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## ► To cite this version:

Thomas Brauge, Erwan Bourdonnais, Sylvain Trigueros, Pierre Cresson, Sabine Debuiche, et al.. Antimicrobial resistance and geographical distribution of *Staphylococcus* sp. isolated from whiting (*Merlangius merlangus*) and seawater in the English Channel and the North sea. *Environmental Pollution*, 2024, 345, pp.123434. 10.1016/j.envpol.2024.123434 . hal-04423681

**HAL Id: hal-04423681**

**<https://hal.science/hal-04423681>**

Submitted on 29 Jan 2024

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1 **Antimicrobial resistance and geographical distribution of**  
2 ***Staphylococcus* sp. isolated from whiting (*Merlangius***  
3 ***merlangus*) and seawater in the English Channel and the**  
4 **North Sea**

5

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8

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19

20 **Keywords: Antimicrobial resistance, marine environment, *Staphylococcus*,**  
21 **environmental parameters.**

22

23 **Abstract**

24

25 *Staphylococcus* is a significant food safety hazard. The marine environment serves as a source  
26 of food for humans and is subject to various human-induced discharges, which may contain  
27 *Staphylococcus* strains associated with antimicrobial resistance (AMR). The aim of this study  
28 was to assess the occurrence and geographical distribution of AMR *Staphylococcus* isolates in  
29 seawater and whiting (*Merlangius merlangus*) samples collected from the English Channel  
30 and the North Sea. We isolated and identified 238 *Staphylococcus* strains, including 12  
31 coagulase-positive (CoPs) and 226 coagulase-negative (CoNs) strains. All CoPs isolates

32 exhibited resistance to at least one of the 16 antibiotics tested. Among the CoNs strains, 52%  
33 demonstrated resistance to at least one antibiotic, and 7 isolates were classified as multi-drug  
34 resistant (MDR). In these MDR strains, we identified AMR genes that confirmed the  
35 resistance phenotype, as well as other AMR genes, such as quaternary ammonium resistance.  
36 One CoNS strain carried 9 AMR genes, including both antibiotic and biocide resistance  
37 genes. By mapping the AMR phenotypes, we demonstrated that rivers had a local influence,  
38 particularly near the English coast, on the occurrence of AMR *Staphylococcus*. The analysis  
39 of marine environmental parameters revealed that turbidity and phosphate concentration were  
40 implicated in the occurrence of AMR *Staphylococcus*. Our findings underscore the crucial  
41 role of wild whiting and seawater in the dissemination of AMR *Staphylococcus* within the  
42 marine environment, thereby posing a risk to human health.

43

## 44 **1 Introduction**

45

46 Antimicrobial resistance (AMR) is a significant public health issue that can increase the  
47 lethality of pathogenic bacteria and lead to economic losses. In 2019, it was estimated that  
48 antibiotic-resistant bacteria were responsible for 1.27 million deaths in Europe alone (Norrby  
49 et al., 2009; Mestrovic et al., 2022). While the accuracy of this estimate is questioned by some  
50 authors, the clinical, economic, and public health burden associated with antimicrobial  
51 resistance is undeniable (O'Neill, 2016). Humans can be exposed to antibiotic-resistant  
52 bacteria through their diet or environment, which aligns with the One Health concept.  
53 *Staphylococcus* sp. is a bacterial agent that is particularly problematic in terms of AMR and is  
54 found in humans, animals, and the environment. Among the *Staphylococcus* genus,  
55 coagulase-positive (CoPS) *S. aureus* is the main pathogen responsible for a variety of clinical  
56 infections in humans, including bacteremia, endocarditis, and various invasive medical device  
57 infections (Tong et al., 2015). Coagulase-negative *Staphylococcus* (CoNS), especially  
58 *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, have also emerged as recurrent  
59 causative agents of nosocomial infections, particularly those associated with indwelling  
60 devices (Becker et al., 2014). The study of CoNS in the context of antimicrobial resistance  
61 and the safety of seafood products is relevant. Indeed, CoNS can act as reservoirs of  
62 antimicrobial resistance genes. Even though they are not the primary pathogens, they can  
63 carry resistance genes that may be transferred to other bacteria, including more virulent  
64 pathogens. This contributes to the dissemination of antimicrobial resistance (Fišarová et al.,

65 2019). Additionally, CoNS can share ecological niches with other bacteria, including  
66 foodborne pathogens. Investigating their presence and antimicrobial resistance can help  
67 understand the complex dynamics of microbial communities in aquatic environments, which  
68 is crucial for assessing potential risks to human health associated with the consumption of  
69 seafood products (Regecova et al., 2014; Chajęcka-Wierzchowska et al., 2015). Seafood can  
70 be contaminated by *Staphylococcus* in two ways: post-capture contamination through human  
71 contact or contaminated surfaces, and pre-capture contamination due to the presence of  
72 *Staphylococcus* in the marine environment. Several studies have highlighted the  
73 contamination of the marine environment with *Staphylococcus*, which is not indigenous to  
74 this environment but rather a consequence of anthropogenic discharges such as wastewater  
75 treatment plants, run-off water, and other discharges from terrestrial environments (Gabutti et  
76 al., 2000; Soge et al., 2009; Goodwin et al., 2012; Akanbi et al., 2017). These anthropogenic  
77 discharges also transport residues of drugs used in human or veterinary medicine, as well as  
78 antimicrobial resistance genes (ARGs), into the marine environment (Marti et al., 2014;  
79 Bourdonnais et al., 2022; Bourdonnais et al., 2023). Additionally, the genome of aquatic  
80 bacteria contains a high abundance of mobile genetic elements that frequently contribute to  
81 the selection and dissemination of ARGs (Stalder et al., 2012). Aquatic bacteria serve as  
82 potential intermediate vectors for the transfer of naturally occurring ARGs from  
83 environmental bacteria to human pathogenic or commensal bacteria, such as *Staphylococcus*.  
84 The presence of numerous AMR genes and the omnipresence of antibiotic residues in the  
85 marine environment, due to anthropization, could significantly contribute to the emergence of  
86 AMR *Staphylococcus*, which poses a threat to human health. Only a few studies have reported  
87 the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) in fish and fishery  
88 products in various regions of the world (Vaiyapuri et al., 2019).

89 Therefore, questions remain regarding the presence of AMR *Staphylococcus* in the marine  
90 environment, the influence of the environment on its occurrence, and the associated risk  
91 posed by AMR *Staphylococcus* in wild fish intended for human consumption, such as  
92 whiting. Whiting is a keystone fish species in the area, known for its high trophic position and  
93 predominant biomass in the fish assemblage (Cresson et al., 2020; Timmerman et al., 2020;  
94 Timmerman et al., 2021). To our knowledge, no data on this subject is available in the  
95 literature. To address this information gap, we investigated the occurrence and geographical  
96 distribution of AMR *Staphylococcus* in areas subject to heterogeneous human impacts and  
97 various environmental conditions. We collected seawater and whiting (*Merlangius*  
98 *merlangus*) samples from the English Channel and the North Sea, which are ideal locations

99 due to their numerous areas with high anthropogenic influences, particularly near the coasts  
100 and in regions with intense fishing activities and maritime traffic.

