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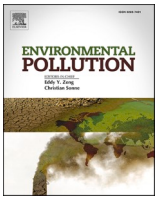

Antimicrobial resistance and geographical distribution of *Staphylococcus* sp. isolated from whiting (*Merlangius merlangus*) and seawater in the English Channel and the North sea☆

Thomas Brauge[a,*], Erwan Bourdonnais[a], Sylvain Trigueros[a], Pierre Cresson[b], Sabine Debuiche[a], Sophie A. Granier[c], Graziella Midelet[a]

[a] *French Agency for Food, Environmental and Occupational Health & Safety, Laboratory for Food Safety, Bacteriology and Parasitology of Fishery and Aquaculture Products Unit, Boulogne-sur-Mer, France*
[b] *Ifremer, Channel/North Sea Fisheries Laboratory, Boulogne-sur-Mer, France*
[c] *French Agency for Food, Environmental and Occupational Health & Safety, Fougères Laboratory, Fougères, France*

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ABSTRACT

Staphylococcus is a significant food safety hazard. The marine environment serves as a source of food for humans and is subject to various human-induced discharges, which may contain *Staphylococcus* strains associated with antimicrobial resistance (AMR). The aim of this study was to assess the occurrence and geographical distribution of AMR *Staphylococcus* isolates in seawater and whiting (*Merlangius merlangus*) samples collected from the English Channel and the North Sea. We isolated and identified 238 *Staphylococcus* strains, including 12 coagulase-positive (CoPs) and 226 coagulase-negative (CoNs) strains. All CoPs isolates exhibited resistance to at least one of the 16 antibiotics tested. Among the CoNs strains, 52% demonstrated resistance to at least one antibiotic, and 7 isolates were classified as multi-drug resistant (MDR). In these MDR strains, we identified AMR genes that confirmed the resistance phenotype, as well as other AMR genes, such as quaternary ammonium resistance. One CoNS strain carried 9 AMR genes, including both antibiotic and biocide resistance genes. By mapping the AMR phenotypes, we demonstrated that rivers had a local influence, particularly near the English coast, on the occurrence of AMR *Staphylococcus*. The analysis of marine environmental parameters revealed that turbidity and phosphate concentration were implicated in the occurrence of AMR *Staphylococcus*. Our findings underscore the crucial role of wild whiting and seawater in the dissemination of AMR *Staphylococcus* within the marine environment, thereby posing a risk to human health.

1. Introduction

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

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* Corresponding author.
E-mail address: thomas.brauge@anses.fr (T. Brauge).

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

Therefore, questions remain regarding the presence of AMR *Staphylococcus* in the marine environment, the influence of the environment on its occurrence, and the associated risk posed by AMR *Staphylococcus* in wild fish intended for human consumption, such as whiting. Whiting is a keystone fish species in the area, known for its high trophic position and predominant biomass in the fish assemblage (Cresson et al., 2020; Timmerman et al., 2020; Timmerman et al., 2021). To our knowledge, no data on this subject is available in the literature. To address this information gap, we investigated the occurrence and geographical distribution of AMR *Staphylococcus* in areas subject to heterogeneous human impacts and various environmental conditions. We collected seawater and whiting (*Merlangius merlangus*) samples from the English Channel and the North Sea, which are ideal locations due to their numerous areas with high anthropogenic influences, particularly near the coasts and in regions with intense fishing activities and maritime traffic.

2. Material and methods

2.1. Marine samples and preparation

Forty-six sampling stations were defined in the English Channel and the North Sea area (Fig. 1). At each sampling station, 10 whiting (*Merlangius merlangus*) and 300 mL of seawater were collected. These samples were collected during the International Bottom Trawl Survey (IBTS) in February 2017 on the R/V Thalassa (Verin, 2017). The IBTS is an ecosystemic survey, initially designed to estimate fish abundance to inform fisheries management indicators but which includes sampling of all ecosystem components. Protocols of the survey are currently being

