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**Microbiota and volatilome profile of fresh and chill-stored deepwater rose shrimp (*Parapenaeus longirostris*)**

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(Article begins on next page)

1 **Microbiota and volatilome profile of fresh and chill-stored deepwater**  
2 **rose shrimp (*Parapenaeus longirostris*)**

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26 **ABSTRACT**

27 Bacterial communities and Volatile Organic Compounds (VOCs) profile of deepwater  
28 rose shrimp (*Parapenaeus longirostris*) stored at 0°C (ice) and 4°C were investigated  
29 using 16S amplicon based sequencing and Solid phase micro-extraction (SPME) -  
30 Gas chromatography/mass spectrometry (GC/MS), respectively. The shelf-life of  
31 shrimps determined by sensory assessment was 5 and 2 days at 0°C and 4°C,  
32 respectively. Based on 16S analysis (culture-independed), the initial microbiota of  
33 shrimps mainly consists of *Photobacterium*, *Candidatus Hepatoplasma*,  
34 *Psychrobacter*, *Acinetobacter* and *Delftia*. *Psychrobacter* and *Carnobacterium*  
35 dominated during storage at both temperatures. *Psychrobacter* was the most dominant  
36 taxon at the end of shelf-life of chill-stored shrimps. A minor microbial population  
37 composed by *Brevundimonas*, *Stenotrophomonas*, *Staphylococcus*, *Legionella*,  
38 *Acinetobacter*, *Bacillus*, *Escherichia-Shigella*, *Enterococcus*, *Enterobacter*, *Klebsiella*  
39 was also detected. Those taxa may be originated from the environment due to an  
40 inadequate hygienic practice during fishing, handling and icing. VOCs such as  
41 ethanol, 3-methyl-1-butanol, 2-ethyl-1-hexanol, 3-hydroxy-2-butanone, indole etc.,  
42 were found to be associated with shrimps at 4°C, while acetone and dimethyl sulfide  
43 with shrimps in ice. Some VOCs, from microbial or chemical origin, increased in  
44 shrimps either at 0°C (i.e. 1-octen-3-ol, *trans*-2-octenal) or at 4°C (i.e. 3-methyl-1-  
45 butanol, indole), while 2-methylbutanal and 3-methylbutanal increased in both  
46 temperatures. A positive correlation between *Psychrobacter* with 2-ethyl-1-hexanol  
47 and *Carnobacterium* with 3-methyl-1-butanol was also observed. Concluding, we  
48 suggest the reinforcement of Good Hygiene Practices on fishing boats during  
49 fishing/handling, the rapid onboard icing and keeping shrimps iced avoiding even  
50 small increase of storage temperature that affects quality parameters (e.g. microbial

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population level, synthesis of microbiota, VOCs profile) in order to provide a product of the highest quality and safety in the market.

**Keywords:** crustaceans, shrimps, spoilage, microbiota, 16S Next Generation Sequencing, Volatile Organic Compounds

## 1. Introduction

Crustaceans are traded and consumed in million tons around the world. The deepwater rose shrimp (*Parapenaeus longirostris*, Lucas 1846) is an important commercial species of the coasts of Italy, Spain, France, Algeria, Tunisia, Greece and Turkey, with Italy (4631 tons) and Spain (4237 tons) to be the countries with the largest catches (FAO, 2019). *P. longirostris* is one of the most landed and highly consumed shrimp species in Greece, where it is consumed as unprocessed. However, rose shrimp is extremely sensitive to deterioration even when it is stored in ice (shelf-life 6 days, Mendes, Gonçalves, Pestana & Pestana, 2005).

Despite the spoilage of shrimp begins with the appearance of melanosis (Ashie, Smith & Simpson, 1996; Lopez-Caballero, Martinez-Alvarez, Gomez-Guillen & Montero, 2006), microorganisms grow quickly and deteriorate its quality. Fresh shrimp spoils due to the rich content of protein, non-protein nitrogen compounds and other nutrients that can allow microbial growth (Don, Xavier, Devi, Nayak & Kannuchamy, 2018; Licciardello, Kharchoufi, Muratore & Restuccia, 2018). Microbial spoilage of shrimp begins immediately after catch and the microbial growth can be controlled by rapid icing of shrimps (Heinsz, Harrison & Leiting, 1988). A consortium of bacteria, the so-called Specific Spoilage Organisms (SSOs), is a small part of the initial total microbiota which grows faster over the other members of the

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76 total microbiota and produces metabolites responsible for the development of off-  
77 odors and off-flavors during storage resulting the organoleptic rejection of the product  
78 (Gram & Huss, 1996). The domination of particular microorganisms or SSOs depends  
79 on various factors such as fish species, geographical origin, synthesis of the initial fish  
80 microbiota, microbial interactions, contamination during fishing, handling, storage  
81 and distribution in combination with the applied storage conditions (Boziaris &  
82 Parlapani, 2016; Parlapani et al., 2018b). The type of the microorganisms that prevail  
83 during storage determines the type of fish spoilage e.g. the production of Volatile  
84 Organic Compounds (VOCs) and the development of specific off-odors and off-  
85 flavors (Boziaris & Parlapani, 2016).

86         The use of 16S Next Generation Sequencing is able to reveal cultivable and  
87 non-cultivable bacterial genera directly from the sample which they may be involved  
88 in spoilage of shrimps (Yang, Xie & Qian, 2017) and other seafood (Chaillou et al.  
89 2015; Kuuliala et al. 2018; Parlapani et al. 2018a; 2018b; 2019; Zotta, Parente,  
90 Ianniello, De Filippis & Ricciardi, 2019). In addition, microorganisms may be  
91 associated with hygiene conditions during fishing, icing, landing and distribution or  
92 potential pathogens coming from various sources of contamination can be also  
93 highlighted (Parlapani et al. 2018b). The derived information can be used to  
94 implement preventive measures for the improvement of microbial quality and safety  
95 of fish during handling and storage.

96         Despite the increasing consumption of deepwater rose shrimp, there is no  
97 study, to our knowledge, about bacterial communities and volatile compounds  
98 production during chill storage. The aim of this study was: (i) to determine microbiota  
99 through 16S amplicon sequencing, (ii) to investigate the VOCs profile using SPME-  
100 GC/MS and (iii) to correlate the volatilome with microbiome and sensory score for

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101 chill-stored shrimps, in order to reveal the initial microbiota of shrimps, the  
102 microbiota changes during storage and the produced VOCs at the time point the  
103 particular microorganisms dominate the shrimp causing spoilage.