101

## 102 **2 Material and methods**

103

### 104 *2.1 Marine samples and preparation*

105

106 Forty-six sampling stations were defined in the English Channel and the North Sea area  
107 (Figure 1). At each sampling station, 10 whiting (*Merlangius merlangus*) and 300 mL of  
108 seawater were collected. These samples were collected during the International Bottom Trawl  
109 Survey (IBTS) in February 2017 on the R/V Thalassa (Verin, 2017). The IBTS is an  
110 ecosystemic survey, initially designed to estimate fish abundance to inform fisheries  
111 management indicators but which includes sampling of all ecosystem components. Protocols  
112 of the survey are currently being evaluated by Ifremer and are validated by the ICES IBTS  
113 Working Group (<https://www.ices.dk/community/groups/pages/ibtswg.aspx>). In addition,  
114 survey's PIs received training about animal well-being and ethics. The physico-chemical  
115 parameters at the water surface, including water temperature, pH, salinity, turbidity, oxygen  
116 saturation, as well as the concentrations of phosphate, nitrite, nitrate, silicate, ammonium,  
117 chlorophyll a, total phaeopigments, total suspended matter, organic, and inorganic suspended  
118 matter, were measured at each sampling station during sample collection.

119 The forty-six seawater samples were collected at a depth of about 30 meters using Niskin  
120 bottles and transferred in sterile containers. All seawater samples were filtered on 0.45 µm  
121 pore-size mixed cellulose ester membranes (Millipore, Burlington, USA) with a Nalgene  
122 individual vacuum filtration system (Thermo Fisher Scientific, Waltham, USA). The 0.45 µm  
123 filter membranes were placed in a tube containing 30 mL of alkaline peptone saline water  
124 (APSW, Oxoid, Basingstoke Hampshire, UK) with 20% (v/v) of glycerol and stored at -20°C  
125 on the ship. Back to the laboratory, the tubes containing the filters were thawed at room  
126 temperature and vortexed for 1 minute. The filter was removed using sterilized forceps. The  
127 tubes were centrifuged for 10 minutes at 5000 ×g and the pellet was remained in 4 mL of the  
128 supernatant. Two milliliters were analyzed to isolate the *Staphylococcus* strains.

129 A total of 460 fish were analyzed. After thawing the fish at room temperature, we swabbed  
130 both surfaces of each fish using a sterile sponge soaked with buffered peptone water (BPW)  
131 (3M, Cergy-Pontoise, France) to study fish skin. The homogenate was recovered and we

132 added 20% (v/v) of glycerol. The fish gills were extracted, diluted by half with BPW (v/m),  
133 mixed and supplemented with 20% (v/v) of glycerol. The fish skin and gill samples were  
134 pooled for each sampling station (10 individuals per sampling station) except for the V0116,  
135 V0117, V0127 and V0133 sample stations (the fish samples were analyzed individually).

136

## 137 2.2 Isolation and identification of bacterial isolates

138

139

140 Each sample was thawed and diluted at 1/10 in BPW and APSW enrichment broths. The  
141 broths were incubated at 30°C overnight and plated on total 3 selective and 2 non-selective  
142 media. The ASPW enrichment broths were plated on non-selective saline nutritive agar (SNA,  
143 2% NaCl) (Biorad, Marne-la-Coquette, France, Oxoid) then on the following selective agars:  
144 ChromID<sup>®</sup> MRSA (Biomérieux, Lyon, France), Chapman (Oxoid) and Thiosulfate Citrate  
145 Bile Saccharose (TCBS, Oxoid). Although the SNA is a non-selective medium, its salt  
146 content (2% NaCl) still influences the preferential growth of certain bacterial groups, thereby  
147 promoting the development of halophilic bacteria. This approach has allowed us to increase  
148 our chances of isolating halophilic bacteria such as staphylococci. The use of a non-selective  
149 saline nutrient agar has facilitated the growth of a diverse range of bacteria without  
150 discrimination, thus optimizing the recovery of microorganisms present in the sample,  
151 including those in low abundance. The BPW enrichment broths were plated on non-selective  
152 Trypticase Soy Agar with 0.6% Yeast extract (TSAYe, Oxoid) and on selective Polymyxin  
153 pyruvate Egg yolk Mannitol Bromothymol blue Agar (PEMBA, Oxoid). PEMBA were  
154 incubated at 30°C for 48h and TSAYE, TCBS, Chapman and ChromID<sup>®</sup> MRSA agars at  
155 37°C for 24h. After incubation, one colony of different morphologies of each medium was  
156 plated on a non-selective agar (TSAYe for the BPW enrichments and SNA for the ASPW  
157 enrichments) and incubated for 24 hours at the same temperature of the bacterial isolation  
158 agars. The bacterial isolates obtained were stored in brain heart infusion (BHI) (Biomérieux)  
159 supplemented with 20% (v/v) of glycerol at -80°C. The strains were then identified by 16  
160 rRNA gene sequencing and MALDI TOF spectroscopy according to the protocols described  
161 by (Brauge et al., 2021).

162

## 163 2.3 Antimicrobial susceptibility test of the Staphylococcus isolates

164

165 *Staphylococcus* isolates from BHI supplemented with 20% (v/v) of glycerol at -80°C were  
166 grown on SNA or TSA Ye for 24h at 37°C. Antimicrobial susceptibility was tested by disk  
167 diffusion following the EUCAST standard (EUCAST, 2012). A 0,5Mc Farland  
168 staphylococcal suspension was plated with a cotton swab on a Muller Hinton Agar (MHA,  
169 Biorad, Marnes La Coquette, France) and the plates were incubated at 35°C for 16-18h. The  
170 antibiotic disks (Biorad) tested were: penicillin G (1 U), cefoxitin (30 µg), ciprofloxacin (5  
171 µg), kanamycin (30 µg), tobramycin (10 µg), gentamycin (10 µg), erythromycin (15 µg),  
172 clindamycin (2 µg), vancomycin (5 µg), linezolid (10 µg), sulfamide (200 µg), trimethoprim  
173 (5 µg), tetracycline (30 µg), chloramphenicol (30 µg), rifampicin (5 µg) and fusidic acid (10  
174 µg). Cefoxitin was used to detect methicillin resistance and the clindamycin disk was  
175 positioned next to the erythromycin disk to visualize MLS<sub>B</sub> mechanism. Zone diameters were  
176 measured with a Biomic V3 (Giles Scientific, Santa Barbara, USA). The *Staphylococcus*  
177 ATCC 25923 strain was used as a quality control.