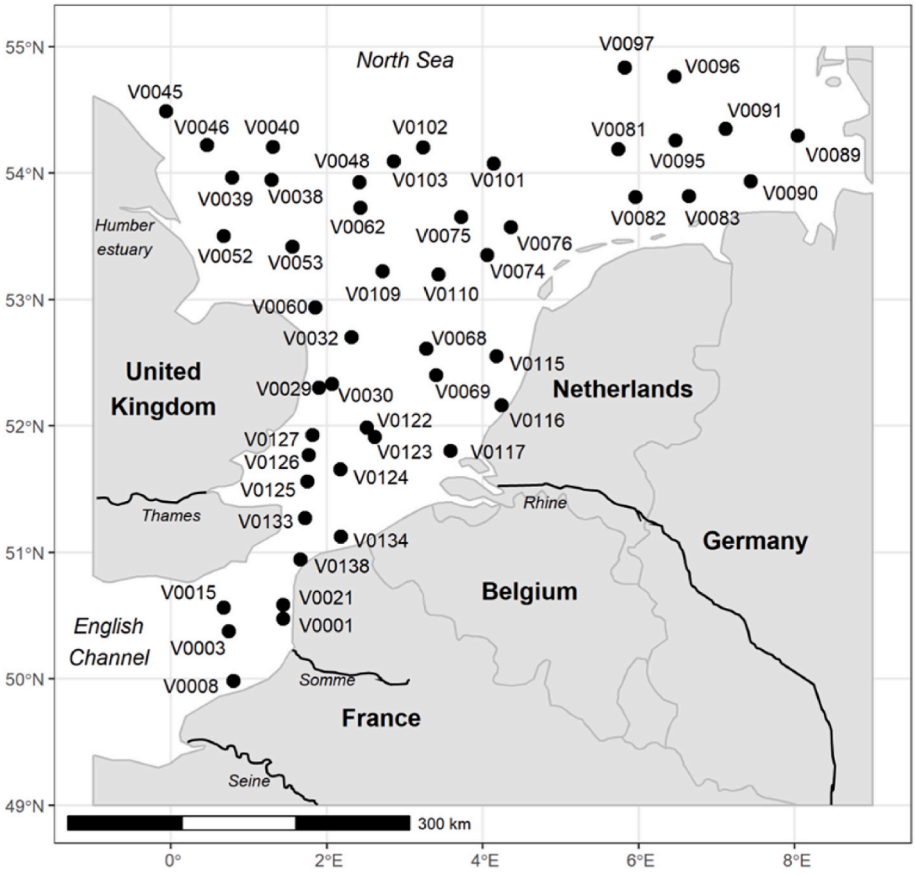

Fig. 1. Sampling stations in the English Channel and the North Sea.

Table 1
Antibiotic resistance phenotypes of coagulase-positive *Staphylococcus* (CoPS, n = 12).

		CFOX		CLND		CLOR		CPRX		ERTH		FSAD		GENT		KANA		LNZD		PENG		RFPN		SULF		TETR		TOBR		TRIM		VNCO	
Sample	Species	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Water	*S. aureus*	0	4	0	4	0	4	0	4	0	4	0	4	0	4	0	12	0	4	4	0	0	4	0	4	0	4	0	4	0	4	0	4
Gills	*S. aureus*	0	4	0	4	0	4	0	4	3	1	3	1	0	4	0	12	0	4	4	0	0	4	0	4	0	4	0	4	0	4	0	4
Skin	*S. aureus*	0	4	0	4	0	4	0	4	1	3	0	4	0	4	0	12	0	4	4	0	0	4	0	4	0	4	0	4	0	4	0	4
Total		0	12	0	12	0	12	0	12	4	8	3	9	0	12	0	12	0	12	12	0	0	12	0	12	0	12	0	12	0	12	0	12

R: resistant; S: susceptible. CFOX: cefoxitin; CLND: clindamycin; CLOR: chloramphenicol; CPRX: ciprofloxacin; ERTH: erythromycin; FSAD: fusidic acid; GENT: gentamycin; KANA: kanamycin; LNZD: linezolid; PENG: penicillin G; RFPN: rifampicin; SULF: sulfamide; TETR: tetracycline; TOBR: tobramycin; TRIM: trimethoprim; VNCO: vancomycin.

evaluated by Ifremer and are validated by the ICES IBTS Working Group (https://www.ices.dk/community/groups/pages/ibtswg.aspx). In addition, survey's PIs received training about animal well-being and ethics. The physico-chemical parameters at the water surface, including water temperature, pH, salinity, turbidity, oxygen saturation, as well as the concentrations of phosphate, nitrite, nitrate, silicate, ammonium, chlorophyll *a*, total phaeopigments, total suspended matter, organic, and inorganic suspended matter, were measured at each sampling station during sample collection.