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## 105 **2. Materials and Methods**

### 106 *2.1 Shrimps provision, handling and storage*

107 Two different batches of the deepwater rose shrimp (*P. longirostris*), that were  
108 caught by trawl fishery from Strymonikos Gulf (mean weight±sd: 12.3±3.0g, 72m  
109 depth, F/V Athanassios) in April (10-04-2017, 17-04-2017) were packaged in  
110 insulated boxes with melted ice (0°C) and transferred to the Laboratory of Marketing  
111 and Technology of Aquatic Products and Foods (Department of Ichthyology and  
112 Aquatic Environment, University of Thessaly, Volos, Greece) within four hours from  
113 capture. The insulated boxes containing 1kg of shrimps each, were stored in  
114 incubators operating at two different temperature conditions; 0°C (shrimps with  
115 melted ice replaced every day) and 4°C.

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### 117 *2.2 Evaluation of shrimp's shelf-life*

118 Sensory evaluation was performed by 5 trained panelists, who evaluated the  
119 appearance of the shell (bright colours, slight blackening on the head, blackening on  
120 head and body), and flesh odor (fresh, marine, musty, ammoniacal, sour, putrid),  
121 according to FAO/WHO (1999). The sensory attributes rating were scored using a 5  
122 to 1 scale with 5 (excellent), 4 (good), 3 (acceptable), 2 (unacceptable) and 1 (spoiled)  
123 scores. A score of 3 was considered as the score for minimum acceptability and the  
124 time point that average score was below 3 (which means that at least one out of the  
125 five panelists scored with 2) was considered as the rejection time point.

126

127 *2.3 Microbiological analysis*

128 Ten grams (10 g) flesh from 10 shrimp individuals were aseptically transferred  
129 into stomacher bags with 90 mL MRD (Maximum Recovery Diluent, 0.1% w/v  
130 peptone, 0.85% w/v NaCl) and homogenized for 2 min using a Stomacher (Bug  
131 Mixer, Interscience, London, UK). Using the spread plate technique, 0.1 mL from 10-  
132 fold serial dilutions were used in order to enumerate the total microbial population as  
133 aerobic plate counts (APC) on TSA (Tryptone Soy Agar) and *Pseudomonas* on  
134 ceftrimide-fucidin-cephaloridine agar (CFC) after incubation at 25°C for 48 h. Using  
135 the pour plating method with overlay technique, 1 mL of serial dilutions in MRD was  
136 used for the enumeration of counts on Iron Agar (IA) by counting only the black  
137 colonies (H<sub>2</sub>S producing bacteria) after incubation at 25°C for 72 h, Lactic Acid  
138 Bacteria (LAB) on De Man, Rogosa, Sharpe agar (MRS) after incubation at 25°C for  
139 72 h and counts on Violet Red Bile Glucose agar (VRBGA) by counting only the red  
140 to dark purple colonies surrounded by a reddish zone (Enterobacteriaceae), after  
141 incubation at 37°C for 24 h. The results were expressed as mean log cfu g<sup>-1</sup> ± standard  
142 deviation (sd) of 4 replicates (two replicates per batch).

143

144 *2.4 16S rRNA meta-barcoding analysis*

145 *DNA extraction, quality evaluation and sequencing*

146 Twenty five (25) grams of flesh per batch (N=20 individuals) were used to  
147 collect bacterial pellets for 16S rRNA amplicon sequencing, as it was described in  
148 Parlapani et al. (2018b). For each sample, 200 µl of each diluted pellet were used for  
149 bacterial DNA extraction with ZR Soil Microbe DNA MicroPrep (ZYMO  
150 RESEARCH; Irvine, CA, USA). DNA concentration was measured on a Qubit 2.0

151 Fluorimeter using the Qubit® dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA).

152 The total DNA (DNA from two batches) was pooled before the analysis.

153 Metagenomic analysis was performed by amplifying the V3–V4 region of the  
154 16S rRNA gene using the Illumina’s 16S Metagenomic Sequencing Library  
155 Preparation (15044223 B) protocol. For the amplification of the V3-V4 region, gene-  
156 specific primers were selected based on the Klindworth et al. (2013), by adding  
157 Illumina overhang adapter nucleotide sequences at the 5’ end. PCR reactions were  
158 performed in a 36-well rotor carousel on a Rotor-Gene Q Thermocycler (Qiagen,  
159 Hilden, Germany) according to Parlapani et al. (2018b).

160 PCR products were purified to remove unincorporated primers and primer-  
161 dimer species using NucleoMag® NGS Bead Suspension (Macherey-Nagel, Düren,  
162 Germany). A second PCR was performed to attach dual indices and Illumina  
163 sequencing adapters in all PCR fragments following the instruction of the Illumina’s  
164 16S Metagenomic Sequencing Library Preparation. All libraries were quantified with  
165 fluorometric quantification using Qubit® dsDNA BR assay kit and their molarity was  
166 calculated in relation to the size of DNA amplicons after indexing. Quantitative PCR  
167 (qPCR) was conducted on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany)  
168 with the KAPA Library Quantification kit for Illumina sequencing platforms (KAPA  
169 BIOSYSTEMS, Woburn, MA, U.S.A.).

170 Sequencing was performed on a MiSeq platform according to the  
171 manufacturer’s instructions, using the MiSeq® reagent kit v3 (2 x 300 cycles)  
172 (Illumina, San Diego, California).

173

174 *Bioinformatics and Data Analysis*



175 Trim Galore wrapper (Krueger, 2015) was used for quality trimming of raw  
176 reads (.fastq files). Sequences were clustered into operational taxonomic units (OTUs)  
177 using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et  
178 al., 2010), with 99% sequence similarity to SILVA 128 release for QIIME database.  
179 OTU picking was executed using the default clustering algorithm UCLUST (Edgar,  
180 2010). Further processing of OTU tables was conducted in R version 3.4.2 (R  
181 Development Core Team, 2010). OTU counts and taxonomic assignments were  
182 merged to a phyloseq object with phyloseq R package (McMurdie & Holmes, 2013).  
183 Sequences have been submitted through the NCBI sequencing submission portal and  
184 deposited under the SRA: SUB6377492, with accession number SAMN12878864.

