calculated as log10(x + 1) to account for counts of 0.

2.5. Metataxonomic analysis

2.5.1. Sample preparation

Samples for metataxonomic analysis were collected at times T1 and T2 (see Fig. 1) to analyse differences between the ultrasound exposure and control group during and after treatment. Prior to sample collection, the fish were euthanised as described above. Skin mucus of two fish per tank ($N = 12$) was collected from the whole body of the fish using a sterile Teflon spatula. For gut samples, eight fish per tank ($N = 48$) were first immerged successively in three baths of sterile distilled water to remove loosely attached microorganisms on the skin. Then the mid- and hindgut were collected using sterile dissection tools. Seawater samples (1 L) were collected from each tank ($N = 6$) and filtered through a 0.2 µm pore size polycarbonate membrane (Whatman). All samples were immediately stored at −80 °C in sterile vials until DNA extraction for metataxonomic analysis.

2.5.2. DNA extraction, PCR and sequencing

DNA from all samples was extracted using the MasterPure Complete DNA Purification Kit (Epicentre) according to the manufacturer's instructions for total DNA extraction. The gut samples were pre-processed as described in Benhaïm et al. (2020) to reduce PCR inhibition. The water sample residues were resuspended by adding 300 µl of Tissue and Cell Lysis Solution to each filter and scraping the surface gently with a sterile scalpel before continuing with the manufacturer's protocol. DNA from each sample was subject to PCR using the universal prokaryotic primer pair S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21(5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013) covering the V3-V4 region of the 16S rRNA gene and yielding an amplicon of approximately 465 base pairs. The PCR reactions were performed with Phusion High-Fidelity DNA polymerase (NEB) according to the manufacturer's recommendations using 10 ng of template DNA in a 25 µl reaction volume. The thermocycler conditions were set to an initial denaturation step at 98 °C for 30 s followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 52 °C for 30 s and extension at 72 °C for 30 s. Final extension was set to 72 °C for 5 min. The libraries were then multiplexed with Nextera XT v2 indices (Illumina), normalised using Sequel-Prep Normalisation Plates (ThermoFisher Scientific) and sequenced on a MiSeq system (Illumina) using v3 chemistry and 2 × 300 cycles.

2.5.3. Bioinformatic analysis

Bioinformatic analysis was performed in R version 4.0.2 (R Core Team, 2020) implemented in RStudio version 1.2.5019 (RStudio Team, 2016). Raw sequence reads were demultiplexed and amplicon sequence variants (ASVs) inferred with the R package DADA2 version 1.16 (Callahan et al., 2016). Briefly, raw reads were filtered with the *filterAndTrim* command set to truncLen = c(260,240), maxEE = 3, trimLeft = 21, truncQ = 2. Error rates were learned with the *learnError* command performed on a subset of 10^8 bases. Taxonomic assignment of ASVs was performed using the *assignTaxonomy* command against a training set of the SILVA database version 138 (Quast et al., 2013). Taxa classified as *Eukarya*, chloroplasts or mitochondria were removed from the dataset. In addition, ASVs that were 20 base pairs shorter or larger the expected amplicon size were removed, as well as one ASV classified as *Escherichia* sp. which was detected in all samples at a similar relative abundance and hence likely a contaminant. Microbial community analysis was performed with packages phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2013). For further taxonomic analysis, ASVs were compared against the NCBI 16S rRNA sequence database using the blast web application (Johnson et al., 2008). Plots were generated with package ggplot2 (Wickham, 2009).

2.6. Statistics analysis

Differences in the SGR was analysed using generalized linear mixed-effects models (GLMM). The explanatory variables included in the full model were "Treatment" (control vs ultrasound) in interaction with "Period" (before, during and after ultrasound treatment). In addition to these fixed effects, the random effects were "Tank nested within treatment" and "individual fish ID". The full model was reduced by backward selection based on the Akaike Information Criterion (AIC) (Zuur et al., 2009). Diagnostics based on residuals of the model were performed to assess the adequacy of the reduced model and compliance with the underlying assumptions. Finally, the effects of the independent variables were estimated from the reduced models and their significance was tested by likelihood ratio tests (LRT) between models respecting marginality of the effects that are supposed to follow a $\chi 2$ distribution under the null hypothesis (type II tests; (Fox and Weisberg, 2011)). This analysis was followed by a post-hoc multiple comparison test (Hothorn et al., 2008) to assess pairwise differences.

