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

3

4

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20 **Highlights**

- 21 • Ultrasound exposure does not reduce growth rate or survival of farmed sea bass.
- 22 • Ultrasound exposure increases *Vibrio* and *Pseudomonas* in gill tissue.
- 23 • Ultrasound exposure increases gut-associated *Pelomonas* in skin mucus.
- 24 • Sea bass gut microbiota remains unaffected by ultrasound exposure.

25

26 **Abstract**

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

39

40 **Keywords:** antifouling; ultrasound; aquaculture; microbiome; sea bass; *Vibrio*

41 1. Introduction

42 Human activities developed within maritime areas are constantly increasing. Among these
43 activities, renewable energies and aquaculture are the most emergent and will grow
44 significantly in the future. The marine environment offers multiple sources to produce
45 renewable energy (wind, wave, tides, currents, temperature and pressure gradients) (Abhinav
46 et al., 2020) and, by 2030, the demand for seafood will exceed the current supply by 40
47 million metric tonnes (FAO, 2018). Consequently, to optimize marine spatial planning, co-
48 location opportunities for renewable energies and aquaculture facilities must be exploited to
49 develop multi-purpose platforms (offshore platforms serving the needs of multiple offshore
50 industries; Abhinav et al., 2020) in future decades. Case studies are essential to explore co-
51 location options and several recent projects were carried out to study the feasibility to
52 combine offshore energy production and aquaculture (Abhinav et al., 2020; Benassai et al.,
53 2014; Dalton et al., 2019; Papandroulakis et al., 2017; Schütz and Slater, 2019; Weiss et al.,
54 2018). These studies have demonstrated that co-location reduces operating costs (Weiss et al.,
55 2018) and that offshore aquaculture is a viable alternative for increasing the global seafood
56 production while offering several advantages such as ample space for expansion, low
57 exposure to pollution and optimal environmental conditions for a wide variety of marine
58 species (Holm et al., 2017).

59 Nevertheless, offshore activities are associated with high technical risks (e.g., mechanical
60 forces, corrosion, extreme conditions, unreliable moorings and biofouling) and the measures
61 put in place to manage these risks must not harm any of the activities developed around the
62 multi-purpose platforms. Biofouling, which refers to the attachment of undesirable molecules
63 and fouling organisms to the submerged surface, is a major problem facing the marine
64 industry. In the marine renewable energy industry, it initiates or accelerates the corrosion
65 process of metal and concrete structures, compromises device performance and requires

66 additional maintenance activities (Blackwood et al., 2017; Loxton et al., 2017; Want et al.,
67 2017). In the aquaculture industry, biofouling hinders nutrient exchange and deforms cages
68 due to extra weight which can lower productivity (Fitridge et al., 2012). Therefore, the use of
69 anti-biofouling agents is of high economic importance. In general, to limit biofouling and help
70 to decrease device maintenance requirements and costs associated with the removal of
71 biofouling, the submerged structures are protected with antifouling solutions, such as paints
72 and coatings (Finnie and Williams, 2010; Gu et al., 2019). However, these paints and coatings
73 contain toxic substances, such as trace metals and booster co-biocides (zinc and copper
74 pyrrithione, chlorothalonil, ziram, zineb, dichlofluanid, diuron) (Turner, 2010), which can be
75 released into the marine environment and impact non-target marine organisms (Chen and
76 Lam, 2017; Soon et al., 2019; Soroldoni et al., 2020). Thus, it is necessary to develop new
77 environmental-friendly antifouling systems.

78 To date, several environmental-friendly antifouling approaches have been considered to
79 combat marine biofouling, including coatings with dynamic surfaces (Xie et al., 2019), foul
80 release coatings (Tian et al., 2020), bioinspired coatings (Li and Guo, 2019), electrolyzing
81 seawater (Wake et al., 2006) and acoustic methods (Legg et al., 2015). Among acoustic
82 treatments studied, ultrasound has been reported to be effective at inhibiting the formation of
83 biofouling on surfaces suspended in freshwater and seawater (Legg et al., 2015; Park and Lee,
84 2018). The most promising results have been obtained for frequencies between 20 and
85 50 kHz. At these frequencies, ultrasound is, for example, capable of causing mortality of
86 numerous crustacean and bivalve larvae (Haque and Kwon, 2018; Legg et al., 2015),
87 removing fouling organisms (Mazue et al., 2011), inhibiting settlement (Guo et al., 2012),
88 inactivating zooplankton (Holm et al., 2008) and reducing biofilm thickness (Bott, 2000).
89 Consequently, ultrasonic treatment could be an interesting alternative to the use of toxic
90 antifouling molecules in order to protect offshore platforms combining energy production and

91 aquaculture. Nevertheless, ultrasound can be considered as an anthropogenic noise, which
92 could have a potential negative impact on marine life and farmed fish. Currently, only one
93 study has been carried out to evaluate the impact of a long-term ultrasound exposure on a
94 commonly farmed fish species (common carp exposed to a dual-frequency of 23 and 46 kHz
95 from an antibiofilm ultrasound device over a 30-day period; Techer et al., 2017). The results
96 of this study did not provide an indication that ultrasound negatively affects the welfare and
97 physiological homeostasis of carp. However, it is essential to expand our knowledge on the
98 impact that ultrasound could have on different farmed or wild fish species. In addition, recent
99 advances in microbiology show that animal health and welfare are closely linked to its host-
100 associated microbiota (Douglas, 2019; Merrifield and Rodiles, 2015). Since ultrasound is
101 known to affect microorganisms, for instance, by impacting their growth, inducing metabolite
102 production or inactivating cells through membrane disruption (Piyasena et al., 2003), it is
103 important to investigate whether ultrasound can impact the microbiome of farmed fish and
104 thereby their growth performance, health or welfare. Indeed, a recent study has demonstrated
105 a correlation between the growth performance of European eel and its associated microbiota
106 (Shi et al., 2020).