#### 186 *2.5 VOCs determination by Headspace SPME-GC/MS analysis*

187 Ten (10) g of flesh from approximately ten shrimps were homogenized. Five  
188 grams of the homogenized sample in duplicate per batch were transferred into a 20  
189 mL glass vial (two replicates per batch, 2x2=4 replicates in total). The SPME-GC/MS  
190 analysis was performed according to Parlapani et al. (2015a). Compounds were  
191 identified by comparing: (i) the linear retention indices (LRI) based on an  
192 homologous series of even numbered n-alkanes (C8–C24, Niles, Illinois, USA) with  
193 those of standard compounds and literature data, and (ii) the MS data with those of  
194 reference compounds and that of MS data obtained from NIST library  
195 (NIST/EPA/NIH Mass Spectral Library with Search Program, data version NIST 05,  
196 software version 2.0d). The Amdis software (version 2.62,  
197 <http://chemdata.nist.gov/mass-spc/amdis/>) was used for the deconvolution of mass  
198 spectra and the identification of target components. The amount of VOCs was

199 expressed in arbitrary units of the peak area of the deconvoluted component  
200 multiplied by  $10^{-6}$ .

201

## 202 *2.6 Statistical analysis*

203 For the 16S data plots were visualized by combining functions provided by the  
204 ggplot2 R package (Wickham, 2009).

205 Alpha diversity indices were calculated using the Chao1 and ACE indices  
206 (Chao & Chiu, 2016) were assessed to measure species richness and the Shannon,  
207 Simpson, Inverse Simpson and Fisher indices (Lande, 1996) were used to assess the  
208 proportional abundances and frequencies of the identified species by the *vegan*  
209 package of R.

210 Abundance estimations for each species were normalized to 100% within each  
211 sample, thus, percentages do not reflect the true biomass fraction of each sample.  
212 Venn diagrams were constructed using the InteractiVenn online tool (Heberle,  
213 Meirelles, da Silva, Telles & Minghim, 2015) to depict pairwise comparisons of  
214 shared, common and/or unique OTUs. Spearman's non-parametric correlations were  
215 used through the R package *psych* to study the relationships between microbial taxa  
216 abundance and volatilome profile and visualized in R using the *corrplot* package of R.

217 Differences of means in microbial populations were subjected to Analysis of  
218 Variance (ANOVA) followed by Tukey's significant difference test, using  
219 STATISTICA 6.0. A probability level of  $p \leq 0.05$  was considered statistically  
220 significant.

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## 222 **3. Results**

### 223 *3.1 Rejection time of chill-stored shrimps*

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224            Sensory attributes of shrimps were evaluated as ‘excellent’ at the beginning of  
225 the experiments (T0, Table 1). Their shell presented bright colors. Flesh odor was  
226 fresh and marine. At the time of minimum acceptability (grade 3), the shrimp odor  
227 was weak "fishy odour", even slight ammonia was produced, and the color was  
228 natural light pink with grey-greenish, and the shell lacked luster sheen. After this  
229 point, the shrimps exhibited unpleasant odor, thus they were graded as unfit and  
230 rejected (Table 1). Therefore, the shelf-life determined by sensory assessment was  
231 estimated to be 5 days at 0°C (ice) and 2 days at 4°C.

232

### 233 *3.2 Microbial population changes*

234            At the beginning of the shrimp’s shelf-life (T0), the APC was found to be  
235  $3.12 \pm 0.58$  log cfu/g, while the populations of *Pseudomonas*, H<sub>2</sub>S producing bacteria  
236 and LAB were found to be  $2.69 \pm 0.61$ ,  $2.04 \pm 0.08$ ,  $3.00 \pm 0.70$  log cfu/g, respectively  
237 (Table 2). During storage, the bacteria were grown faster at 4°C reaching 4 to 5 logs  
238 for all the population monitored after one day of storage ( $p \leq 0.05$ , T1, Table 2) and  
239 reached up to 6.50 logs after two days of storage (T2). In respect of ice-stored  
240 shrimps, APC was found to be  $6.59 \pm 0.36$  log cfu/g after five days of storage (T5),  
241 while the populations of *Pseudomonas*, H<sub>2</sub>S producing bacteria and LAB reached  
242  $6.11 \pm 0.53$ ,  $4.96 \pm 0.63$  and  $5.23 \pm 0.47$  log cfu/g respectively (Table 2).  
243 Enterobacteriaceae remained below the detection limit of 1 log cfu/g throughout the  
244 experiment.

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### 246 *3.3 NGS analysis*

#### 247 *3.3.1 Illumina MiSeq data analysis*

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248 After sequencing 500,206 reads were obtained and after the quality filtering  
249 465,369 sequences were assigned to 4,026 unique OTUs Table S1. In particular,  
250 90.6%, 90.4%, 89.4%, 88.5% and 85.1% of reads were assigned to the Phylum, Class,  
251 Order, Family and Genus level, respectively. The most diverse community was  
252 observed for T2-0°C storage conditions in which the highest number of unique OTUs  
253 was observed among the studied samples (Table S1). That was also verified by the  
254 Chao1 index (Table S2).

### 256 3.3.2. Microbiota composition

257 A simple microbiota was observed in all the samples analysed with the  
258 predominance of *Photobacterium* (18.0%) as the main microbes in fresh shrimps  
259 (T0), while *Psychrobacter* and *Carnobacterium* dominated together in shrimps during  
260 storage at both temperatures (Table 3). *Carnobacterium* was recorded at abundances  
261 of 62.4 % at 0° and 20.5% at 4°C at the middle stages of storage, while the  
262 *Psychrobacter* at abundances of 34.0 at 0°C and 52.5% at 4°C (Table 3).  
263 *Psychrobacter* became the most dominant microorganism at the end of shelf-life of  
264 shrimps (57.7% and 48.0% in samples at 0°C and 4°C, respectively), while  
265 *Carnobacterium* was also found at 29.8% and 41.2% at 0°C and 4°C, respectively  
266 (Table 3). Other genera like *Candidatus Hepatoplasma*, *Pseudomonas*, *Acinetobacter*,  
267 *Delftia*, *Brevundimonas*, *Stenotrophomonas*, *Bacillus*, *Vibrio*, *Vagococcus* were also  
268 found but at lower abundances (up to 11.3%) in fresh or chill-stored samples (Table  
269 3), while other 167 genera including *Staphylococcus*, *Legionella*, *Bacillus*,  
270 *Escherichia-Shigella*, *Enterococcus*, *Enterobacter*, *Klebsiella* etc. were found at  
271 abundances of less than 1% in all cases (Table S3).