178

#### 179 2.4 Whole genome sequencing of multi-drug resistant strains

180

181

182 Among all the strains identified as resistant, we specifically targeted and sequenced in wole  
183 genome the strains of *Staphylococcus* classified as multi-drug resistant (MDR). The  
184 designation of MDR among the *Staphylococcus* strains was applied when these strains  
185 exhibited phenotypic resistance to at least three drugs belonging to three different antibiotic  
186 classes (Gandolfi-Decristophoris et al., 2013). This categorization denoted a comprehensive  
187 resistance profile, indicating the ability to withstand the effects of multiple antimicrobial  
188 agents. Following the classification as MDR, the *Staphylococcus* strains were cultured on  
189 Mueller-Hinton Agar (MHA) media. The genomic DNA was then extracted from few  
190 colonies using the DNeasy<sup>®</sup> Blood & Tissue Kit (Qiagen, Hilden, Germany) following  
191 manufacturer's protocol for Gram positive strains. DNA quality control and sequencing was  
192 carried out by Genoscreen society (Lille, France). The ARGs were identified with the  
193 Resfinder database from the CGE server (<http://genomicepidemiology.org/>, last access March  
194 22<sup>nd</sup> 2023). The presence of the ARGs was validated when it had a 100% percent identity.

195

#### 196 2.5 Data analysis and statistics

197

198 Antibiotic susceptibility test results were analyzed to define the phenotypic resistances  
199 according to the clinical thresholds established in the CLSI (CLSI, 2015, 2016) and EUCAST  
200 (EUCAST, 2018) guidelines and according to (Briet, 2018). All data analysis were performed  
201 as described in our previous study (Bourdonnais et al., 2022), using RStudio Software version  
202 1.4.1717 (RStudio, Inc, Boston, USA). The occurrence of the antimicrobial resistance  
203 phenotypes was plotted on a map, using the leaflet and ggplot2 packages of the software.  
204 Correlations between the environmental factors and the number of *Staphylococcus* strains  
205 with phenotypic resistances were determined with Pearson's rank correlation coefficient and  
206 plotted using the corrplot package. Statistical analysis of correlations (*p*-values) was  
207 performed with a significance test. Only the Pearson correlation coefficients with a significant  
208 *p*-value ( $p < 0.05$ ) were considered.

209

### 210 **3 Results**

211

212 Among the 1,742 bacteria isolated from seawater and whiting samples, we identified  
213 238 isolates as belonging to the *Staphylococcus* genus at the species level. Twelve strains of  
214 *Staphylococcus* were coagulase positive (CoPS) and were identified as *S. aureus* (Table 1).  
215 Four of these strains were isolated from seawater, four from fish skin and four from fish gills  
216 samples. We observed that 100% of the CoPS were resistant to penicillin G. We identified  
217 three CoPS strains from fish gill samples that exhibited resistance to both erythromycin and  
218 fusidic acid, representing a resistance prevalence of 25%. In comparison, erythromycin  
219 resistance has been observed at a prevalence of approximately 30% in *S. aureus* strains  
220 isolated from fish imported from Egypt, India, Yemen, and Turkey (Ertas Onmaz et al., 2015;  
221 Obaidat et al., 2015). Fusidic acid resistance was also noted in 22% of *S. aureus* strains  
222 isolated from raw shrimp in China (Dai et al., 2023). Additionally, the induction of  
223 clindamycin resistance by erythromycin was observed in one CoPS strain (B3PA-RCPE17-  
224 62à71PEPE1), indicating a MLSB phenotype. This particular strain was isolated from a fish  
225 skin sample.

226

227 **Table 1: Antibiotic resistance phenotypes of coagulase-positive *Staphylococcus* (CoPS, n**  
228 **= 12).** R: resistant; S: susceptible. CFOX: cefoxitin; CLND: clindamycin; CLOR:  
229 chloramphenicol; CPRX: ciprofloxacin; EARTH: erythromycin; FSAD: fusidic acid; GENT:  
230 gentamycin; KANA: kanamycin; LNZD: linezolid; PENG: penicillin G; RFPN: rifampicin;



231 SULF: sulfamide; TETR: tetracycline; TOBR: tobramycin; TRIM: trimethoprim; VNCO:  
232 vancomycin.

233

234 Two hundred and twenty six coagulase-negative *Staphylococcus* (CoNS) were isolated  
235 (Table 2) with 46 strains isolated from seawater, 63 strains from fish skin and 117 strains  
236 from fish gills. The predominantly identified CoNS *Staphylococcus* species were *S. pasteurii*  
237 (n = 89) and *S. warneri* (n = 85) followed by *S. epidermidis* (n = 19), *S. equorum* (n = 13), *S.*  
238 *haemolyticus* (n = 9), *S. saprophyticus* (n = 5), *S. hominis* (n = 4) and *S. cohnii* (n = 2). We  
239 noticed that 52.2% of these strains had phenotypic resistance to at least one antibiotic. All  
240 samples combined (fish skin, gills and seawater), the highest number of resistance was  
241 associated with erythromycin (40.7% of the CoNS), followed by fusidic acid (12.4% of the  
242 CoNS) and tetracycline (10.2% of the CoNS). Resistances were detected to tobramycin in  
243 3.5%, to cefoxitin in 2.2% and to kanamycin in 0.4% of the CoNS. No phenotypic resistance  
244 to ten other antibiotics tested was observed for these strains and there was no specific  
245 resistance for the type of samples analyzed. We identified 7 MDR strains among the CoNS.  
246 Two strains isolated from seawater had three phenotypic resistances, one (*S. hominis* B3PA-  
247 RCPE-6-FE-AL1) to fusidic acid, cefoxitin, tobramycin and the other (*S. pasteurii* B3PA-  
248 RCPE-24FEPE1) to erythromycin, fusidic acid, tobramycin. Another CoNS strain (*S. warneri*  
249 B3PA-RCPE-17-7280OUE(2)) with all three resistances to erythromycin, fusidic acid and  
250 tetracycline was isolated from fish gills. We also observed resistance to 4 antibiotics  
251 (erythromycin, fusidic acid, tobramycin and tetracycline) for 2 strains of *S. hominis* (B3PA-  
252 RCPE-17-418OUE(3) and B3PA-RCPE-17-424OUE(1)) isolated from fish gills and one  
253 strain of *S. warneri* (B3PA-RCPE-14FEPE1) isolated from seawater. Finally, one strain of *S.*  
254 *haemolyticus* B3PA-RCPE2FEMRSA1 resistant to 5 antibiotics (erythromycin, fusidic acid,  
255 cefoxitin, tobramycin and kanamycin) was also isolated from seawater. In order to assess the  
256 genetic basis of these phenotypic resistances, we sequenced the total genome of the seven  
257 MDR strains and identified the resistance genes present in their genomes with 100% identity  
258 (Table 3). Ten antibiotic resistance genes confirming phenotypic resistance results were  
259 identified in the MDR *Staphylococcus* strains. These genes were consistent with the observed  
260 phenotypic resistances: the *fusC* (4 strains) and *fusB* (3 strains) genes coding for fusidic acid  
261 resistance, *mecA* (2 strains) for cefoxitin resistance, *aadD* (5 strains) for tobramycin  
262 resistance, *mphC* (3 strains), *msrA* (3 strains) and *ermC* (1 strain) for erythromycin resistance,  
263 *tet(K)* (4 strains) for tetracycline resistance and *aph(3')-III* (1 strain) for kanamycin resistance.  
264 Moreover other resistance genes were also identified: *blaZ* gene encoding penicillin resistance