Plate counts (HPC, *Pseudomonas* spp. and *Vibrio* spp.) were subject to Student's Two Sample *t*-test at time T1 (control: $n = 6$; US: $n = 6$) and Welch's Two Sample *t*-test at time T2 (control: $n = 3$; US: $n = 3$). Results were considered significant when the *p*-value was below 0.05.

For metataxonomic analysis, differential abundance of taxa between groups was compared with DESeq2 (Love et al., 2014) implemented in the R package animalcules version 1.4.0 (Zhao et al., 2020a) at genus level taxonomic assignment with default parameters and a minimum count cut-off of 10. Results were considered significant when the adjusted *p*-value was below 0.05. Significance of alpha-diversity indices (number of observed ASVs and Shannon diversity index) between groups was determined using Student's Two Sample *t*-test. Beta-diversity was plotted using Non-metric Multi-dimensional Scaling (NMDS) of Bray-Curtis dissimilarities and weighted UniFrac distances and significance of results between groups was determined using PERMANOVA implemented in the vegan package with 999 permutations.

3. Results

3.1. Growth performance and survival

The average individual body weight of the fish increased from 13.47 ± 3.73 g in the control group and 13.36 ± 3.77 g in the ultrasound exposure group at the beginning of the experiment (386 dph) to 97.77 ± 23.57 g and 102.03 ± 25.33 g, in each of the group respectively, at the end of the experiment (589 dph). The average SGR in the control group was 1.13 ± 0.15, 0.77 ± 0.1 and 0.61 ± 0.11% bodyweight d^{-1} before, during and after ultrasound exposure period, respectively (Fig. 2). In the ultrasound exposed group, the average SGR was 1.17 ± 0.16, 0.78 ± 0.1 and 0.61 ± 0.1% bodyweight d^{-1} before, during and after ultrasound exposure, respectively. No significant difference was detected between the SGR of the control and ultrasound exposed group over the entire duration of the experiment ($\chi 2 = 2.5$, df = 1, $p = 0.11$) using GLMM. However, the post hoc test showed that the SGR was significantly higher in the ultrasound exposed group before activation of the ultrasound transducer ($Z = 2.8$, $p = 0.025$). No significant differences between the groups were observed by the post hoc test during ($Z = 0.33$, $p = 0.99$) and after ($Z = 0.06$, $p = 1$) activation of the ultrasound transducer. No mortality was recorded in either group over the course of the experiment.

3.2. Cultivation-based microbiological analyses

Bacterial counts of gills, skin mucus and tank seawater at times T0, T1 and T2 are presented in Fig. 3 and Table 1. Ultrasound exposure had an impact on the cultivable bacterial flora on the gills at time T1, with the counts of HPC, *Vibrio* spp. and *Pseudomonas* spp. being significantly higher for ultrasound exposed fish than for the control fish. The mean increase was log 0.45 CFU g^{-1}, log 0.62 CFU g^{-1} and log 0.55 CFU g^{-1} for HPC, *Pseudomonas* spp. and *Vibrio* spp., respectively. At time T2, the mean counts remained higher for fish in the ultrasound exposure group than for the control fish, although the differences between the two groups were not significant anymore. For fish in the control group, the mean counts of *Pseudomonas* spp. and *Vibrio* spp. on the gills remained constant throughout the experiment (about log 3.7 CFU g^{-1} and log 3.8 CFU g^{-1}, respectively), while the mean concentration of HPC was lower at time T2 (log 4.8 CFU g^{-1}) than at times T0 and T1 (log 5.3 CFU g^{-1}).

The counts of cultivable bacteria on the skin mucus was not significantly impacted by ultrasound exposure (Fig. 3B, Table 1). HPC increased from T0 to T1 but remained relatively stable between T1 and T2 as well as between the ultrasound exposed and control group. At T1, *Vibrio* spp. and *Pseudomonas* spp. were detected in 3 and 5 out of 12 skin mucus samples, respectively, and at T2 both taxa were detected in 4 out of 6 samples each. During ultrasound exposure (time T1) no counts of *Vibrio* spp. were detected in the control group compared to a mean count of log 2.8 CFU g^{-1} in the ultrasound exposed group.

Counts of cultivable bacteria in the tank seawater were not significantly impacted by ultrasound exposure (Fig. 3C, Table 1). The concentration of HPC and *Vibrio* spp. remained constant and similar between the two groups (US and Control), throughout the study (about log 4.8 CFU mL^{-1} and log 2.3 CFU mL^{-1} for HPC and *Vibrio* spp., respectively). The mean counts of *Pseudomonas* spp. remained the same at all three times for the control group, while it increased by log 0.8 CFU mL^{-1} between T1 and T2 for the ultrasound exposed group.