273 3.4 VOCs produced in chill-stored shrimps

274 A total of 41 VOCs were identified (11 alcohols, 12 aldehydes, 11 ketones, 3  
275 esters and other 4 compounds: dimethyl sulfide, methylene chloride, carbon disulfide  
276 and indole). By comparing the presence of the VOCs between T0 and samples stored  
277 in ice or in refrigerate condition it was possible to observe that samples stored at 4°C  
278 were characterized by the presence of ethanol, 3-methyl-1-butanol, 2-ethyl-1-hexanol,  
279 *trans*-2-nonenal, *trans*-2,*cis*-6-nonadienal, 3-hydroxy-2-butanone, 3-octen-2-one and  
280 indole, while acetone and dimethyl sulfide were mainly associated with ice storage  
281 (Fig 1a). The compounds 1-heptanol, 1-nonanol, octanal, 3-pentanone and methylene  
282 chloride were associated with the fresh shrimps (T0, Fig 2a). Some compounds were  
283 also increased in shrimps stored at 0°C (i.e. 1-octen-3-ol and *trans*-2-octenal) or at  
284 4°C (i.e. 3-methyl-1-butanol, *trans*-2,*cis*-6-nonadienal and indole), while the  
285 compound 2-methylbutanal and 3-methylbutanal were increased at both 0 and 4°C  
286 (Fig 1b).

288 3.5 Correlation between Microbiome and Volatilome in rose shrimps

289 Spearman's correlation between the microbial OTUs and VOCs clearly  
290 indicated the correlation of the most abundant bacterial species in rose shrimps with  
291 the concentration of metabolites produced during their chill storage ( $P < 0.05$ , Fig 2).  
292 *Psychrobacter* and *Carnobacterium* which found to be the most abundant genera  
293 during storage at both temperatures, they were positively correlated with some VOCs.  
294 In particular, *Psychrobacter* was positively correlated with 2-ethyl-1-hexanol, 2-  
295 nonanone and 2-octanone, and *Carnobacterium* with 3-methyl-1-butanol and 2-  
296 pentanone. Positive correlations were also found between *Candidatus Hepatoplasma*  
297 and 1-heptanol, 1-nonanol, octanal, 3-pentanone, *Photobacterium* and 2-butanone,

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298 *Vagococcus* and 3-methyl-1-butanol, 2-methylbutanal, 3-methylbutanal, 2-octanone,  
299 2-nonanone, *Vibrio* and 2-butanone, ethyl acetate (Fig 2). On the other hand,  
300 *Psychrobacter* was negatively correlated with dimethyl sulphide, while *Delftia* and  
301 *Lactobacillus* with 3-methyl-1-butanol (Fig 2).

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#### 303 **4. Discussion**

304 The 16S sequencing coupled with VOCs profile analysis were used to  
305 investigate microbial spoilage of the deepwater rose shrimp caught from Greek  
306 seawaters. The correlation of the microbiome with volatilome and their link with  
307 sensory score allow us to understand how the quality of shrimp is being deteriorated  
308 during storage and how it is eventually rejected.

309 Fresh shrimp remains one of the most delicious and delicatessen food in  
310 restaurants, catering and all-inclusive hotels along Greek coasts, inland and islands.  
311 However, the shelf-life of the deepwater rose shrimp is short as it was determined in  
312 this study (5 days in ice and 2 days at 4°C). *Pseudomonas*, H<sub>2</sub>S producing bacteria  
313 and LAB are usually responsible for the quality loss of chill-stored shrimp such as the  
314 tropical brackish water shrimp *Penaeus notialis* (Dabadé et al., 2015). In the present  
315 study, these organisms reached the levels of 5-6 logs at the end of fish shelf-life, with  
316 *Pseudomonas* to dominate the shrimp using the classical microbiological approach.  
317 However, *Pseudomonas* was found at very low abundances using the 16S NGS  
318 analysis (culture independed approach). This indicates that organisms other than  
319 *Pseudomonas* might grew on CFC culture medium. The microbiota grown on the  
320 selective culture media has to be investigated using molecular methodologies in future  
321 studies.

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322 Microorganisms such as *Acinetobacter*, *Bacillus* and *Delftia*, which were  
323 found during the first days of storage, are usually associated with the handling  
324 conditions and contamination occurs from the human skin, equipment, soil, washing  
325 water or ice, etc. (de Amorim & Nascimento, 2017; Heinsz et al., 1988; Parlapani et  
326 al., 2018). Other bacteria coming from human and environmental sources like  
327 *Enterobacter*, *Escherichia*, *Shigella*, *Klebsiella*, *Legionella*, *Enterococcus* and  
328 *Staphylococcus* are also involved in the inadequate attention to hygiene during  
329 fishing, handling and icing affecting fish quality and safety. The fact that these genera  
330 were found at abundances less than 1% herein does not minimize the possibility of  
331 pathogens presence. All of them contain human pathogens, foodborne or not, might  
332 be found in raw shrimps. In that case, a likely consumption of undercooked shrimps  
333 could cause foodborne infections causing socioeconomic hardship in the Greek  
334 market, like legal penalties, shutdown of business, and tarnished reputation. On the  
335 other hand, the detection of them using 16S NGS does not indicate potential risk.  
336 NGS is not able to differentiate living and dead cells, thus overestimating the living  
337 taxa in microbial communities (Emerson et al., 2017). To minimize contamination in  
338 order to maximize the quality and safety of fishery products, the findings derived  
339 from this and previous studies (Parlapani et al. 2018a; 2018b; 2019) could be used to  
340 develop approaches for rapid analysis like the High Resolution Melting analysis  
341 (HRM) or the multiplex PCR using specific genes as targets for the identification of  
342 pathogenic species/strains contained into the genera identified using 16S meta-  
343 barcoding. In addition, we could strengthen extension activities (education,  
344 communication and outreach) for individuals, communities and industries involved in  
345 the primary production in Greece.