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

112

113 2. Material and methods

114 2.1 Study species and animal maintenance

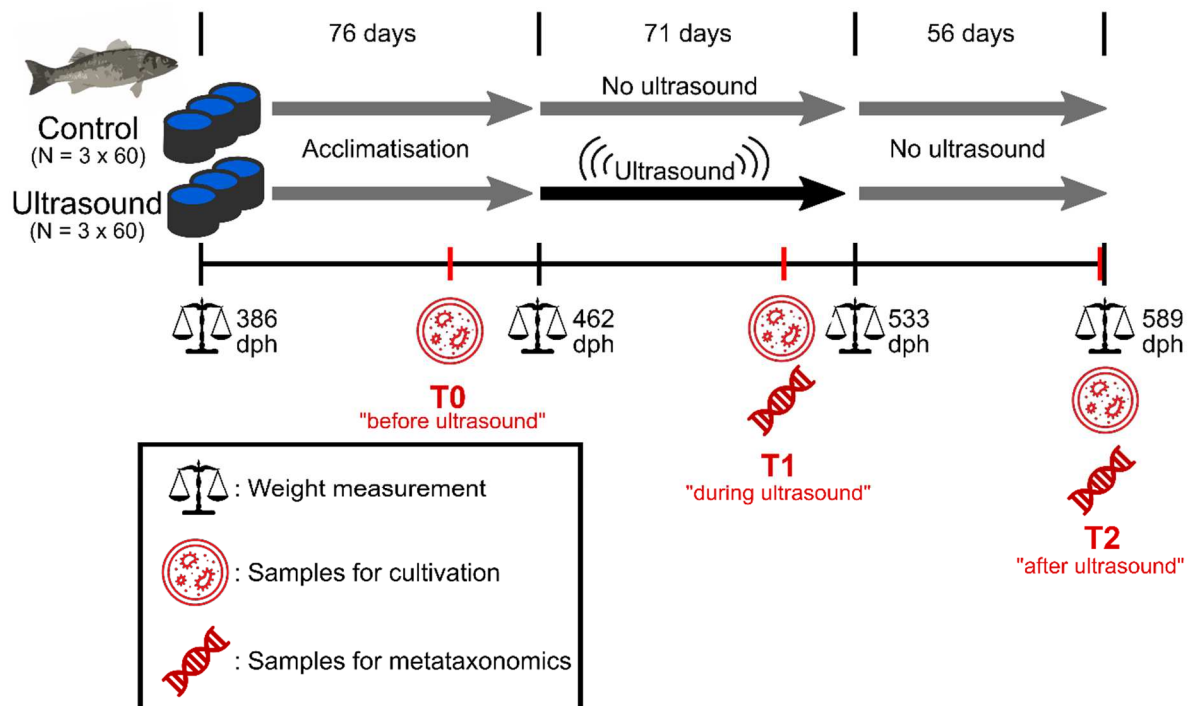
115 European sea bass, *Dicentrarchus labrax* L. (Moronidae, Perciformes), used in this
116 experiment were sourced from a seabass aquaculture broodstock at the Ifremer aquaculture
117 station (Palavas-les-Flots, France). A batch of 569 individuals was transferred to the
118 experimental station at the CNAM Intechmer (Tourlaville, France) at 240 days-post-hatching
119 (dph). After a 56-day acclimation period, all individuals were tagged with passive integrated
120 transponder (PIT tag) at 296 dph to track individual fish weight. This operation was
121 performed under benzocaine anaesthesia (200 ppm). A sub-sample of 360 individuals was
122 randomly split into six 600 litre tanks (N = 60 individuals per tank) supplied with filtered
123 seawater in a recirculating system (flow rate: 0.4 m³h⁻¹ and 100% water renewal per day).
124 Mean water temperature, oxygen concentration and salinity were measured throughout the
125 experiment with average values being 20.4 ± 1.7 °C, 7.21 ± 2.8 mg L⁻¹, and 35 ± 0 g L⁻¹, for
126 each parameter respectively. The light was switched on for 14 h and off for 10 h of each 24-h
127 cycle (light switched on at 07:00 U.T. + 1), with twilight transition periods of 30 min. The
128 individuals were hand-fed twice a day (8:30 a.m. and 4:00 p.m.), with commercial food (Le
129 Gouessant Aquaculture). The size of the pellets and the rations were adjusted after each
130 weight measurement according to the manufacturer's recommendations.

131

132 2.2 Ultrasound equipment and experiment set-up

133 Ultrasonic frequencies were generated using ultrasound transducers (20 - 80 kHz, Model 2
134 Harsonic for Propellers, Harsonic) connected to a frequency generator (Virtual Bench,
135 VB8012, National instruments). The LabVIEW (NXG 2017, National instrument) software
136 was programmed to generate randomly alternated frequency sequence loops between 20 and
137 80 kHz. At 462 dph, an ultrasound transducer was placed at the centre-bottom of each tank to

138 avoid any biases due to the presence of the device in the tank. However, only the transducers
 139 in three tanks were activated (Ultrasound exposure group, US) while the three others were
 140 kept inactive (Control group). The ultrasound devices remained active for 71 days and were
 141 turned off at 533 dph (see Figure 1). The frequencies generated by the transducers in the three
 142 tanks were measured at 512, 514 and 517 dph using a wideband hydrophone (Hydrophone
 143 HTI-99-HF, Scorpions Oceanics). The 999 first frequencies generated by the transducers were
 144 recorded (See Supplemental Figure S1) giving average frequencies of 26.2 ± 49.7 kHz, $26.7 \pm$
 145 45.0 kHz and 26.0 ± 17.5 kHz in each of the tanks with active devices, respectively.
 146



147
 148 *Figure 1: Overview of experimental timeline, weight measurements and sample collection.*
 149

150 2.4 Growth performance

151 Individual fish weight was assessed four times during the experiment (Figure 1). Before each
 152 measurement, the fish were fasted for 24 hours then anesthetized with Benzocaine (Sigma
 153 Aldrich, 200 ppm). Individual growth performance was assessed by calculating the specific

154 growth rate (SGR, % increase in body weight per day) for the periods before (386 – 462 dph,
155 76 days), during (462 – 533 dph, 71 days) and after (533 – 589 dph, 56 days) ultrasound
156 exposure. Individual fish weight and SGR are presented as averages with standard deviations.