265 through the production of a  $\beta$ -lactamase in all the MDR strains, *bleO* gene (bleomycin  
266 resistance) in 6 MDR strains and the *fosD* gene (fosfomycin resistance) in the *S. warneri*  
267 B3PA-RCPE-14FEPE1 strain isolated from seawater. Other resistance genes were also  
268 identified in the *S. haemolyticus* B3PA-RCPE2FEMRSA1 strain isolated from seawater such  
269 as the *vgaA* gene coding for resistance to streptogramin A and relative compounds as well as  
270 the *qacA* gene involved in resistance to quaternary ammoniums. This latest strain carried 9  
271 antimicrobial genes. And, the induction of resistance to clindamycin by erythromycin was  
272 observed for three CoNS strain (*S. haemolyticus* B3PA-RCPE2FEMRSA1 isolated in  
273 seawater sample at the V0003 station, *S. epidermidis* B3PA-RCPE17-72à80OUTSA(2)  
274 isolated in seawater sample at the V00039 station and *S. warneri* B3PA-RCPE17-  
275 134à144PECH(1)) isolated in seawater sample at the V0060 station), which is a marker of a  
276 phenotype MLS<sub>B</sub>. For the MDR *S. haemolyticus* B3PA-RCPE2FEMRSA1 strain, we  
277 identified the *ermC* gene.

278

279 **Table 2: Antibiotic resistance phenotypes of coagulase-negative *Staphylococcus* (CoNS, n**  
280 **= 226).** R: resistant; S: susceptible. CFOX: cefoxitin; CLND: clindamycin; CLOR:  
281 chloramphenicol; CPRX: ciprofloxacin; EARTH: erythromycin; FSAD: fusidic acid; GENT:  
282 gentamycin; KANA: kanamycin; LNZD: linezolid; PENG: penicillin G; RFPN: rifampicin;  
283 SULF: sulfamide; TETR: tetracycline; TOBR: tobramycin; TRIM: trimethoprim; VNCO:  
284 vancomycin.

285

286 **Table 3: Phenotypic and genotypic resistances of multidrug resistant strains of**  
287 ***Staphylococcus*.** FSAD: fusidic acid; CFOX: cefoxitin; TOBR: tobramycin; EARTH:  
288 erythromycin; TETR: tetracycline; KANA: kanamycin.

289

290 The geographical distribution of all antimicrobial resistant CoPS and CoNS strains  
291 was investigated (Figure 2). Regarding the number of isolated strains, a significantly higher  
292 number of AMR *Staphylococcus* strains were located at the sampling stations near the  
293 Thames, Humber and Rhine estuaries. Erythromycin-resistant strains were widely distributed  
294 in the English Channel and the North Sea extending from coastal to offshore stations without  
295 distinction. Similar observations have been made for fusidic acid resistance in these seas.  
296 Penicillin G and cefoxitin-resistant strains were isolated from the samples collected near the  
297 French and English coasts, especially off the Humber estuary and the mouth of the Thames.  
298 The kanamycin-resistant *Staphylococcus* strain was located between the French and English

299 coasts in the English Channel. Tobramycin and tetracycline resistant-strains were mostly  
300 isolated from the samples collected along the English coast, near the Thames and Humber  
301 estuaries, but also in the open sea in the Northern North Sea. We identified a tetracycline-  
302 resistant strain along the Dutch coast close to the port of Rotterdam and the Rhine estuary. It  
303 is clear that the diversity of antimicrobial resistance was more important at stations located  
304 near the French and English coasts, more precisely in estuaries of the Thames, Humber and  
305 Seine. In contrast, few antimicrobial resistance diversity was observed along the German and  
306 Dutch coasts. Moreover, the MDR strains of *Staphylococcus* were isolated from the samples  
307 collected in the middle of the English Channel (sampling station V0003), at the mouth of the  
308 Thames (V0029 and V0133), off the Humbert estuary (V0039 and V0052) but also from the  
309 northern coast of the Netherlands (V0082).

310 In order to evaluate the potential relationships between the occurrence of the  
311 phenotypic resistance of *Staphylococcus* and the environmental parameters, we performed a  
312 correlation analysis (Figure 3). For all the nature of samples (seawater and fish), we observed  
313 that the number of tobramycin-resistant strains was moderately and positively correlated with  
314 the number of strains with a resistance to erythromycin, fusidic acid and ceftiofur. The  
315 number of tetracycline-resistant strains was positively correlated with the number of strains  
316 resistant to erythromycin and fusidic acid as well as the turbidity, the phosphate concentration  
317 and the inorganic suspended matter (ISMP). Regarding the number of kanamycin-resistant  
318 strains, we observed a positive correlation with the number of ceftiofur and gentamicin-  
319 resistant strains. Finally, a positive correlation was also observed between the number of  
320 strains resistant to penicillin G and fusidic acid.

321

## 322 **4 Discussion**

323

324 In this study, we investigated the occurrence and geographical distribution of AMR  
325 *Staphylococcus* in whiting (*Merlangius merlangus*), a widely harvested and consumed wild  
326 fish, as well as in seawater samples from the English Channel and the North Sea. These areas  
327 experience significant anthropogenic pressures, both directly and through river discharges  
328 (Tappin and Millward, 2015; Schöneich-Argent et al., 2020; Bourdonnais et al., 2022;  
329 Bourdonnais et al., 2023; Mauffret et al., 2023). Initially, we isolated and identified 238  
330 *Staphylococcus* strains before assessing their resistance to 16 antibiotics crucial for human  
331 health, using the disk diffusion method following the EUCAST guidelines. Methicillin-

332 resistant *Staphylococcus* represents a major concern, as these strains are typically resistant not  
333 only to all  $\beta$ -lactams but also to a wide range of other antibiotics. The prevalence of  
334 methicillin-resistant *Staphylococcus aureus* (MRSA) varies significantly across institutions  
335 and geographical regions. In Europe, a North-South gradient is generally observed, with  
336 MRSA strains being less common in Scandinavian hospitals (<2%) compared to  
337 Mediterranean ones (>40%) (Stefani and Varaldo, 2003). Our study conducted in northern  
338 waters seems to support this trend, as no MRSA was detected. Specifically, the cefoxitin  
339 phenotypic resistance was not observed in the 12 *S. aureus* (CoPS) isolates in our samples,  
340 and all isolates were resistant to penicillin G, which aligns with previous observations in  
341 beach sand and bathing seawater samples Akanbi et al. (2017). It has been reported by Daurel  
342 and Leclercq (2008) that over 80% of *S. aureus* produce a penicillinase enzyme. We observed  
343 the  $MLS_B$  phenotype in one CoPS strain and three CoNS strains. In the MDR CoNS strains,  
344 we detected the presence of the *ermC* gene, which confirmed the underlying phenotypic  
345 mechanism in the *S. haemolyticus* B3PA-RCPE2FEMRSA1 strain. The *ermC* gene that we  
346 identified in the MDR *S. haemolyticus* strain has previously been detected in marine  
347 sediments collected in the Arctic region, but its presence has been associated with human  
348 activities (Tan et al., 2018).