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346 The 16S data also revealed herein the presence of *Candidatus Hepatoplasma*,  
347 *Photobacterium* and *Psychrobacter* at the beginning of deepwater rose shrimp shelf-  
348 life. From those, *Candidatus Hepatoplasma* has been described as symbionts of the  
349 terrestrial isopods *Porcellio scaber* and *Oniscus asellus* (Wang, Stingl, Anton-  
350 Erxleben, Zimmer & Brune, 2004; Wang, Brune & Zimmer, 2007), while the other  
351 two bacteria are usually found in fresh fish (Gram & Huss 1996). *Photobacterium* has  
352 been also reported as spoiler of fish under particular storage conditions.  
353 *Photobacterium* (*P. phosphoreum* or *Photobacterium* sp.) usually predominate in fish  
354 such as the cod, the halibut and the cold-smoked salmon under elevated CO<sub>2</sub>  
355 concentrations (Modified Atmosphere Packaging – MAP) or vacuum (Gram &  
356 Dalgaard, 2002; Hovda, Sivertsvik, Lunestad, Lorentzen & Rosnes, 2007; Kuuliala et  
357 al. 2018; Olofsson, Ahrne & Molin, 2007; Stohr, Joffraud, Cardinal & Leroi, 2001).  
358 The facultative fermentative metabolism of *Photobacterium* and the competitive  
359 interactive environment defined by the presence of other bacteria in fish flesh stored  
360 under aerobic conditions (Gram & Dalgaard, 2002) could may explain the absence of  
361 its growth increase during the storage of the deepwater rose shrimp. *Psychrobacter*  
362 and *Carnobacterium* were observed to co-dominate during storage in this study, at the  
363 first two days at 4°C, and the last two days at 0°C. This means that *Psychrobacter* and  
364 *Carnobacterium* bacteria dominate the shrimps stored at the higher temperature (4°C),  
365 in a shorted time than the ice-stored shrimps (0°C). *Psychrobacter* usually dominate  
366 the chill-stored crustaceans like the brown shrimp (Broekaert, Heyndrickx, Herman,  
367 Devlieghere & Vlaemynck, 2013a; Broekaert, Nosedá, Heyndrickx, Vlaemynck &  
368 Devlieghere, 2013b), the Pacific white shrimp (Yang et al., 2017), the Norway lobster  
369 (Bekaert, Devriese, Maes & Robbens, 2015), but also the fish like the gilt-head sea  
370 bream (Parlapani et al., 2018a), the thawed hake, the thawed plaice fillets (Zotta et



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371 al., 2019) or even the cephalopods like the thawed cuttlefish (Parlapani et al., 2018b).  
372 This bacterium might causes the spoilage of the brown shrimp through the break  
373 down of lipids and the hydrolysis of proteins and amino acids (Broekaert et al.,  
374 2013b). On the other hand, *Carnobacterium* was found to dominate the peeled brown  
375 shrimp stored under MAP at 4°C (Calliauw et al., 2016a), or it was detected in  
376 shrimps stored in MAP (Dalgaard et al., 2003) or in fish species stored in air  
377 (Parlapani et al., 2018a; Zotta et al., 2019).

378         The compound 3-methyl-1-butanol has been associated with the metabolic  
379 activity of *Carnobacterium* in inoculated cooked and peeled tropical shrimp *Penaeus*  
380 *vannamei* stored under MAP at 8°C (Jaffres et al., 2011). In our study, this compound  
381 was positively correlated to the presence of the *Carnobacterium* and it was increased  
382 in shrimps stored at 4°C. *C. divergens* and *C. maltaromaticum* are known to cause  
383 sensory spoilage of cooked and peeled shrimps stored under MAP at 5°C, producing  
384 ammonia, tyramine, alcohols (including 3-methyl-1-butanol), aldehydes and ketones  
385 (including 2-pentanone, Laursen, Leisner, & Dalgaard, 2006). Other compounds such  
386 as the ethanol, the dimethyl sulfide and the ethyl acetate were being increased in  
387 cooked peeled gray/brown shrimp *Crangon crangon* stored under MAP (Nosedá et  
388 al., 2012). The ethyl acetate, which showed a positive correlation with *Vibrio* herein,  
389 has been also reported to the brown shrimp (Kuuliala et al., 2018). *Vagococcus*  
390 (particularly *V. salmoninarum*) was found to produce 2-nonanone in inoculated brown  
391 shrimp stored under MAP at 4°C (Calliauw, Horemans, Broekaert, Michiels &  
392 Heyndrickx, 2016b). From the aforementioned compounds, only the 3-methyl-1-  
393 butanol increased in this study. Additionally, the 1-octen-3-ol and the *trans*-2-octenal  
394 were increased in the deepwater rose shrimps stored at 0°C, the *trans*-2,*cis*-6-  
395 nonadienal and indole in those stored at 4°C, while the 2-methylbutanal and the 3-

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396 methylbutanal they were increased in the shrimps stored at both temperatures. The 1-  
397 octen-3-ol, *trans*-2-octenal and *trans*-2,*cis*-6-nonadienal are associated with chemical  
398 oxidations in fish (Duflos, Coin, Cornu, Antinelli & Malle, 2006; Iglesias & Medina,  
399 2008), while the 2-methylbutanal and 3-methylbutanal are products of microbial  
400 activity (Parlapani, Mallouchos, Haroutounian & Boziaris, 2017). The compounds 3-  
401 methyl-1-butanol, 1-octen-3-ol, *trans*-2-octenal, 2-methylbutanal, 3-methylbutanal  
402 and indole they also exhibited an increasing trend in chill-stored blue crabs and  
403 proposed as potential spoilage markers of that product (Parlapani et al., 2019). The  
404 indole has been associated with the spoilage of *P. longirostris* (Mendes et al., 2005;  
405 Mendes, Huidobro & Caballero, 2002) and it has been found as a product of various  
406 bacteria like *Vibrio* spp. and *Morganella morganii* isolated from the wild shrimps  
407 *Litopenaeus setiferus* and *Litopenaeus brasiliensis* (Benner, Staruszkiewicz & Otwell,  
408 2004). Herein, no correlation between indole (which found at very low relative  
409 concentrations at 4°C) and bacteria was observed. This may occurs since indole is not  
410 produced in significant quantities by bacteria in shrimps stored at low temperatures  
411 (Mendes et al., 2002). Other compounds such as hexanol, heptanol, hexanal, heptanal,  
412 octanal and nonanal found in rose shrimp are known as aroma compounds and are  
413 mainly coming from enzymatic reactions, oxidation or autoxidation of lipids  
414 (Alasalvar, Taylor & Shahidi, 2005; Duflos et al., 2006).