3.3. Metataxonomic analysis

The sea bass gut microbial community was dominated by the classes *Gammaproteobacteria* and *Alphaproteobacteria*, representing 77.1% and 8.9% of the mean relative abundance respectively across the control gut samples from T1 and T2 (Fig. 4). One ASV, assigned to the genus *Pelomonas* of the *Gammaproteobacteria* class, was the dominant taxon with a mean relative abundance of 57.8% and the only ASV present in all gut samples (Fig. 5). Alignment of its partial 16S rRNA gene sequence showed closest sequence similarity to *Pelomonas aquatica* (99.7% sequence identity to GenBank accession NR_042614.1). Apart from *Pelomonas*, the variability of genera between samples was high with only six additional genera (*Acinetobacter*, *Brevundimonas*, *Curvibacter*, *Acidibacter* and *Sphingomonas*) detected in over half of the control gut samples, together accounting for 14.1% of the mean relative abundance (Fig. 5). At time T1, none of the genera had a significantly differential abundance in the gut microbial community between the ultrasound

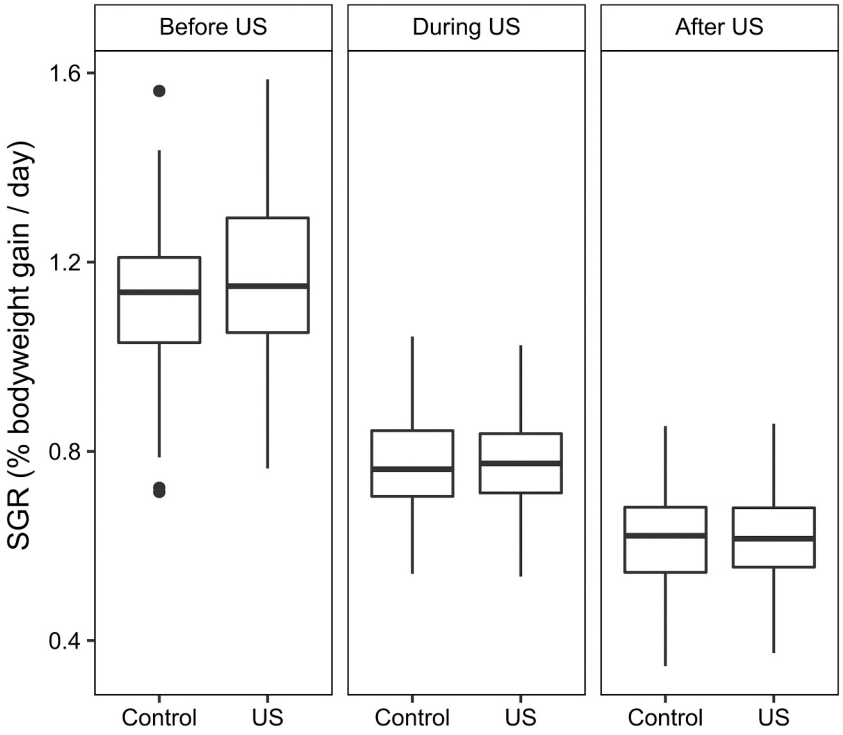

Fig. 2. Specific growth rate (SGR) of sea bass in the control group (Control, $N = 180$) and ultrasound exposure group (US, $N = 180$) for the periods before (386–462 dph), during (462–533 dph) and after (533–589 dph) ultrasound (US) exposure.