157

158 2.5 Cultivation-based microbiological analyses

159 2.5.1 Sample preparation

160 Samples for cultivation-based microbiological analysis were collected before ultrasound
161 exposure (T0), after 55 days of ultrasound exposure (T1) and 55 days after cessation of
162 ultrasound exposure (T2) (see Figure 1). At each occasion, two sea bass and 100 mL seawater
163 were sampled from each experimental tank. Sea bass were caught one at a time, euthanized
164 using a lethal dose of 300 ppm benzocaine placed individually in sterile stomacher bags (400
165 mL, 180×300 mm, Stomacher, Seward) and immediately transported to the laboratory in a
166 cooler with ice packs. Cooler and ice packs were previously cleaned with 70% ethanol.
167 For the gill samples, whole gills were weighed and placed in a sterile stomacher bag (80 mL,
168 100×150 mm, Stomacher, Seward) with 20 mL sterile seawater. They were then mashed for 3
169 min using a stomacher (Stomacher, Lab-Blender 400, Seward), in order to obtain the initial
170 suspension. Each initial suspension was then serially diluted up to 10²-fold in sterile seawater.
171 At times T0 and T2, the gills of the two sea bass from the same tank were mixed to form a
172 single sample, while at time T1, the gills of the two sea bass from the same tank were
173 analysed separately. Therefore, in total, six microbiological analyses were performed at T0,
174 twelve microbiological analyses were performed at T1 (six analyses for control condition and
175 six analyses for ultrasound condition) and six microbiological analyses were performed at T2
176 (three analyses for control condition and three analyses for ultrasound condition).
177 For the skin mucus samples, 1 g of skin mucus was collected at the lateral line using a sterile
178 Teflon spatula and suspended in a tube containing 9 mL of sterile seawater. This suspension

179 was homogenized using a vortex mixer (initial suspension). Each initial suspension was then
180 serially diluted up to 10¹-fold in sterile seawater. Sample pooling was performed as for the
181 gill samples resulting in the same number of samples at T0 (n = 6), T1 (n = 12) and T2 (n =
182 6), respectively.

183 For the tank seawater, water samples from each tank were collected using a 250 mL sterile
184 vial and serially diluted up to 10²-fold in sterile seawater.

185

186 2.5.2 Enumeration of cultivable bacteria by plate count method

187 100 µL of all initial suspensions and dilutions were plated in triplicates onto marine agar
188 plates (Difco) for heterotrophic plate counts (HPC) (Lyons et al., 2007); onto *Pseudomonas*
189 agar base supplemented with cephalothin, fucidin, ceftrimide supplement (Merck) and
190 modified according to Stanbridge and Board (1994) (CFC agar modified) to enumerate
191 *Pseudomonas* spp.; and onto thiosulfate citrate bile salts sucrose agar plates (TCBS agar;
192 Merck) to enumerate *Vibrio* spp. (Bolinches et al., 1988). All plates were incubated for 48 h
193 to 72 h at 22 °C. After incubation, plates showing 30-300 colonies on marine agar and 15-150
194 colonies on CFC agar and TCBS agar were used to calculate the Colony Forming Units
195 (CFU) per g of gill, per g of skin mucus and per mL of seawater. Counts of CFU are presented
196 as logarithmic values and calculated as log₁₀(x + 1) to account for counts of 0.

197

198 2.6 Metataxonomic analysis

199 2.6.1 Sample preparation

200 Samples for metataxonomic analysis were collected at times T1 and T2 (see Figure 1) to
201 analyse differences between the ultrasound exposure and control group during and after
202 treatment. Prior to sample collection, the fish were euthanised as described above. Skin mucus
203 of two fish per tank (N = 12) was collected from the whole body of the fish using a sterile

204 Teflon spatula. For gut samples, eight fish per tank (N = 48) were first immersed successively
205 in three baths of sterile distilled water to remove loosely attached microorganisms on the skin.
206 Then the mid- and hindgut were collected using sterile dissection tools. Seawater samples (1
207 L) were collected from each tank (N = 6) and filtered through a 0.2 µm pore size
208 polycarbonate membrane (Whatman). All samples were immediately stored at -80°C in sterile
209 vials until DNA extraction for metataxonomic analysis.

210

211 2.7.2 DNA extraction, PCR and sequencing

212 DNA from all samples was extracted using the MasterPure Complete DNA Purification Kit
213 (Epicentre) according to the manufacturer's instructions for total DNA extraction. The gut
214 samples were pre-processed as described in Benhaïm et al. (2020) to reduce PCR inhibition.
215 The water sample residues were resuspended by adding 300µl of Tissue and Cell Lysis
216 Solution to each filter and scraping the surface gently with a sterile scalpel before continuing
217 with the manufacturer's protocol. DNA from each sample was subject to PCR using the
218 universal prokaryotic primer pair S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3')
219 and S-D-Bact-0785-a-A-21(5'- GACTACHVGGGTATCTAATCC-3') (Klindworth et al.,
220 2013) covering the V3-V4 region of the 16S rRNA gene and yielding an amplicon of
221 approximately 465 base pairs. The PCR reactions were performed with Phusion High-Fidelity
222 DNA polymerase (NEB) according to the manufacturer's recommendations using 10 ng of
223 template DNA in a 25 µl reaction volume. The thermocycler conditions were set to an initial
224 denaturation step at 98 °C for 30 s followed by 30 cycles of denaturation at 98 °C for 10 s,
225 annealing at 52 °C for 30 s and extension at 72 °C for 30 s. Final extension was set to 72 °C
226 for 5 min. The libraries were then multiplexed with Nextera XT v2 indices (Illumina),
227 normalised using Sequel-Prep Normalisation Plates (ThermoFisher Scientific) and sequenced
228 on a MiSeq system (Illumina) using v3 chemistry and 2 x 300 cycles.

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2.7.3 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).

2.8 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

254 Information Criterion (AIC) (Zuur et al., 2009). Diagnostics based on residuals of the model
255 were performed to assess the adequacy of the reduced model and compliance with the
256 underlying assumptions. Finally, the effects of the independent variables were estimated from
257 the reduced models and their significance was tested by likelihood ratio tests (LRT) between
258 models respecting marginality of the effects that are supposed to follow a χ^2 distribution
259 under the null hypothesis (type II tests; (Fox and Weisberg, 2011)). This analysis was
260 followed by a post-hoc multiple comparison test (Hothorn et al., 2008) to assess pairwise
261 differences.