415 Overall, the inadequate hygiene during fishing/handling can cause microbial  
416 contamination from human, foodstuffs and environmental sources. In this way, the  
417 initial total microbial population increases, the quality of fish product is lost rapidly  
418 and the product might not be safe to consume. For these reasons, fishermen have to  
419 reinforce the Good Hygiene Practices regarding the potential sources of microbial  
420 contamination during handling which usually reduce quality and shorten shelf-life of

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421 fish. Moreover, the shrimps have to be stored and distributed in ice (rapid onboard  
422 icing and keeping fish iced until marketing or consumption). To reinforce the  
423 competitiveness of fishery products at local, national or international commercial  
424 level, the research outputs should be communicated through websites, workshops,  
425 public dialogues, newsletters, and one-on-one consultations in order to create  
426 awareness among academia, fishery sectors and society.

427

## 428 **Conclusions**

429 The 16S meta-barcoding revealed microorganisms associated with safety or spoilage  
430 of the deepwater rose shrimp from Greek seawaters. The simulated storage conditions  
431 showed how the higher temperature causes the fast domination of the shrimps by the  
432 spoilers *Psychrobacter* and *Carnobacterium* resulting to their rapid quality  
433 deterioration (shelf-life: 2 days at 4°C vs 5 days in ice). In addition, compounds  
434 associated with microbial activity e.g. 3-methyl-1-butanol, 2-ethyl-1-hexanol and 2-  
435 nonanone presented a positive correlation with the dominant organisms e.g.  
436 *Psychrobacter* or *Carnobacterium* in rose shrimp. From them, 3-methyl-1-butanol  
437 increased during storage. Other compounds associated with chemical reactions e.g. 1-  
438 octen-3-ol, *trans*-2-octenal and *trans*-2,*cis*-6-nonadienal were also increased. This  
439 indicates that not only microbial action, but also chemical mechanisms might be  
440 responsible for the quality loss of chill-stored rose shrimp. Based on our findings, we  
441 could suggest preventive measures to improve the quality and safety of rose shrimp,  
442 particularly fishermen have to reinforce the Good Hygiene Practices during fishing  
443 and handling, to accomplish rapidly onboard icing and keep shrimps iced avoiding  
444 even small changes in storage temperature that affect the microbial population level,  
445 synthesis of microbiota, VOCs profile and eventually the shelf-life of the product. The

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446 results of this study will be used to prepare a guide for the proper handling and  
447 storage conditions of the fishing products produced by the small-scale fishery sector  
448 in Greece in order to provide a product of the highest quality and safety in the market.  
449

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704 **Table 1.** Sensory score of deepwater rose shrimp stored at 0°C (ice) and 4°C.

	0°C (ice)				4°C	
Sensory score	T0	T2	T4	T5	T1	T2
<i>Shell appearance</i>	Excellent	Good	Acceptable	Unacceptable	Acceptable	Unacceptable
<i>Flesh odor</i>	Excellent	Excellent	Good	Unacceptable	Good	Unacceptable

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707 **Table 2.** Population changes (mean log cfu g<sup>-1</sup> ± sd) of four replicates during storage

708 (in days, T) of shrimps stored at 0°C (ice) and 4°C.

	0°C (ice)				4°C	
	T0	T2	T4	T5	T1	T2
<b>APC</b>	3.12±0.58 <sup>a</sup>	4.32±0.38 <sup>b</sup>	5.92±0.38 <sup>c</sup>	6.59±0.36 <sup>d</sup>	4.85±0.62 <sup>b</sup>	6.44±0.46 <sup>c</sup>
<i>Pseudomonas</i>	2.69±0.61 <sup>a</sup>	3.44±0.43 <sup>b</sup>	5.39±0.46 <sup>c</sup>	6.11±0.53 <sup>d</sup>	4.46±0.51 <sup>e</sup>	5.97±0.47 <sup>c</sup>

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<b>H<sub>2</sub>S bacteria</b>	2.04±0.08 <sup>a</sup>	2.82±0.57 <sup>b</sup>	4.14±0.73 <sup>c</sup>	4.96±0.63 <sup>d</sup>	4.15±0.49 <sup>c</sup>	5.14±0.10 <sup>d</sup>
<b>LAB</b>	3.00±0.70 <sup>a</sup>	3.69±0.85 <sup>a</sup>	4.43±0.33 <sup>b</sup>	5.23±0.47 <sup>c</sup>	4.29±0.61 <sup>b</sup>	5.08±0.48 <sup>c</sup>

709 Mean values with different letters are significantly different (p<0.05). Mean  
710 population values along different time (T0-T5) or temperature values (0 and 4°C), are  
711 compared using ANOVA.

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718 **Table 3.** The Relative abundance (%) of the dominant genera assigned to 16S rRNA  
719 sequences, in the deepwater rose shrimp stored in 0 and 4°C.

OTUs (%)	0°C (ice)				4°C	
	T0	T2	T4	T5	T1	T2
<i>Psychrobacter</i>	8.10	20.9	34.0	57.7	52.5	48.0
<i>Carnobacterium</i>	1.60	8.70	62.4	29.8	20.5	41.2
<i>Pseudomonas</i>	0.30	7.30	0.60	0.70	11.3	0.60
<i>Photobacterium</i>	18.0	0.10	0.00	0.00	0.00	0.20
<i>Acinetobacter</i>	5.20	0.90	0.20	5.30	0.10	0.10
<i>Delftia</i>	4.20	6.70	0.10	0.10	0.10	0.00
<i>Candidatus Hepatoplasma</i>	10.0	0.00	0.00	0.00	0.00	0.00
<i>Vagococcus</i>	0.00	0.00	0.60	2.00	1.90	5.30
<i>Stenotrophomonas</i>	1.10	7.00	0.00	0.20	0.20	0.30
<i>Brevundimonas</i>	6.50	1.50	0.10	0.30	0.10	0.10
<i>Bacillus</i>	0.30	6.90	0.00	0.30	0.00	0.10
<i>Vibrio</i>	5.40	0.00	0.00	0.00	0.00	0.20
<i>Exiguobacterium</i>	0.10	0.00	0.00	0.00	4.40	0.00
<i>Lactobacillus</i>	4.30	0.10	0.00	0.00	0.10	0.00
<i>Paucimonas</i>	1.50	2.20	0.00	0.10	0.00	0.00
<i>Planococcus</i>	0.10	3.20	0.00	0.30	0.20	0.10