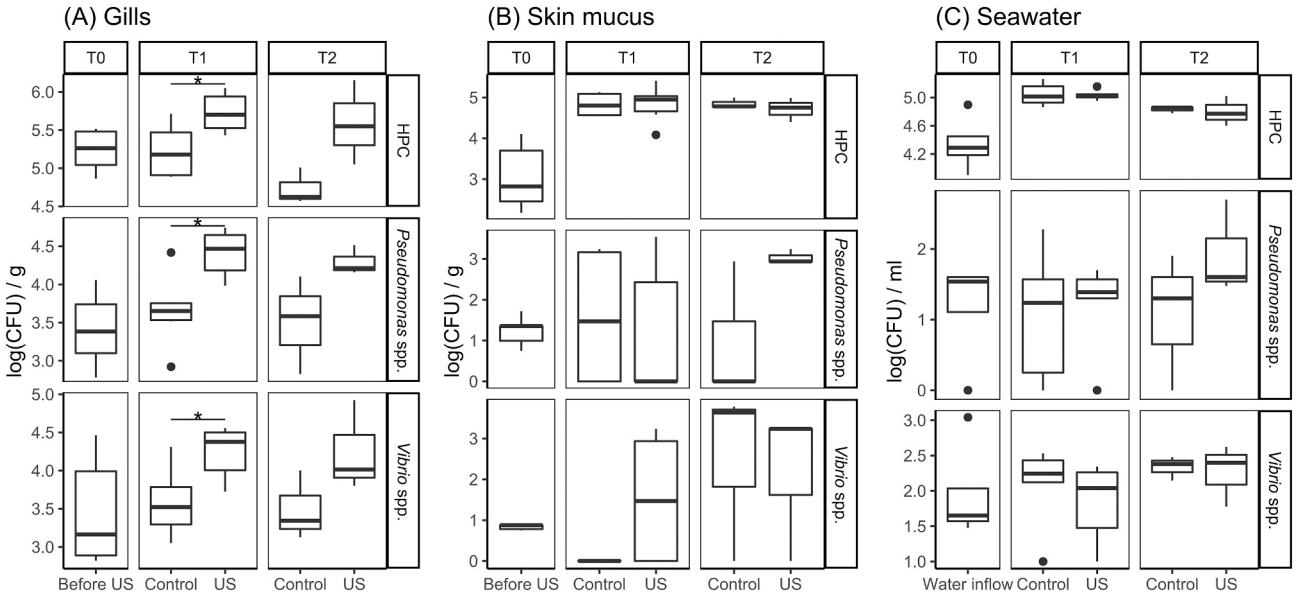

Fig. 3. Heterotrophic plate counts (HPC) and counts of *Pseudomonas* spp. and *Vibrio* spp. at times T0, T1 and T2 in sea bass gills (A), skin mucus (B) and seawater (C) of the control group (Control) and ultrasound exposed group (US), presented as log CFU per g for gills and skin mucus samples and per ml for seawater samples.

exposed and the control group. However, at time T2, the genus *Brevinema* was significantly decreased and *Photobacterium* significantly increased in the ultrasound exposed group compared to the control (adjusted p-value <0.001). No significant differences were detected between the number of observed ASVs or the Shannon diversity index in the control and ultrasound exposed group at T1 and T2, nor between Bray-Curtis dissimilarities and weighted UniFrac distances. (Fig. 4 and Supplemental Fig. S2).

The skin microbial community of the sea bass differed significantly from the gut environment ($R^2 = 0.38$, p-value < 0.001, PERMANOVA on Bray-Curtis distances, Fig. 4) and shared more taxa with the seawater in the rearing tanks, with the exceptions of the genera *Pelomonas* and *Acinetobacter* which were abundant both the gut and skin, but largely absent from the seawater (Fig. 5). *Gammaproteobacteria*, *Bacteroidia*, *Verrucomicrobiae* and *Alphaproteobacteria* were the most abundant classes of the skin microbiome with a mean relative abundance of 43.3%, 26.2%, 14.4% and 9.6% respectively (Fig. 4). At time T1, the genus *Pelomonas* was significantly increased (adjusted p-value < 0.05) in the skin microbiome of the ultrasound exposed fish compared to the control group. Whereas a separation of their respective clusters in the NMDS

Table 1
Statistical analysis of CFU counts between the control group (Control) and the ultrasound exposure group (US). Counts are presented as log CFU per g for gills and skin mucus samples and per ml for seawater samples. Values in bold indicate significant differences ($p < 0.05$) between groups for each timepoint.