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

A total of 460 fish were analyzed. After thawing the fish at room temperature, we swabbed both surfaces of each fish using a sterile sponge soaked with buffered peptone water (BPW) (3M, Cergy-Pontoise, France) to study fish skin. The homogenate was recovered and we added 20% (v/v) of glycerol. The fish gills were extracted, diluted by half with BPW (v/m), mixed and supplemented with 20% (v/v) of glycerol. The fish skin and gill samples were pooled for each sampling station (10 individuals per sampling station) except for the V0116, V0117, V0127 and V0133 sample stations (the fish samples were analyzed individually).

2.2. Isolation and identification of bacterial isolates

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

Table 2
Antibiotic resistance phenotypes of coagulase-negative *Staphylococcus* (CoNS, n = 226).

		CFOX		CLND		CLOR		CPRX		ERTH		FSAD		GENT		KANA		LNZD		PENG		RFPN		SULF		TETR		TOBR		TRIM		VNCO	
Sample	Species	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Water	*S. cohnii*	2	0	0	2	0	2	0	2	2	0	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2
	S. epidermidis	0	2	0	2	0	2	0	2	0	2	1	1	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2
	S. haemolyticus	1	7	0	8	0	8	0	8	8	0	1	7	0	8	1	7	0	8	0	8	0	8	0	8	0	8	1	7	0	8	0	8
	S. hominis	1	1	0	2	0	2	0	2	0	2	2	0	0	2	0	2	0	2	0	2	0	2	0	2	0	2	1	1	0	2	0	2
	S. pasteuri	0	7	0	7	0	7	0	7	7	0	1	6	0	7	0	7	0	7	0	7	0	7	0	7	0	7	1	6	0	7	0	7
	S. saprophyticus	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
	S. warneri	0	24	0	24	0	24	0	24	5	19	1	23	0	24	0	24	0	24	0	24	0	24	0	24	0	24	2	22	2	22	0	24	0	24
Subtotal		4	42	0	46	0	46	0	46	23	23	6	40	0	46	1	45	0	46	0	46	0	46	0	46	0	46	2	44	5	41	0	46	0	46
Gills	*S. epidermidis*	0	15	0	15	0	15	0	15	7	8	2	13	0	15	0	15	0	15	0	15	0	15	0	15	3	12	0	15	0	15	0	15
	S. equorum	0	2	0	2	0	2	0	2	1	1	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2
	S. haemolyticus	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
	S. hominis	0	2	0	2	0	2	0	2	2	0	2	0	0	2	0	2	0	2	0	2	0	2	0	2	2	0	2	0	0	2	0	2
	S. pasteuri	0	47	0	47	0	47	0	47	23	24	11	36	0	47	0	47	0	47	0	47	0	47	0	47	12	35	1	46	0	47	0	47
	S. saprophyticus	1	2	0	3	0	3	0	3	2	1	3	0	0	3	0	3	0	3	0	3	0	3	0	3	0	3	0	3	0	3	0	3
	S. warneri	0	47	0	47	0	47	0	47	10	37	2	45	0	47	0	47	0	47	0	47	0	47	0	47	1	46	0	47	0	47	0	47
Subtotal		1	116	0	117	0	117	0	117	45	72	20	97	0	117	0	117	0	117	0	117	0	117	0	117	18	99	3	114	0	117	0	117
Skin	*S. epidermidis*	0	2	0	2	0	2	0	2	2	0	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2
	S. equorum	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11	0	11
	S. pasteuri	0	35	0	35	0	35	0	35	21	14	2	33	0	35	0	35	0	35	0	35	0	35	0	35	3	32	0	35	0	35	0	35
	S. saprophyticus	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
	S. warneri	0	14	0	14	0	14	0	14	1	13	0	14	0	14	0	14	0	14	0	14	0	14	0	14	0	14	0	14	0	14	0	14
Subtotal		0	63	0	63	0	63	0	63	24	39	2	61	0	63	0	63	0	63	0	63	0	63	0	63	3	60	0	63	0	63	0	63
Total		5	221	0	226	0	226	0	226	92	134	28	198	0	226	1	225	0	226	0	226	0	226	0	226	23	203	8	218	0	226	0	226