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<i>Rhizobium</i>	1.00	2.30	0.00	0.10	0.10	0.00
<i>Aerococcus</i>	0.10	0.20	0.00	0.10	2.60	0.30

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728 **Legends to Figures**

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730 **Figure 1. Panel A. Abundance of VOCs (relative concentrations, area x 10<sup>-6</sup>**  
731 **under the chromatographic peak) during storage of fresh shrimp (T0, red bars)**  
732 **at Time stored in ice (green bars) or 4°C (blue bars). Panel B. Shift in volatilome**  
733 **profile during storage at different storage conditions (T2, T4 and T5 for ice-stored**  
734 **shrimps and T1\_4°C and T2\_4°C for shrimps stored at 4°C). Boxes represent the**  
735 **interquartile ranges (IQRs) between the first and third quartiles, and the lines inside**  
736 **represent the medians (2nd quartiles). Whiskers denote the lowest and the highest**  
737 **values within IQRs from the first and third quartiles, respectively. Circles represent**  
738 **outliers beyond the whiskers.**

739

740 **Figure 2. Spearman's correlation between the bacterial OTUs and VOCs in**  
741 **deepwater rose shrimp. The intensity of the colors indicates the degree of correlation**  
742 **between the bacterial OTUs. The blue color shows a positive correlation, while the**  
743 **red colour shows a negative correlation between OTUs and VOCs.**

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754 **FIG 1-2 (attached file)**

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Figure 1a  
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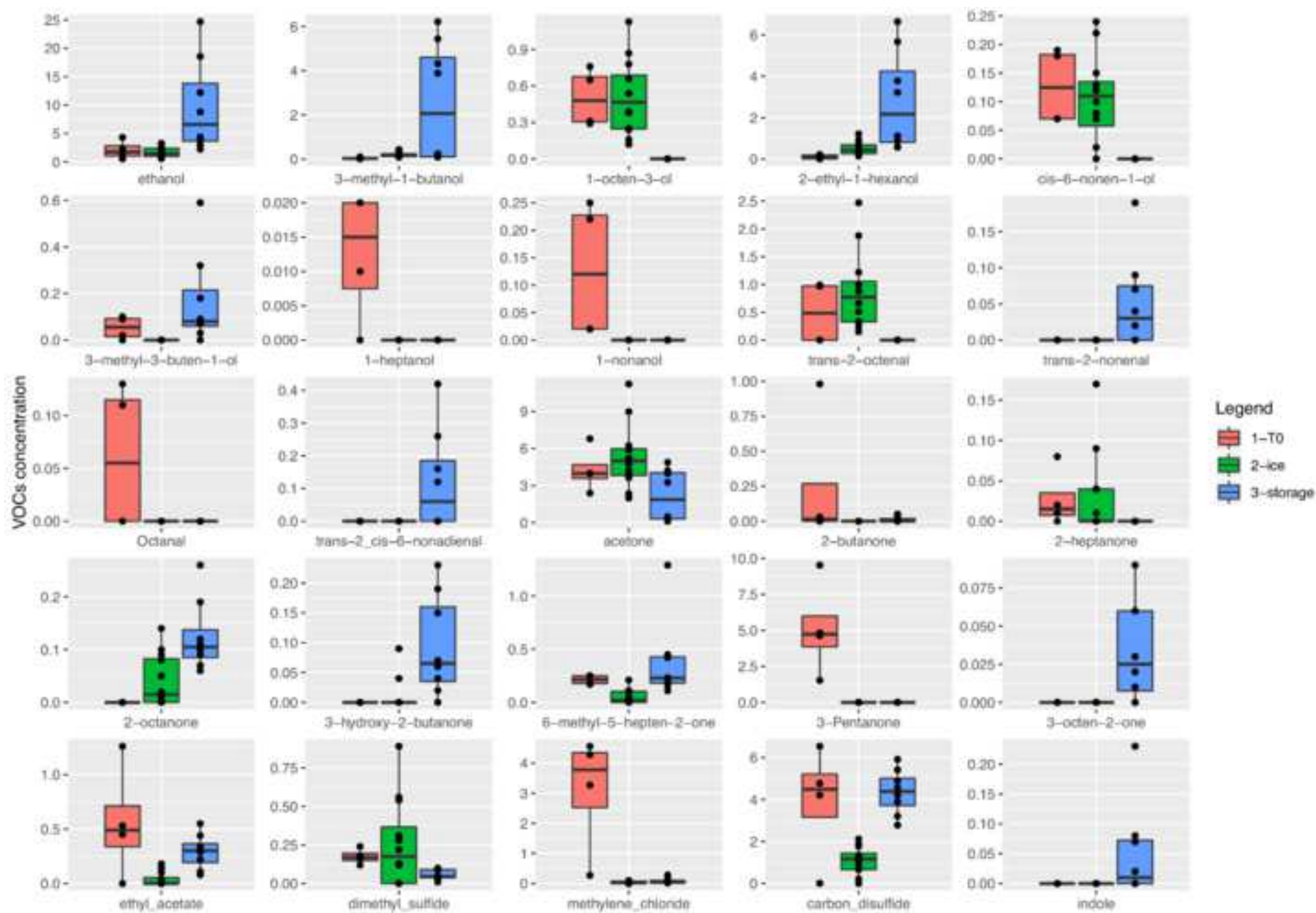
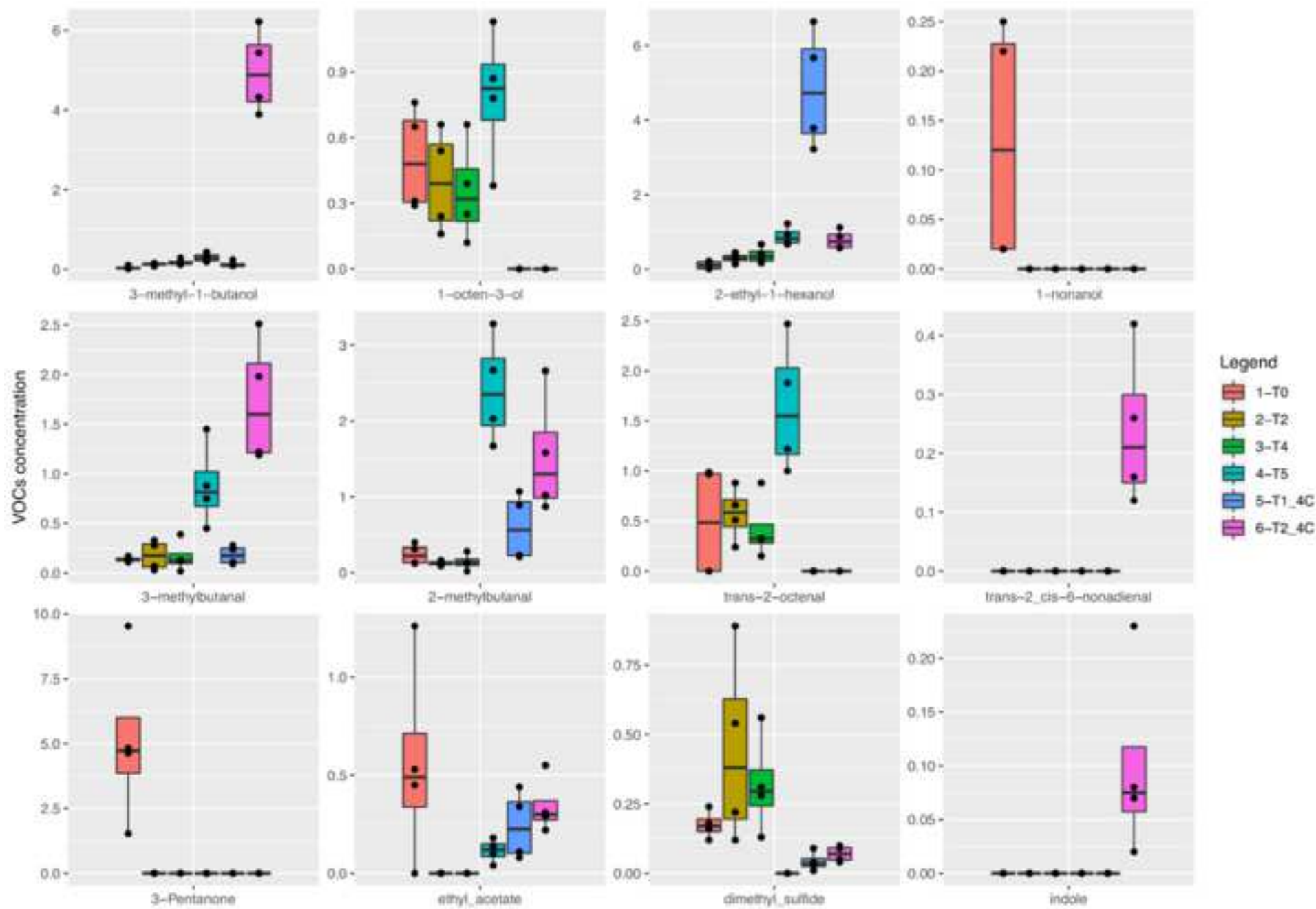