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

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

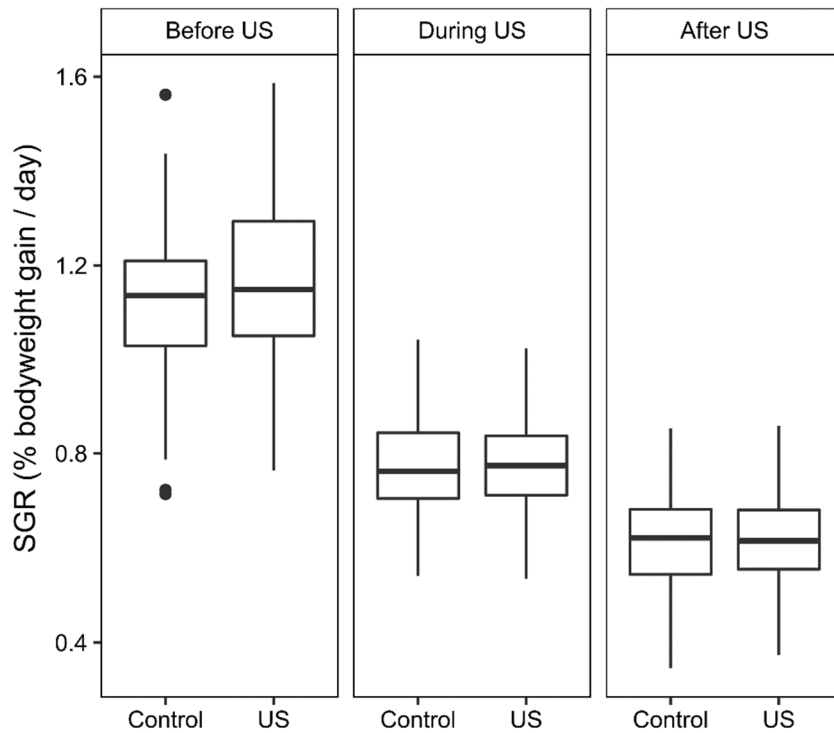
275

276 3. Results

277 3.1 Growth performance and survival

278 The average individual body weight of the fish increased from 13.47 ± 3.73 g in the control
279 group and 13.36 ± 3.77 g in the ultrasound exposure group at the beginning of the experiment
280 (386 dph) to 97.77 ± 23.57 g and 102.03 ± 25.33 g, in each of the group respectively, at the
281 end of the experiment (589 dph). The average SGR in the control group was 1.13 ± 0.15 , 0.77
282 ± 0.1 and 0.61 ± 0.11 % bodyweight d^{-1} before, during and after ultrasound exposure period,
283 respectively (Figure 2). In the ultrasound exposed group, the average SGR was 1.17 ± 0.16 ,
284 0.78 ± 0.1 and 0.61 ± 0.1 % bodyweight d^{-1} before, during and after ultrasound exposure,
285 respectively. No significant difference was detected between the SGR of the control and
286 ultrasound exposed group over the entire duration of the experiment ($\chi^2 = 2.5$, $df = 1$, $p =$
287 0.11) using GLMM. However, the post hoc test showed that the SGR was significantly higher
288 in the ultrasound exposed group before activation of the ultrasound transducer ($Z = 2.8$, $p =$
289 0.025). No significant differences between the groups were observed by the post hoc test
290 during ($Z = 0.33$, $p = 0.99$) and after ($Z = 0.06$, $p = 1$) activation of the ultrasound transducer.
291 No mortality was recorded in either group over the course of the experiment.

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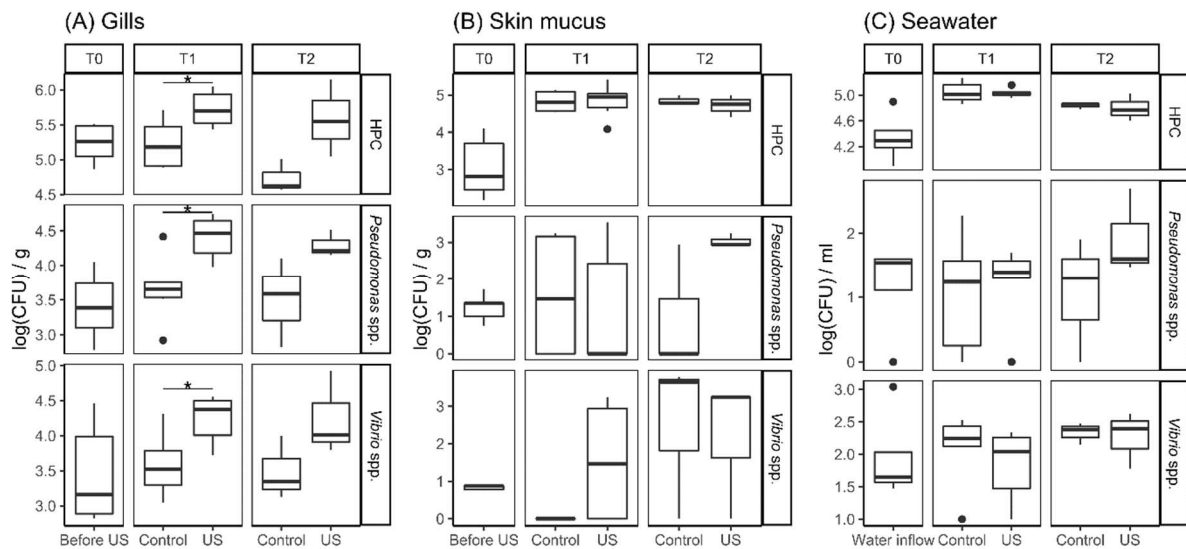
294 *Figure 2: Specific growth rate (SGR) of sea bass in the control group (Control, N = 180) and*
 295 *ultrasound exposure group (US, N = 180) for the periods before (386 – 462 dph), during (462*
 296 *– 533 dph) and after (533 – 589 dph) ultrasound (US) exposure.*

297

298 3.3 Cultivation-based microbiological analyses

299 Bacterial counts of gills, skin mucus and tank seawater at times T0, T1 and T2 are presented
 300 in Figure 3 and Table 1. Ultrasound exposure had an impact on the cultivable bacterial flora
 301 on the gills at time T1, with the counts of HPC, *Vibrio* spp. and *Pseudomonas* spp. being
 302 significantly higher for ultrasound exposed fish than for the control fish. The mean increase
 303 was $\log 0.45 \text{ CFU g}^{-1}$, $\log 0.62 \text{ CFU g}^{-1}$ and $\log 0.55 \text{ CFU g}^{-1}$ for HPC, *Pseudomonas* spp.
 304 and *Vibrio* spp., respectively. At time T2, the mean counts remained higher for fish in the
 305 ultrasound exposure group than for the control fish, although the differences between the two
 306 groups were not significant anymore. For fish in the control group, the mean counts of
 307 *Pseudomonas* spp. and *Vibrio* spp. on the gills remained constant throughout the experiment