349  
350 Furthermore, only 2.2% of the CoNS strains (2 *S. cohnii*, 1 *S. haemolyticus*, 1 *S. hominis*  
351 isolated from seawater samples, and 1 *S. saprophyticus* isolated from fish gills) showed  
352 resistance to cefoxitin, indicating methicillin resistance. These values are lower than those  
353 reported in other studies. The prevalence of MRSA in fish and fishery products varied  
354 between 1% and 60%, depending on the sample type and origin (Vaiyapuri et al., 2019) and  
355 1.0% of wild freshwater fish samples in Germany were found to be positive for MRSA  
356 (EFSA and ECDC, 2023). In the case of the MDR *S. haemolyticus* B3PA-RCPE2FEMRSA1  
357 and *S. hominis* B3PA-RCPE-6-FE-AL1 strains isolated from seawater samples, we identified  
358 the *mecA* gene, which is responsible for methicillin resistance and can be carried by the  
359 mobile chromosomal cassette *SCCmec*, as described by Gomez et al. (2017). *S.*  
360 *hamemolyticus* is associated with nosocomial infections such as bacteremia and infective  
361 endocarditis. Methicillin-resistant *Staphylococcus* strains have previously been isolated from  
362 mackerel, salmon, tuna, flounder, and yellowtail sashimi (Hammad et al., 2012). We also  
363 identified one *S. saprophyticus* strain showing phenotypic resistance to cefoxitin. This species  
364 is commonly implicated in certain types of CoNS clinical infections, such as skin and urinary  
365 tract infections (Silva et al., 2020). Similar to previous studies that identified the *mecA* and

366 *aph(3')-III* genes in marine *S. aureus* strains and river waters (Levin-Edens et al., 2012;  
367 Paulus et al., 2020), we observed that the *S. haemolyticus* B3PA-RCPE2FEMRSA1 strain  
368 also carried these two antimicrobial resistance genes. However, we did not observe  
369 phenotypic penicillin resistance in any of the 7 MDR CoNS strains, but we identified the  
370 presence of the *blaZ* gene for further whole-genome sequencing analysis. Similarly, Pimenta  
371 et al. (2023) demonstrated that a species of *S. haemolyticus* isolated from a clinical sample  
372 harbored the *blaZ* genes and *femA* plasmid but showed a negative result for phenotypic  
373 resistance to cefoxitin and penicillin G. Two hypotheses could explain this result: the first is  
374 that *blaZ* might not be expressed due to genetic mutations in the regulatory regions of the  
375 gene. Indeed, the *blaZ* gene is regulated by two other genes, the *blaRI* antirepressor and the  
376 *blal* repressor. Following exposure to beta-lactams, *blaRI*, a transmembrane sensor-  
377 transducer, undergoes autocatalytic cleavage, promoting the cleavage of the repressor gene,  
378 *blal*, thereby allowing transcription of *blaZ*. A mutation in these regions could affect the  
379 expression of *blaZ* and thus the expression of phenotypic resistance (El Feghaly et al., 2012;  
380 Pimenta et al., 2023). Another hypothesis could be that penicillin resistance might not be  
381 consistently detected using commonly employed susceptibility testing methods. In support of  
382 this, El Feghaly et al. (2012) demonstrated that 9.5% of clinically susceptible *S. aureus*  
383 isolates to penicillin G carried the *blaZ* gene. They proposed that enhancing interpretation  
384 criteria for the disc diffusion zone size from 29 to 35 mm could improve the sensitivity of  
385 phenotypic penicillin resistance testing. Furthermore, our investigation revealed the presence  
386 of the *bleO* gene in 6 out of the 7 MDR CoNS strains, indicating resistance to bleomycin.  
387 Numerous studies have shown that over 80% of CoPS strains resistant to aminoglycosides  
388 also exhibit resistance to bleomycin.

389

390 Regarding the CoNS isolates, we identified resistance to erythromycin (40.7%), fusidic  
391 acid (12.4%), tetracycline (10.2%), tobramycin (3.5%), cefoxitin (2.2%) and kanamycin  
392 (0.4%) which were lower than those reported in clinical settings (Archer and Climo, 1994;  
393 Schmitz et al., 1999; Schreckenberger et al., 2004; Koksai et al., 2009). It is widely accepted  
394 that higher levels of antibiotic resistance in clinical isolates are due to constant exposure to  
395 antibiotics compared to the marine environment. The open marine environment allows for  
396 dilution and dispersion of antibiotic residues and antimicrobial resistance (Stefani and  
397 Varaldo, 2003). However, the environment can also contribute to the development of  
398 antibiotic resistance in microorganisms due to human/animal therapeutics, wastewater,  
399 agriculture, and industrial use of antibiotics (Larsson and Flach, 2021). Bourdonnais et al.

400 (2022) highlighted the presence of the *sul1* and *int11* genes in seawater from the English  
401 Channel and the North Sea with concentrations of up to 3.5 log gene copies / L of seawater.  
402 Marine environment is thus a reservoir of antimicrobial resistance, notably as it is subjected to  
403 anthropogenic discharges. Therefore, the wide distribution of AMR *Staphylococcus* in the  
404 marine environment can be considered as a source of concern. Two hundred and thirty eight  
405 strains of *Staphylococcus* were isolated from the samples collected in the English Channel  
406 and the North Sea, and 54.6% showed at least one antibiotic resistance with 3% of multi-  
407 resistant strains. We also revealed that the number of resistant *Staphylococcus* and the  
408 diversity of resistance associated with them varied between the different sampling stations in  
409 the English Channel and the North Sea. The highest prevalence of antibiotic resistance was  
410 found along the English coast. Furthermore, the majority of MDR *Staphylococcus* was  
411 isolated in these areas (6/7 strains). Several hypotheses can be put forward to explain this  
412 occurrence. The first would be the anthropogenic impact associated to river discharges from  
413 the Thames and the Humber effluents. These two rivers are the largest sources of freshwater  
414 that England discharges into the North Sea. Previous observations have shown the presence of  
415 numerous antimicrobial residues (erythromycin, clarithromycin and azithromycin)  
416 downstream of the Thames source (White et al., 2019). In addition, Xu et al. (2019) quantified  
417 the antimicrobial resistance genes (ARGs) *bla*<sub>TEM</sub>, *tetA*, *sul1*, *sul2*, *ermB*, *tetQ*, *tet(X)*, *drfA1*,  
418 *drfA2* and *int11* in these waters with abundances ranging from 10<sup>5</sup> to 10<sup>8</sup> copies / L. We did  
419 not identify these genes in the genomic DNA of the MDR *Staphylococcus* strains isolated  
420 from the fish and seawater samples collected near these two areas. To our knowledge, this  
421 was the first time that the *mphC*, *aadD*, *fusC* and *fusB* genes were identified in the marine  
422 environment. We identified the *tetK* gene in MDR CoNS 4 strains isolated in fish gills. In  
423 Chile, the *tetK* resistance gene was identified in *Pseudoalteromonas*, *Shewanella*, *Cobetia* and  
424 *Vibrio* strains isolated from sediment and water samples from an aquaculture site but also  
425 from the marine environment (Tomova et al., 2015). The prevalence of resistance to fusidic  
426 acid varies between 4% and 30% depending on the country (Briet, 2018). Resistance to this  
427 antibiotic is acquired either by mutation of the *fusA* target or by acquisition of a *fusB* plasmid  
428 determinant (Briet, 2018). In our study, the MDR *S. hominis* and *S. haemolyticus* strains  
429 carried the *fusC* gene and the MDR *S. warneri* and *S. pasteurii* strains carried the *fusB* gene  
430 that conferred the resistance at fusidic acid confirming by phenotypic and genotypic  
431 resistance. For one *S. warneri* strain, the *fusD* gene was described in more by genotypic  
432 method. In addition, we have identified the *qacA* gene in a *S. haemolyticus* B3PA-  
433 RCPE2FEMRSA1 strain isolated from the English Channel seawater. This gene codes for a