Figure 1b  
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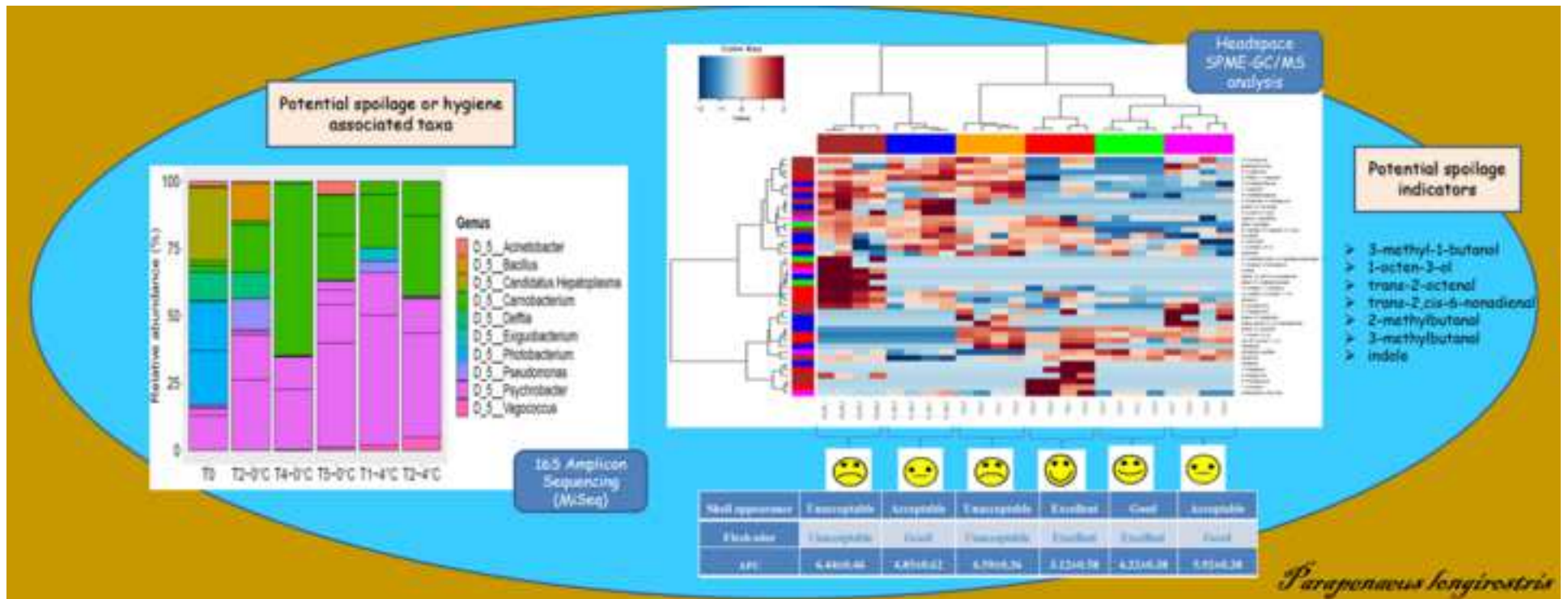




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**Foteini F. Parlapani:** Conceptualization, Methodology, Formal analysis, Investigation, Writing-Review and Editing, Project administration

**Ilario Ferrocino:** Methodology, Formal analysis, Investigation, Writing-Review and Editing

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Volos, 05-10-2019

Dear Editor,

authors declare no competing interests.

Sincerely Yours,

Dr Foteini F. Parlapani