	T0 (N = 6)	T1 (N = 12)				T2 (N = 6)			
	Mean log CFU	Mean log CFU (control)	Mean log CFU (US)	*t*-Value[a]	*p*-Value[a]	Mean log CFU (control)	Mean log CFU (US)	*t*-Value[b]	*p*-Value[b]
Gills									
HPC	5.31	**5.35**	**5.79**	−2.46	0.03*	4.78	5.80	−1.41	0.29
Pseudomonas spp.	3.62	**3.88**	**4.49**	−2.75	0.02*	3.76	4.33	−2.26	0.10
Vibrio spp.	3.92	**3.79**	**4.34**	−2.57	0.03*	3.66	4.52	−1.14	0.37
Skin mucus									
HPC	3.60	4.90	5.00	−0.50	0.63	4.86	4.77	−0.52	0.63
Pseudomonas spp.	1.34	2.86	2.94	−0.21	0.84	2.46	3.06	−2.12	0.10
Vibrio spp.	0.85	<DL	2.76	−2.00	0.10	3.54	3.06	1.22	0.33
Seawater									
HPC	4.50	5.07	50.4	0.41	0.70	4.84	4.83	0.03	0.98
Pseudomonas spp.	1.45	1.65	1.43	0.61	0.57	1.53	2.28	−1.00	0.42
Vibrio spp.	2.48	2.27	2.05	1.23	0.25	2.36	2.39	−0.15	0.90

T0: before US exposure.
T1: during US exposure.
T2: after US exposure.
DL: detection level.
[a] Student's two sample *t*-test.
[b] Welch's two sample *t*-test.
* $p < 0.05$.

plot based on Bray-Curtis distances was apparent (Fig. 4), possibly due to the differential abundance of *Pelomonas* between groups, there was no significant difference between communities based on PERMANOVA. At time T2, no significant difference in the relative abundance of *Pelomonas* was detected between the ultrasound exposed and control group anymore. The average number of observed ASVs in the control group was significantly higher than in the ultrasound exposed group at T1 ($p < 0.01$) (Supplemental Fig. S2). Conversely, at T2, the average number of observed ASVs in the ultrasound exposed group was higher than in the control, however, these results were not statistically significant. The seawater microbial community consisted mainly of *Bacteroidia*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobiae* which together represent 93.2% of the relative abundance in the control samples at time T1 and T2 (Fig. 4). No genera were significantly differentially abundant between the ultrasound exposed and control group and no significant differences were detected in the number of ASVs or the Shannon diversity index between both groups (Supplemental Fig. S2).

The genera *Pseudomonas* and *Vibrio*, which were quantified by cultivation, were only minor taxa in the analysed microbial communities. *Pseudomonas* represented 0.5% and 0.4% of the relative abundance in the gut and skin mucus samples, respectively, and was absent in the seawater. *Vibrio* represented 0.5% and 0.1% of the relative abundance in the skin mucus and seawater samples, respectively, and was absent in the gut (data not shown). No significant differences in the relative abundance of these genera were detected between the ultrasound exposed and control group at time T1.

4. Discussion

Overall, sea bass growth performances were in the range of those observed in previous studies at a similar age and under comparable conditions (Benhaïm et al., 2011). A faster SGR in the ultrasound exposure group led to a higher average weight at the end of the experiment compared to the control group. However, this could not be attributed to ultrasound exposure, as the increase in SGR occurred before activation of the ultrasound transducers. A previous study on the toxicity of ultrasound towards farmed carp (*Cyprinus carpio*), showed that fish welfare and physiological homeostasis was unaffected by ultrasound exposure over a 30-day period (Techer et al., 2017). The present study assessed the impact of ultrasound on the growth performance and survival of a marine fish species over a 71-day period. Here, we confirm that ultrasound exposure does not have a negative impact on fish growth and survival. An additional 56-day period with the transducers kept inactive confirmed no long-term effect of the ultrasound treatment on the growth or survival of sea bass.

Whereas growth and survival were unaffected, our results show that ultrasound exposure can impact the fish microbiota. Concerning the cultivable microbiota, CFU analysis showed that the gills harboured an increased number of heterotrophic bacteria as well as potentially pathogenic bacteria, such as *Pseudomonas* spp. and *Vibrio* spp. after ultrasound exposure. Higher levels of these bacteria, even after inactivating the transducers, indicate that ultrasound could have a long-term impact on the gill microbiota. Several *Pseudomonas* and *Vibrio* species are known to cause serious diseases in farmed fish and lead to significant economic losses (Novriadi, 2016; Toranzo et al., 2005). Among *Pseudomonas*, the species *P. anguilliseptica* is considered the most significant pathogen for farmed fish (Toranzo et al., 2005). Within the genus *Vibrio*, many species have been described as fish pathogens, including *V. anguillarum*, *V. ordalii*, *V. salmonicida*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi* (Novriadi, 2016), *V. toranzoniae* (Lasa et al., 2015) and *V. tapetis* (Vercauteren et al., 2018), and vibriosis is a well-known cause of mortality in aquaculture (Novriadi, 2016). Fish gills perform vital functions (e.g., respiration, osmoregulation, pH balance, ammonia excretion, hormone regulation, detoxification) and their mucus harbours a complex community of commensal microorganisms that play a major part in maintaining homeostasis and protection against pathogens (Reverter et al., 2017). Although pathogenic bacteria can form a part of the mucus microbiome in healthy farmed fish (Rosado et al., 2019), they can also cause disease if there is a shift in their abundance (Hess et al., 2015; Rosado et al., 2019). Consequently, an increase in the abundance of *Pseudomonas* spp. and *Vibrio* spp. within the gills could potentially increase the risk of pathologies. However, it is well established that cultivable bacteria represent only a negligible part (<2%) of complex bacterial communities associated with various environmental and animal habitats