308 (about $\log 3.7 \text{ CFU g}^{-1}$ and $\log 3.8 \text{ CFU g}^{-1}$, respectively), while the mean concentration of
 309 HPC was lower at time T2 ($\log 4.8 \text{ CFU g}^{-1}$) than at times T0 and T1 ($\log 5.3 \text{ CFU g}^{-1}$).
 310
 311 The counts of cultivable bacteria on the skin mucus was not significantly impacted by
 312 ultrasound exposure (Figure 3B, Table 1). HPC increased from T0 to T1 but remained
 313 relatively stable between T1 and T2 as well as between the ultrasound exposed and control
 314 group. At T1, *Vibrio* spp. and *Pseudomonas* spp. were detected in 3 and 5 out of 12 skin
 315 mucus samples, respectively, and at T2 both taxa were detected in 4 out of 6 samples each.
 316 During ultrasound exposure (time T1) no counts of *Vibrio* spp. were detected in the control
 317 group compared to a mean count of $\log 2.8 \text{ CFU g}^{-1}$ in the ultrasound exposed group.
 318



319
 320 *Figure 3: Heterotrophic plate counts (HPC) and counts of Pseudomonas spp. and Vibrio spp.*
 321 *at times T0, T1 and T2 in sea bass gills (A), skin mucus (B) and seawater (C) of the control*
 322 *group (Control) and ultrasound exposed group (US), presented as log CFU per g for gills and*
 323 *skin mucus samples and per ml for seawater samples.*

324

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

331

332 *Table 1: Statistical analysis of CFU counts between the control group (Control) and the*
 333 *ultrasound exposure group (US). Counts are presented as log CFU per g for gills and skin*
 334 *mucus samples and per ml for seawater samples. Values in bold indicate significant*
 335 *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 ¹	p-value ¹	Mean log CFU (Control)	Mean log CFU (US)	t-value ²	p-value ²	
Gills										
HPC	5.31	5.35	5.79	-2.46	0.03*	4.78	5.80	-1.41	0.29	
<i>Pseudomonas</i> spp.	3.62	3.88	4.49	-2.75	0.02*	3.76	4.33	-2.26	0.10	
<i>Vibrio</i> 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	
<i>Pseudomonas</i> spp.	1.34	2.86	2.94	-0.21	0.84	2.46	3.06	-2.12	0.10	
<i>Vibrio</i> spp.	0.85	< DL	2.76	-2.00	0.10	3.54	3.06	1.22	0.33	
Seawater										
HPC	4.50	5.07	5.04	0.41	0.70	4.84	4.83	0.03	0.98	
<i>Pseudomonas</i> spp.	1.45	1.65	1.43	0.61	0.57	1.53	2.28	-1.00	0.42	
<i>Vibrio</i> spp.	2.48	2.27	2.05	1.23	0.25	2.36	2.39	-0.15	0.90	

¹ Student's Two Sample t-test

² Welch's Two Sample t-test

* p < 0.05

T0: Before US exposure

T1: During US exposure

T2: After US exposure

DL: Detection level

336

337

338 3.4 Metataxonomic analysis

339 The sea bass gut microbial community was dominated by the classes *Gammaproteobacteria*
340 and *Alphaproteobacteria*, representing 77.1% and 8.9% of the mean relative abundance
341 respectively across the control gut samples from T1 and T2 (Figure 4). One ASV, assigned to
342 the genus *Pelomonas* of the *Gammaproteobacteria* class, was the dominant taxon with a mean
343 relative abundance of 57.8% and the only ASV present in all gut samples (Figure 5).
344 Alignment of its partial 16S rRNA gene sequence showed closest sequence similarity to
345 *Pelomonas aquatica* (99.7% sequence identity to GenBank accession NR_042614.1). Apart
346 from *Pelomonas*, the variability of genera between samples was high with only six additional
347 genera (*Acinetobacter*, *Brevundimonas*, *Curvibacter*, *Acidibacter* and *Sphingomonas*)
348 detected in over half of the control gut samples, together accounting for 14.1% of the mean
349 relative abundance (Figure 5). At time T1, none of the genera had a significantly differential
350 abundance in the gut microbial community between the ultrasound exposed and the control
351 group. However, at time T2, the genus *Brevinema* was significantly decreased and
352 *Photobacterium* significantly increased in the ultrasound exposed group compared to the
353 control (adjusted p-value < 0.001). No significant differences were detected between the
354 number of observed ASVs or the Shannon diversity index in the control and ultrasound
355 exposed group at T1 and T2, nor between Bray-Curtis dissimilarities and weighted UniFrac
356 distances. (Figure 4 and Supplemental Figure S2).