434 subunit of the QacA multidrug efflux pump conferring resistance to antimicrobial compounds  
435 such as quaternary ammoniums. This gene has been identified in several MDR *S.*  
436 *haemolyticus* strains isolated from dairy cows, a human with septicemia and also in a  
437 persistent strain from a veterinary clinic floor despite disinfection (Anthonisen et al., 2002).  
438 The *qacA* gene was located on a plasmid with the *blaZ* gene, coding for a  $\beta$ -lactamase and  
439 identified in the 7 MDR *Staphylococcus* strains in our study. Disinfectant resistant bacteria  
440 surviving disinfection thus represent a threat to the food industry and to the public health.  
441 This *S. haemolyticus* B3PA-RCPE2FEMRSA1 strain, isolated in seawater sample, carried 9  
442 ARGs (*ermC* (ERTH), *fusC* (FSAD), *mecA* (CFOX), *aadD* (TOBR), *aph(3')-III* (KANA),  
443 *blaZ* (penicillin), *bleO* (bleomycin), *vga(A)* (streptogramin A), *qacA* (quaternary  
444 ammoniums)) with resistance to antibiotics but also to disinfectant in food industry. The  
445 transfer of AMR genes between CoNS and *S. aureus* has been reported that CoNS may act as  
446 a AMR gene reservoir for CoPS due to their ability to exchange genetic material inter/intra  
447 species (Rossi et al., 2020).

448

449 Another study conducted in the estuarine waters of the Humber revealed that *Vibrio*  
450 *parahaemolyticus* strains were phenotypically resistant to various antibiotics such as  
451 kanamycin, gentamycin, cefazolin and tetracycline (Daramola et al., 2009), indicating a  
452 potential source of AMR in the Humber estuary. We can also postulate that this  
453 contamination by AMR could come from man-made structures in the English Channel and the  
454 North Sea. Indeed, various ports (Port of Hull, Green Port Hull, Immingham) and offshore  
455 installations (oil and gas platforms, wind farms) are present in these areas. These different  
456 areas can be a source of AMR contamination due to the human activities or to the marine  
457 animals concentration such as birds which are recognized as a vector of ARGs in the North  
458 sea (Vanermen et al., 2015). These could have an additional impact on the occurrence of  
459 resistance in the marine environment, but to our knowledge, no studies have been carried out  
460 on the impact of these artificial structures on the occurrence of antimicrobial resistance.

461

462 A high abundance of AMR *Staphylococcus* (especially to erythromycin) was also observed  
463 near the mouths of the Rhine and the Meuse, two large rivers flowing into the North Sea. This  
464 is in agreement with the observations of Blaak et al. (2012) who found several *Enterococcus*  
465 *faecium* strains resistant to erythromycin, tetracycline and ampicillin in the Meuse, Rhine and  
466 New Meuse rivers. These *E. faecium* strains may have been responsible for horizontal transfer  
467 of ARGs to *Staphylococcus* strains. More recently, Paulus et al. (2020) have demonstrated the

468 presence of resistance genes to erythromycin (*ermB* and *ermF*), sulfonamide (*sulI*),  
469 tetracycline (*tetM*), aminoglycosides (*aph(III)*) and carbapenem (*bla<sub>OXA</sub>*) along the entire  
470 length of the Rhine in Switzerland, Germany and the Netherlands due to potential pollution  
471 by pharmaceutical industries in the Lower Rhine. The impact of these river effluents on the  
472 pollution of the North Sea is cumulative with that of the city and harbor of Rotterdam, the  
473 largest harbor in Europe and therefore the source of much anthropogenic pollution. However,  
474 there is no information on the contamination of effluents from port activities with ARGs or  
475 antibiotic residues.

476

477 Few antibiotic resistances has been observed in the North Sea environment. This area, in  
478 contrast to the others, is quite far from the coast and thus from its influence, but has a  
479 concentration of offshore platforms for oil and gas exploitation (Fujii, 2015). This  
480 dissemination of AMR *Staphylococcus* within the marine environment may raise questions  
481 about the involvement of heavy maritime traffic and their various discharges. Indeed,  
482 previous studies have shown that ballast water from ships can contain antibiotic resistant  
483 bacteria and ARGs (Brinkmeyer, 2016; Lv et al., 2020).

484

485 A further consideration is the impact of environmental factors that may favor the spread  
486 and persistence of AMR *Staphylococcus*. Thus, in this study we looked at environmental  
487 factors related to phenotypic resistance of *Staphylococcus*. We showed that resistance to  
488 tobramycin and tetracycline was strongly correlated with turbidity and suspended matter.  
489 Both descriptors are proxies of organic matter and of aggregation of microorganisms.  
490 Similarly, Bourdonnais et al. (2022) observed that turbidity values were positively correlated  
491 with the abundance of the *sulI* and *intI1* genes, and with the abundance of the bacterial  
492 population in the North Sea and the English Channel seawaters. Chen et al. (2020) showed  
493 that the occurrence of ARGs in an estuary in China was correlated with turbidity with a higher  
494 prevalence of the ARGs in areas with high turbidity. Although not carried by the same strains,  
495 co-occurrence of penicillin G and cefoxitin resistance was observed in several strains isolated  
496 from samples along the English coast. A plausible explanation lies in the fact that  
497 *Staphylococcus* strains are exposed to similar environmental conditions or medical treatments  
498 along the English coast, potentially creating a common selective pressure that favors the  
499 development of resistance mechanisms to both penicillin G and cefoxitin. This situation may  
500 result from the frequent use of these antibiotics in the region. Indeed, the study by Curtis et al.  
501 (2019) clearly indicated that antibiotic prescription trends in the community in England from