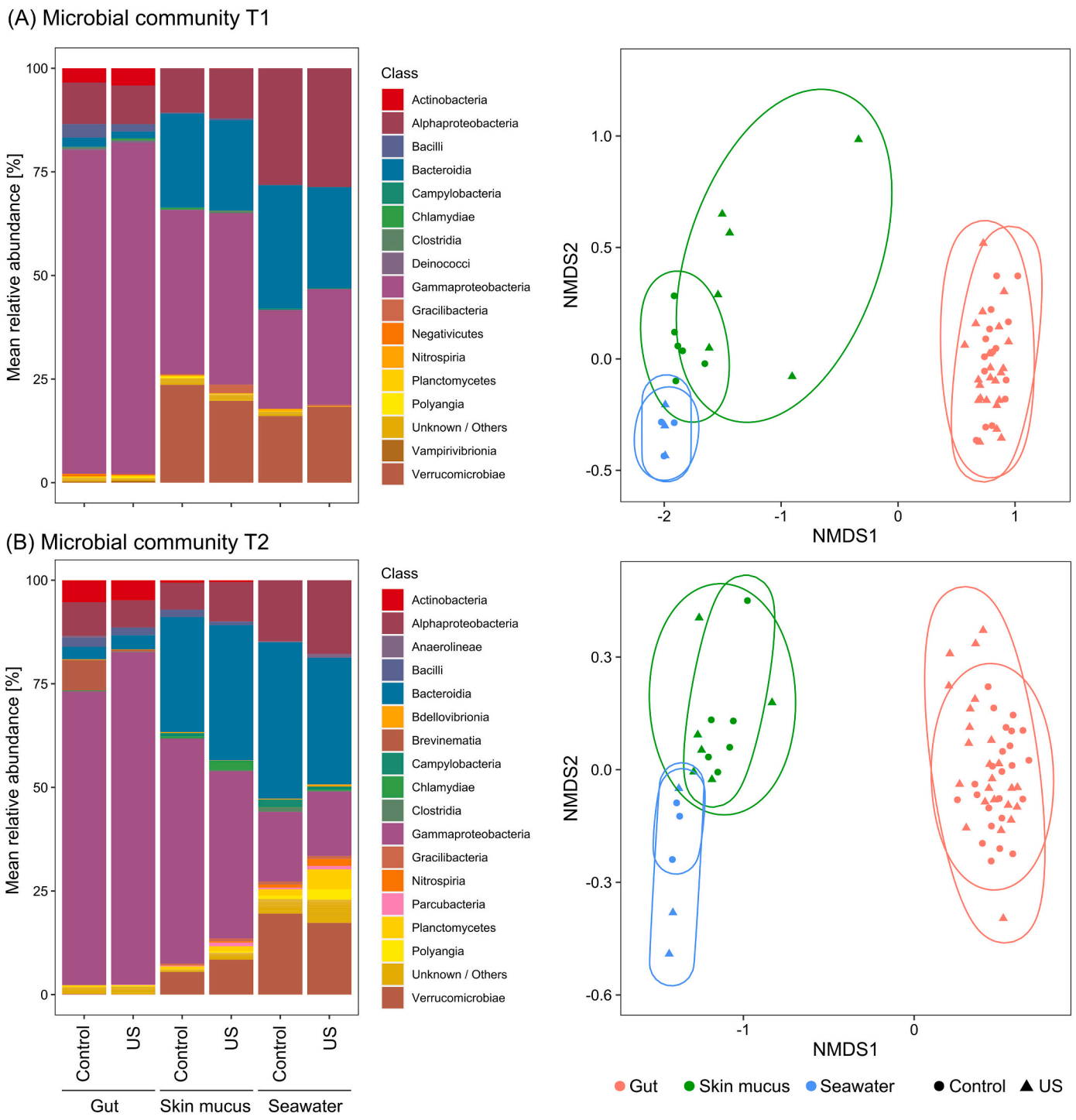

Fig. 4. Microbial community profiles at class level assignment (left) and non-metric multidimensional scaling (NMDS) plot of Bray-Curtis distances (right) of gut, skin mucus and seawater microbiota at time T1 (A) and T2 (B) from fish in the control group (Control) or ultrasound exposed group (US). NMDS ordination is based on normalised data and stress values <0.1.