357

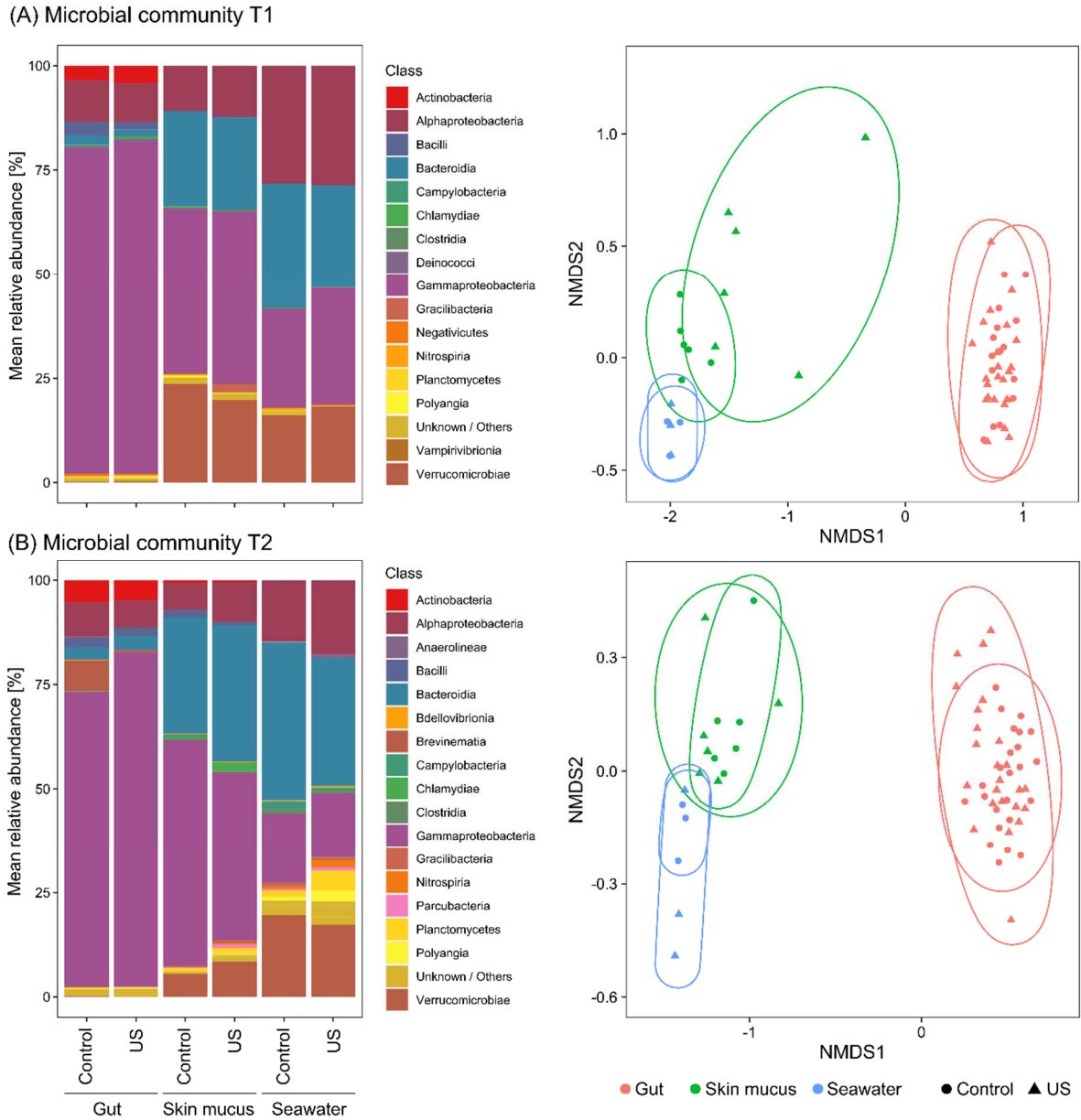
358 The skin microbial community of the sea bass differed significantly from the gut environment
359 ($R^2 = 0.38$, p-value < 0.001, PERMANOVA on Bray-Curtis distances, Figure 4) and shared
360 more taxa with the seawater in the rearing tanks, with the exceptions of the genera *Pelomonas*
361 and *Acinetobacter* which were abundant both the gut and skin, but largely absent from the
362 seawater (Figure 5). *Gammaproteobacteria*, *Bacteroidia*, *Verrucomicrobiae* and

363 *Alphaproteobacteria* were the most abundant classes of the skin microbiome with a mean
364 relative abundance of 43.3%, 26.2%, 14.4% and 9.6% respectively (Figure 4). At time T1, the
365 genus *Pelomonas* was significantly increased (adjusted p-value < 0.05) in the skin
366 microbiome of the ultrasound exposed fish compared to the control group. Whereas a
367 separation of their respective clusters in the NMDS plot based on Bray-Curtis distances was
368 apparent (Figure 4), possibly due to the differential abundance of *Pelomonas* between groups,
369 there was no significant difference between communities based on PERMANOVA. At time
370 T2, no significant difference in the relative abundance of *Pelomonas* was detected between
371 the ultrasound exposed and control group anymore. The average number of observed ASVs in
372 the control group was significantly higher than in the ultrasound exposed group at T1 (p <
373 0.01) (Supplemental Figure S2). Conversely, at T2, the average number of observed ASVs in
374 the ultrasound exposed group was higher than in the control, however, these results were not
375 statistically significant. The seawater microbial community consisted mainly of *Bacteroidia*,
376 *Alphaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobiae* which together represent
377 93.2% of the relative abundance in the control samples at time T1 and T2 (Figure 4). No
378 genera were significantly differentially abundant between the ultrasound exposed and control
379 group and no significant differences were detected in the number of ASVs or the Shannon
380 diversity index between both groups (Supplemental Figure S2).