1998 to 2016 were predominantly represented by penicillins, cephalosporins, and other beta-lactams. The potential presence of antibiotic residues in English effluents could exert selective pressure on *Staphylococcus* strains, prompting them to evolve and adapt to their local environment. If resistance to penicillin G and cefoxitin confers a selective advantage in this particular environment, these resistance characteristics can coexist in different strains. Several studies have identified the *blaZ* gene, encoding  $\beta$ -lactamase, as responsible for resistance to penicillin G and/or cefoxitin in *Staphylococcus* strains (Mok et al., 2023; Pimenta et al., 2023). Despite the absence of phenotypic resistance, genetic analysis of the 7 multidrug-resistant *Staphylococcus* strains in our study confirmed the significant presence of this gene. The lack of phenotypic results for beta-lactams despite the presence of the *blaZ* gene suggests that other, currently unknown, resistance genes may be responsible for resistance to cefoxitin and penicillin G. This aligns with the observations of Lienen et al. (2021), who demonstrated that 14 out of 15 non-golden *Staphylococcus* isolates lacking the *blaZ*, *mecA*, and *mecC* genes exhibited reduced sensitivity to several beta-lactam antibiotics, including cefoxitin (MIC 4 to 8 mg/L) and penicillin (MIC 0.25 to 0.5 mg/L). Two kanamycin-resistant strains were also resistant to gentamycin and tobramycin. Gentamycin is the most commonly active aminoglycoside on *Staphylococcus*. This resistance is mainly due to the acquisition of aminoglycoside inactivating enzymes which can be encoded by the *aadD* or *aph(3')-III* genes, two genetic determinants identified in this study by WGS approach and which also allow resistance to kanamycin and tobramycin (KTG phenotype).

522

## 523 **5 Conclusion**

524

This study provided, for the first time, information on the presence of AMR *Staphylococcus* in wild fish destined for human consumption and in seawater from the North Sea and the English Channel. Two hundred and thirty eight *Staphylococcus* strains were isolated from seawater and fish samples, of which 52.2% showed at least one antibiotic resistance and 2.9% were multi-resistant. Only 12 *Staphylococcus aureus* strains were identified. All of them were resistant to penicillin G. In contrast, 226 CoNS were isolated and 3% of these strains were resistant to cefoxitin. The next most common resistances found were to erythromycin (40.7%), fusidic acid (12.4%) and tetracycline (10.2%). We had observed the phenotype MLS<sub>B</sub> for 1 CoPS and 3 CoNS and the KTG phenotype for two CoNS. One strain, *S. haemolyticus* B3PA-RCPE2FEMRSA1 isolated in seawater sample, carried 9 ARGs (*ermC*

535 (ERTH), *fusC* (FSAD), *mecA* (CFOX), *aadD* (TOBR), *aph(3')-III* (KANA), *blaZ* (penicillin),  
536 *bleO* (bleomycin), *vga(A)* (streptogramin A), *qacA* (quaternary ammoniums)) with resistance  
537 to antibiotics but also to disinfectant in food industry. This isolate was a potential vector of  
538 ARGs in clinic and in food industry. The geographical distribution of AMR *Staphylococcus*  
539 clearly showed an influence of river and port discharges on the occurrence of these strains and  
540 the diversity of resistances, especially near the English coasts. This study showed for the first  
541 time the occurrence of AMR *Staphylococcus* in the North Sea and the English Channel, and  
542 the carriage by wild fish thus representing a health risk for consumers.

543

## 544 **6 Conflict of interests**

545

546 The authors declare that the research was conducted in the absence of any commercial or  
547 financial relationships that could be construed as a potential conflict of interest.

548

## 549 **7 Funding**

550

551 This study was supported by CPER MARCO and ECOANTIBIO

552

## 553 **8 Acknowledgments**

554

555 We would like to thank IFREMER, and the crew of R/V Thalassa, the PI and scientific staff  
556 of IBTS survey for their help for fish and seawater sampling, and for the provision of  
557 environmental parameters.

558

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560

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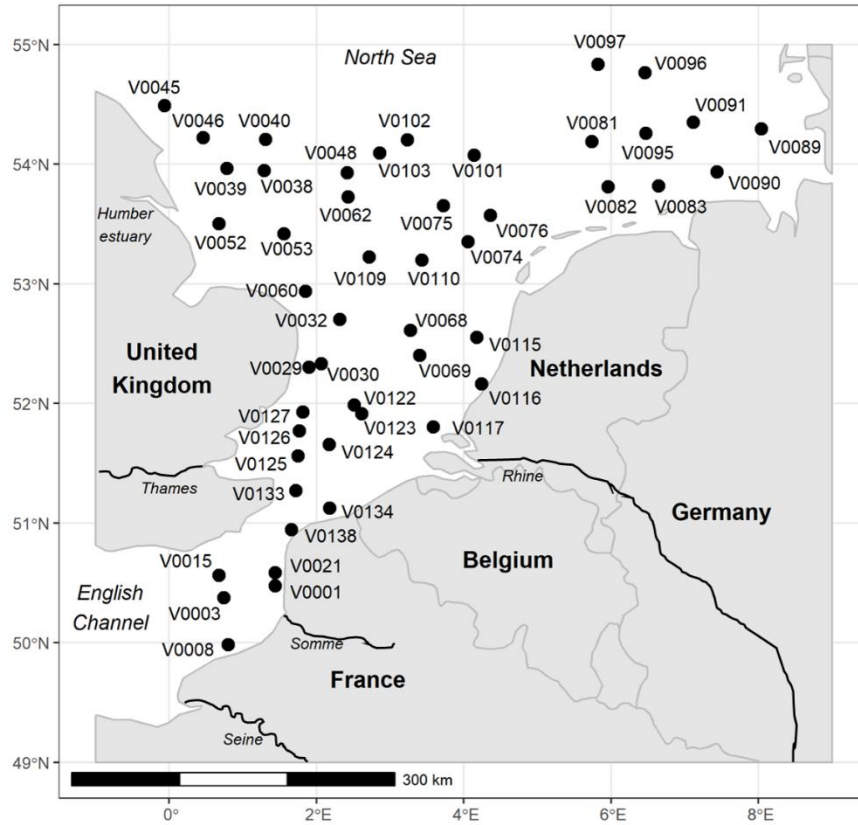
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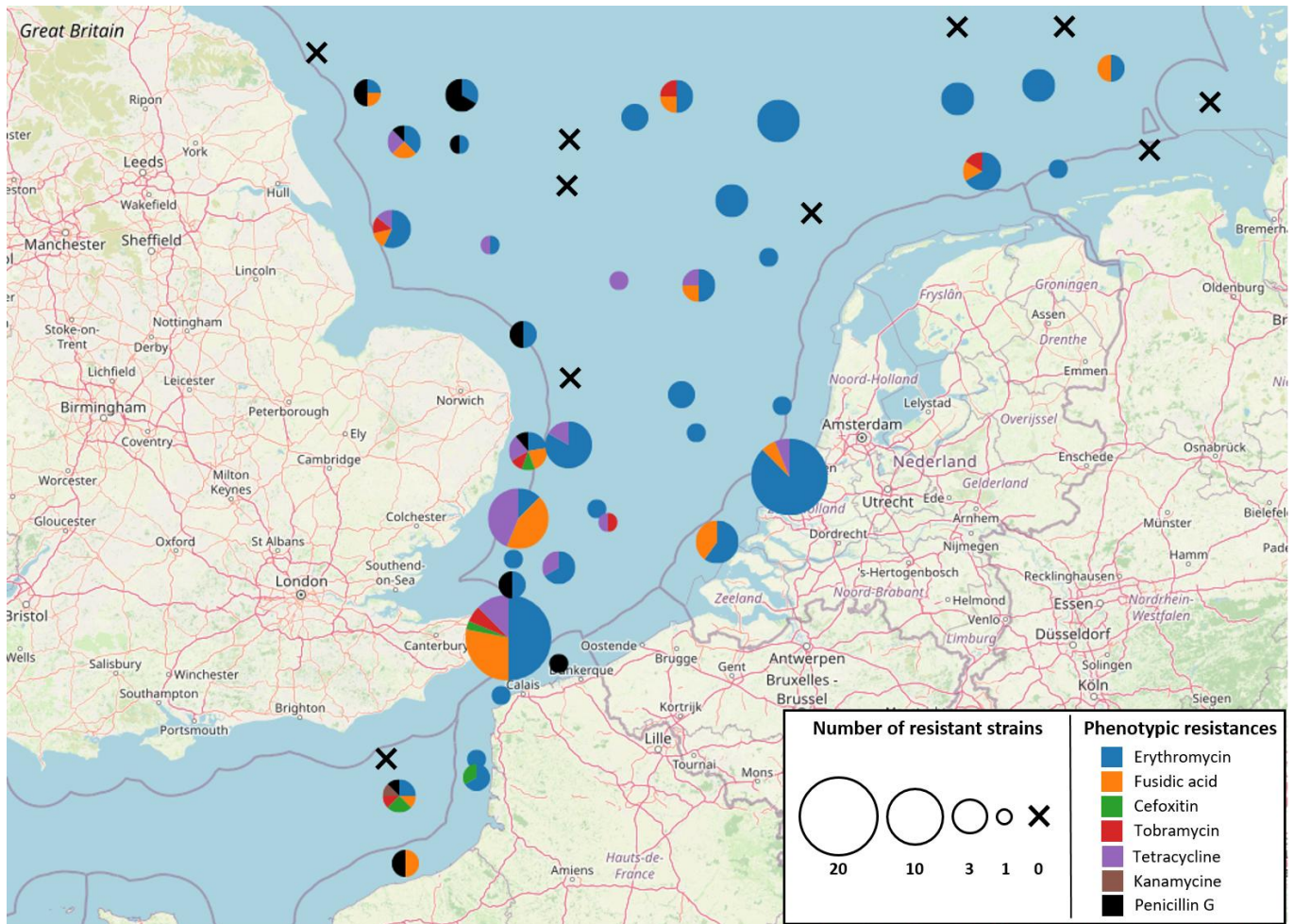
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772 Figure 1: Sampling stations in the English Channel and the North Sea.