(Vartoukian et al., 2010). Their enumeration therefore does not provide a functional implication of the whole gill microbiome (Legrand et al., 2020), but rather warrants further research into putative pathogenicity of the bacteria propagated by ultrasound exposure.

At time T1, ultrasound exposure also seemed to facilitate the colonization of the skin mucus by *Vibrio* spp. as shown by cultivation-based analysis. As *Vibrio* only constituted a minor taxon in the skin microbial community and was not detected in all samples, these results could not be confirmed by metataxonomic analysis. However, a significant increase in the relative abundance of *Pelomonas* in the skin microbial community during ultrasound exposure further highlights the potential disruption of the skin microbiota by ultrasound. *Pelomonas* has previously been detected in the gut of farmed sea bass (Nikouli et al., 2018) and is a dominant member of the gut microbiome of other fish species (Nikouli et al., 2020; Rasheeda et al., 2017; Zhao et al., 2020b). *Pelomonas* has also been identified on the skin of brook charr (Boutin et al., 2013) and the gills of rainbow trout (Lowrey et al., 2015), however at low relative abundances. As the gut and skin environment in the current

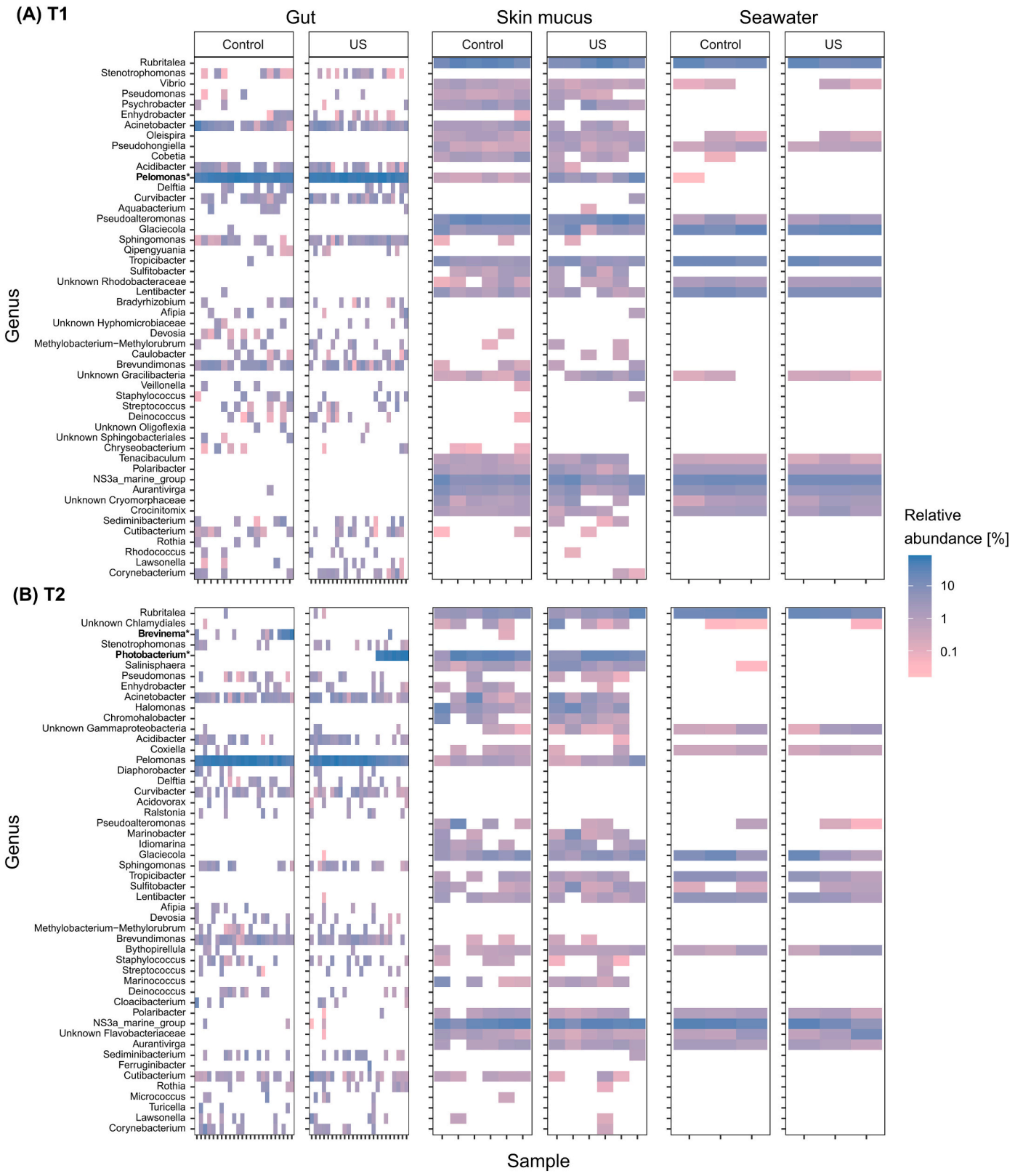

Fig. 5. Heatmap showing the relative abundance of the 50 most abundance genera across gut, skin mucus and seawater samples with logarithmic colour scale. Genera which showed significantly differential abundance between the control group (Control) and ultrasound exposed group (US) in one of the sample types are highlighted in bold with an asterisk.

study shared the same *Pelomonas*-associated ASV, it appears that this gut-associated bacterium is a low-abundant opportunist on the skin. The near absence of *Pelomonas* in the seawater further demonstrates that it is not a transient bacterium in the farming environment. A previous study has shown that stress may induce changes in skin mucus microbiota, such as a decrease in the abundance of probiotic-like bacteria and an increase in opportunistic pathogenic bacteria (Boutin et al., 2013). As such, these results could demonstrate a state of stress in fish subjected to ultrasound.

The sea bass gut microbial profiles detected in this study showed similarities to previous reports on farmed sea bass and sea bream, with a dominance of the phylum *Proteobacteria* (Kokou et al., 2020; Kormas et al., 2014; Nikouli et al., 2018; Rimoldi et al., 2020). High inter-individual variability of the gut microbiome has also been reported, possibly being linked to life history and genetic background (Gatesoupe et al., 2016), or