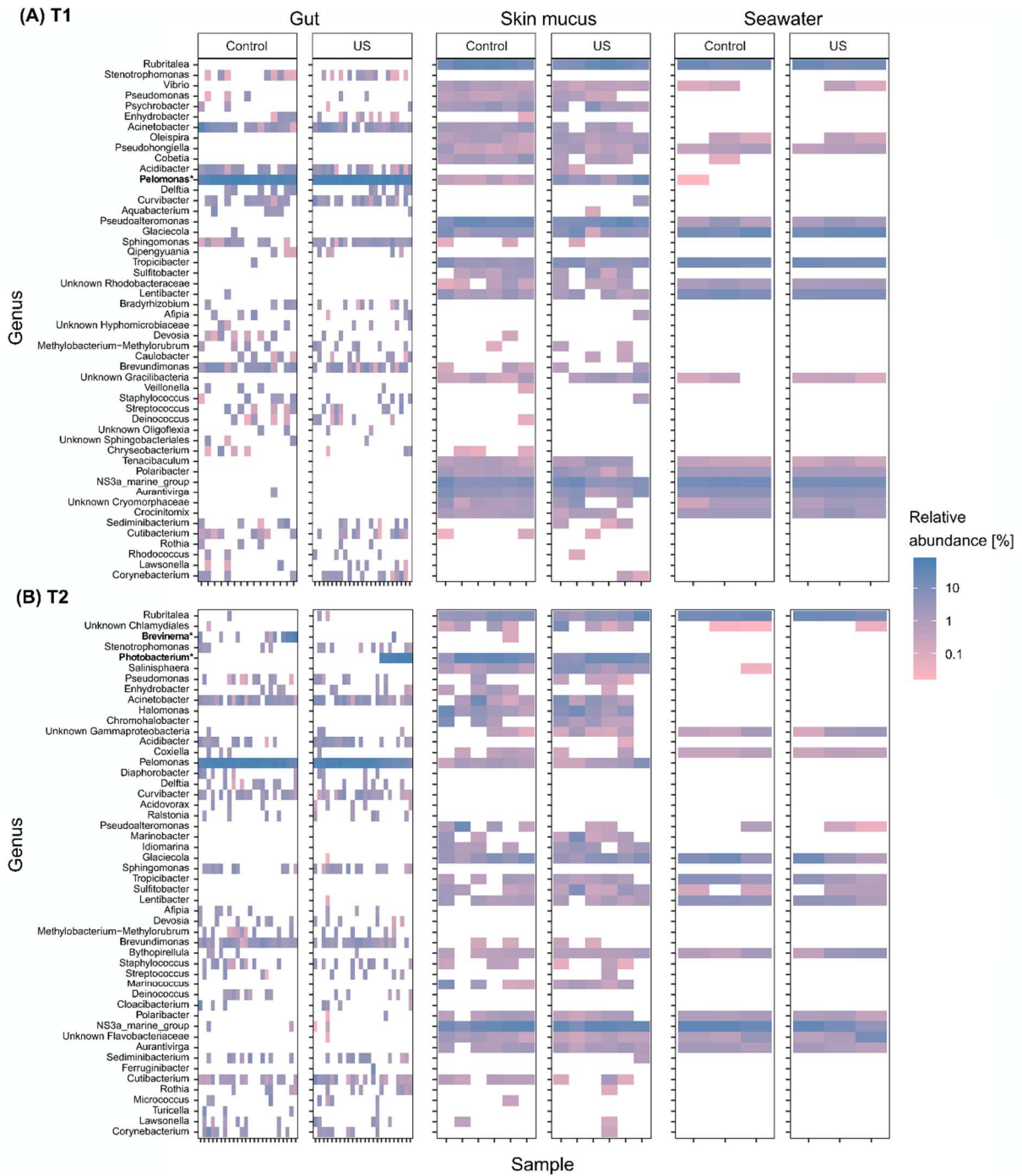
381

382 The genera *Pseudomonas* and *Vibrio*, which were quantified by cultivation, were only minor
383 taxa in the analysed microbial communities. *Pseudomonas* represented 0.5% and 0.4% of the
384 relative abundance in the gut and skin mucus samples, respectively, and was absent in the
385 seawater. *Vibrio* represented 0.5% and 0.1% of the relative abundance in the skin mucus and
386 seawater samples, respectively, and was absent in the gut (data not shown). No significant

387 differences in the relative abundance of these genera were detected between the ultrasound
 388 exposed and control group at time T1.
 389



390
 391 *Figure 4: Microbial community profiles at class level assignment (left) and non-metric*
 392 *multidimensional scaling (NMDS) plot of Bray-Curtis distances (right) of gut, skin mucus and*
 393 *seawater microbiota at time T1 (A) and T2 (B) from fish in the control group (Control) or*
 394 *ultrasound exposed group (US). NMDS ordination is based on normalised data and stress*
 395 *values < 0.1.*



397

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

402 4. Discussion

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

415

416 Whereas growth and survival were unaffected, our results show that ultrasound exposure can
417 impact the fish microbiota. Concerning the cultivable microbiota, CFU analysis showed that
418 the gills harboured an increased number of heterotrophic bacteria as well as potentially
419 pathogenic bacteria, such as *Pseudomonas* spp. and *Vibrio* spp. after ultrasound exposure.
420 Higher levels of these bacteria, even after inactivating the transducers, indicate that ultrasound
421 could have a long-term impact on the gill microbiota. Several *Pseudomonas* and *Vibrio*
422 species are known to cause serious diseases in farmed fish and lead to significant economic
423 losses (Novriadi, 2016; Toranzo et al., 2005). Among *Pseudomonas*, the species
424 *P. anguilliseptica* is considered the most significant pathogen for farmed fish (Toranzo et al.,
425 2005). Within the genus *Vibrio*, many species have been described as fish pathogens,
426 including *V. anguillarum*, *V. ordalii*, *V. salmonicida*, *V. vulnificus*, *V. parahaemolyticus*, *V.*

427 *alginoliticus*, *V. harveyi* (Novriadi, 2016), *V. toranzoniae* (Lasa et al., 2015) and *V. tapetis*
428 (Vercauteren et al., 2018), and vibriosis is a well-known cause of mortality in aquaculture
429 (Novriadi, 2016). Fish gills perform vital functions (e.g., respiration, osmoregulation, pH
430 balance, ammonia excretion, hormone regulation, detoxification) and their mucus harbours a
431 complex community of commensal microorganisms that play a major part in maintaining
432 homeostasis and protection against pathogens (Reverter et al., 2017). Although pathogenic
433 bacteria can form a part of the mucus microbiome in healthy farmed fish (Rosado et al.,
434 2019), they can also cause disease if there is a shift in their abundance (Hess et al., 2015;
435 Rosado et al., 2019). Consequently, an increase in the abundance of *Pseudomonas* spp. and
436 *Vibrio* spp. within the gills could potentially increase the risk of pathologies. However, it is
437 well established that cultivable bacteria represent only a negligible part (< 2%) of complex
438 bacterial communities associated with various environmental and animal habitats (Vartoukian
439 et al., 2010). Their enumeration therefore does not provide a functional implication of the
440 whole gill microbiome (Legrand et al., 2020), but rather warrants further research into
441 putative pathogenicity of the bacteria propagated by ultrasound exposure.

442

443 At time T1, ultrasound exposure also seemed to facilitate the colonization of the skin mucus
444 by *Vibrio* spp. as shown by cultivation-based analysis. As *Vibrio* only constituted a minor
445 taxon in the skin microbial community and was not detected in all samples, these results could
446 not be confirmed by metataxonomic analysis. However, a significant increase in the relative
447 abundance of *Pelomonas* in the skin microbial community during ultrasound exposure further
448 highlights the potential disruption of the skin microbiota by ultrasound. *Pelomonas* has
449 previously been detected in the gut of farmed sea bass (Nikouli et al., 2018) and is a dominant
450 member of the gut microbiome of other fish species (Nikouli et al., 2020; Rasheeda et al.,
451 2017; R. Zhao et al., 2020). *Pelomonas* has also been identified on the skin of brook charr

452 (Boutin et al., 2013) and the gills of rainbow trout (Lowrey et al., 2015), however at low
453 relative abundances. As the gut and skin environment in the current study shared the same
454 *Pelomonas*-associated ASV, it appears that this gut-associated bacterium is a low-abundant
455 opportunist on the skin. The near absence of *Pelomonas* in the seawater further demonstrates
456 that it is not a transient bacterium in the farming environment. A previous study has shown
457 that stress may induce changes in skin mucus microbiota, such as a decrease in the abundance
458 of probiotic-like bacteria and an increase in opportunistic pathogenic bacteria (Boutin et al.,
459 2013). As such, these results could demonstrate a state of stress in fish subjected to
460 ultrasound.