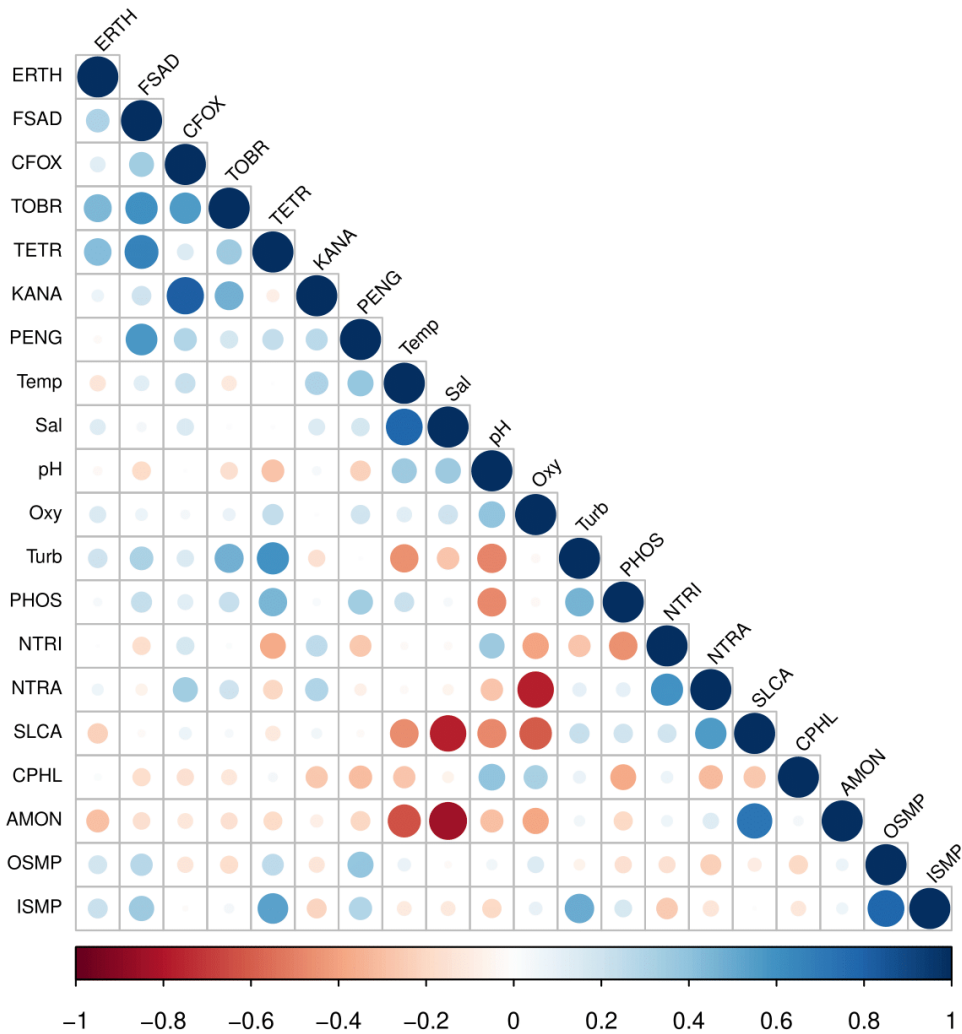


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774 Figure 2: Geographical distribution of antimicrobial resistant *Staphylococcus* strains (CoNS and  
 775 CoPS).

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778 Figure 3: Pearson correlation matrix between the phenotypic resistances of coagulase-negative and  
 779 coagulase-positive *Staphylococcus* strains and the environmental parameter values. ERTH:  
 780 erythromycin; FSAD: fusidic acid; CFOX: ceftiofur; TOBR: tobramycin; TETR: tetracycline; KANA:  
 781 kanamycin; PENG: penicillin G. Temp: temperature; Sal: salinity; Oxy: oxygen saturation; Turb:  
 782 turbidity; PHOS phosphate; NTRA: nitrate; NTRI: nitrite; SLCA: silicate; CPHL: chlorophyll a;  
 783 TPHP: total phaeopigments; AMON: ammonium; OSMP: organic suspended matter; ISMP:  
 784 inorganic suspended matter.

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