pointing towards insufficient replicate sampling (Panteli et al., 2020). Compared to the gills and skin mucus, the absence of differentially abundant taxa between the ultrasound exposed and control group in the present study indicate that ultrasound exposure does not directly impact the fish gut microbial community. Differences in the relative abundance of *Brevinema* and *Photobacterium* eight weeks after the end of the ultrasound exposure may be attributed to a belated or long-term effect of ultrasound exposure, especially as members of *Photobacterium* are known fish pathogens (Bakopoulos et al., 2003; Terceti et al., 2016). However, this could also be an artefact of the high inter-individual variability in the gut microbiota and to support this observation it would be necessary to define a temporally stable core gut microbiota in farmed sea bass.

In conclusion, our study shows that close-proximity and continuous ultrasound exposure can impact the surface microbial community of farmed sea bass. The skin and gill mucus layers act as a protective barrier against the external environment and are essential in protecting the fish from pathogenic organisms (Merrifield and Rodiles, 2015). In addition, commensal microorganisms can prevent the colonization of the skin by pathogens (Pérez-Sánchez et al., 2011). Hence, even though an adverse effect of ultrasound exposure was not evident on the growth performance and survival of sea bass, alterations to the microbiome could lead to a higher incidence of disease and mortality in the long-term and therefore warrants further investigation. As these results were generated under controlled laboratory conditions and the fish were kept in close proximity to the ultrasound source, further studies should be conducted to determine the impact of ultrasonic antifouling control on the fish microbiota under in situ conditions, while assessing the potential benefits of this technology compared to traditional antifouling control strategies.

CRediT authorship contribution statement

Stephen Knobloch: Formal analysis, Investigation, Visualization, Writing – original draft. **Joris Philip:** Formal analysis, Investigation, Data curation, Writing – original draft. **Sébastien Ferrari:** Investigation, Data curation. **David Benhaïm:** Investigation, Funding acquisition, Formal analysis, Writing – original draft. **Martine Bertrand:** Funding acquisition, Writing – review & editing. **Isabelle Poirier:** Investigation, Supervision, Funding acquisition, Writing – original draft.

Declaration of competing 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112072.

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