461

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

477

478 In conclusion, our study shows that close-proximity and continuous ultrasound exposure can
479 impact the surface microbial community of farmed sea bass. The skin and gill mucus layers
480 act as a protective barrier against the external environment and are essential in protecting the
481 fish from pathogenic organisms (Merrifield and Rodiles, 2015). In addition, commensal
482 microorganisms can prevent the colonisation of the skin by pathogens (Pérez-Sánchez et al.,
483 2011). Hence, even though an adverse effect of ultrasound exposure was not evident on the
484 growth performance and survival of sea bass, alterations to the microbiome could lead to a
485 higher incidence of disease and mortality in the long-term and therefore warrants further
486 investigation. As these results were generated under controlled laboratory conditions and the
487 fish were kept in close proximity to the ultrasound source, further studies should be conducted
488 to determine the impact of ultrasonic antifouling control on the fish microbiota under *in situ*
489 conditions, while assessing the potential benefits of this technology compared to traditional
490 antifouling control strategies.

491

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495

496 **Competing interest statement**

497 The authors declare that there are no competing interests.

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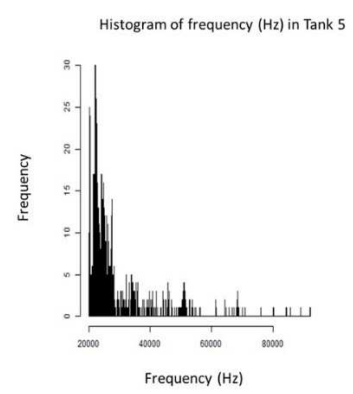
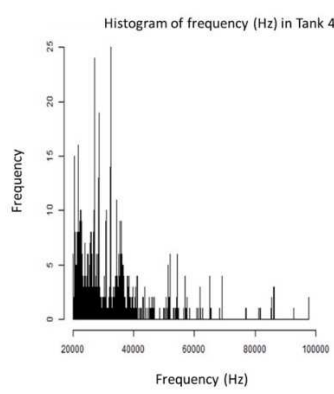
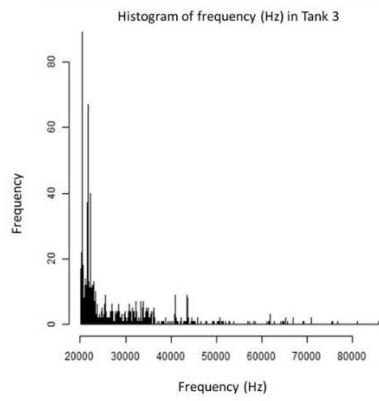
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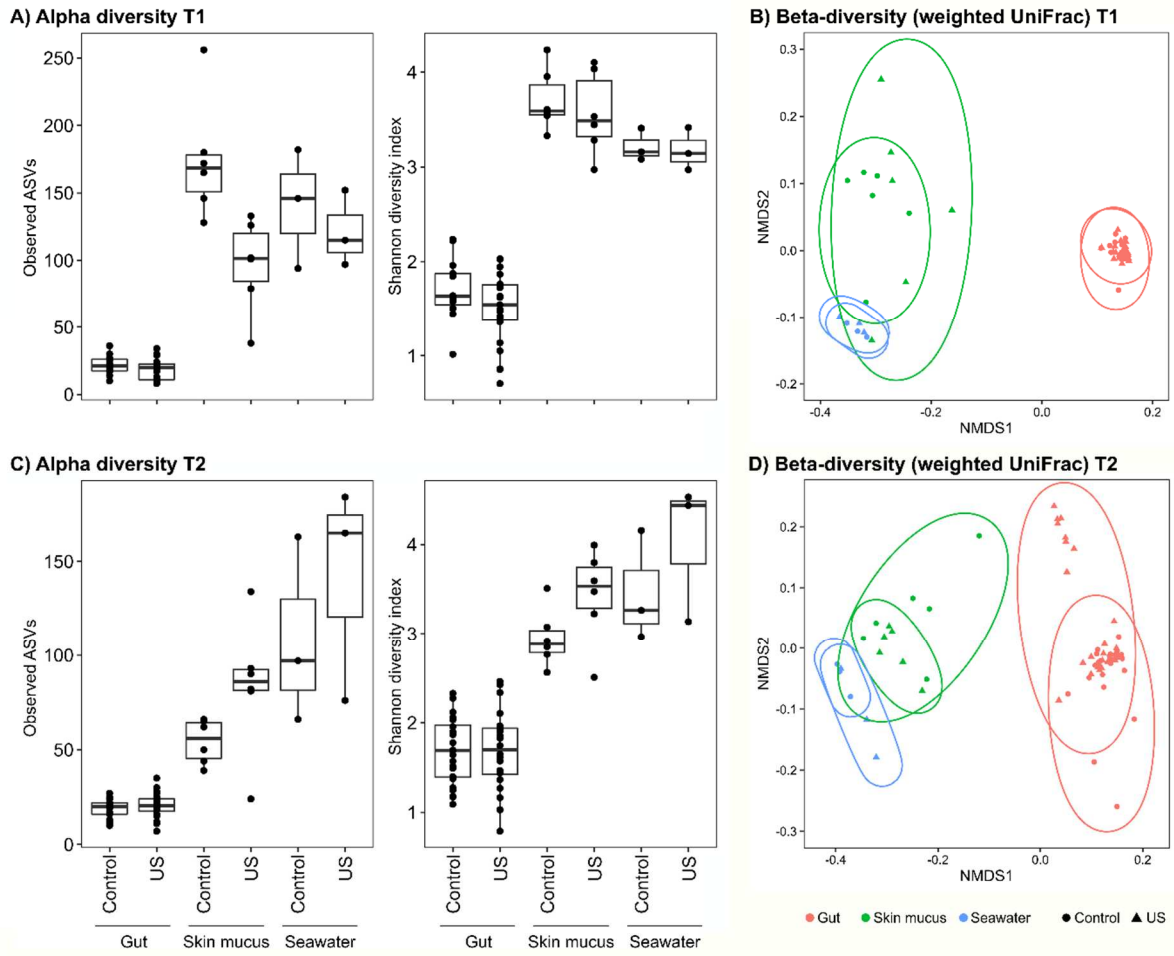
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759 **Supplemental Figure S1:** Histogram of frequencies for the three tanks with ultrasound
760 exposure (high quality image submitted separately).

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763 **Supplemental Figure S2:** Alpha diversity measures for gut, skin mucus and seawater
 764 microbial communities (A and C). NMDS of weighted UniFrac distances (stress values <
 765 0.08).

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