R: resistant; S: susceptible. CFOX: cefoxitin; CLND: clindamycin; CLOR: chloramphenicol; CPRX: ciprofloxacin; ERTH: erythromycin; FSAD: fusidic acid; GENT: gentamycin; KANA: kanamycin; LNZD: linezolid; PENG: penicillin G; RFPN: rifampicin; SULF: sulfamide; TETR: tetracycline; TOBR: tobramycin; TRIM: trimethoprim; VNCO: vancomycin.

Table 3
Phenotypic and genotypic resistances of multidrug resistant strains of *Staphylococcus*.

Species Number identification	Sample/ Station	Phenotypic resistances	Genotypic resistances confirming phenotype results	Other genotypic resistance genes
S. hominis B3PA-RCPE-6-FE-AL1	Seawater (V0029)	FSAD, CFOX and TOBR	*fusC* (FSAD), *mecA* (CFOX), *aadD* (TOBR)	*blaZ* (penicillin), *bleO* (bleomycin)
S. hominis B3PA-RCPE-17-418OUPE(3)	Fish gills (V0133)	ERTH, FSAD, TOBR and TETR	*mphC/msrA* (ERTH), *fusC* (FSAD), *aadD* (TOBR), *tet(K)* (TETR)	*blaZ* (penicillin), *bleO* (bleomycin)
S. hominis B3PA-RCPE-17-424ouPE(1)	Fish gills (V0133)	ERTH, FSAD, TOBR and TETR	*mphC/msrA* (ERTH), *fusC* (FSAD), *aadD* (TOBR), *tet(K)* (TETR)	*blaZ* (penicillin), *bleO* (bleomycin)
S. warneri B3PA-RCPE-14FEPE1	Seawater (V0052)	ERTH, FSAD, TOBR and TETR	*mphC/msrA* (ERTH), *fusB* (FSAD), *aadD* (TOBR), *tet(K)* (TETR)	*blaZ* (penicillin), *bleO* (bleomycin), *fosD* (fosfomycin)
S. warneri B3PA-RCPE-17-72à80OUPE(2)	Fish gills (V0039)	ERTH, FSAD and TETR	*msrA* (ERTH), *fusB* (FSAD), *tet(K)* (TETR)	*blaZ* (penicillin)
S. haemolyticus B3PA-RCPE2FEMRSA1	Seawater (V0003)	ERTH, FSAD, CFOX, TOBR and KANA	*ermC* (ERTH), *fusC* (FSAD), *mecA* (CFOX), *aadD* (TOBR), *aph(3′)-III* (KANA),	*blaZ* (penicillin), *bleO* (bleomycin), *vga(A)* (streptogramin A), *qacA* (quaternary ammoniums)
S. pasteuri B3PA-RCPE-24FEPE1	Seawater (V0082)	ERTH, FSAD and TOBR	*msrA* (ERTH), *fusB* (FSAD), *aadD* (TOBR)	*blaZ* (penicillin), *bleO* (bleomycin)

FSAD: fusidic acid; CFOX: cefoxitin; TOBR: tobramycin; ERTH: erythromycin; TETR: tetracycline; KANA: kanamycin.

2.3. Antimicrobial susceptibility test of the Staphylococcus isolates

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

2.4. Whole genome sequencing of multi-drug resistant strains

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

2.5. Data analysis and statistics

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

3. Results

Among the 1742 bacteria isolated from seawater and whiting samples, we identified 238 isolates as belonging to the *Staphylococcus* genus at the species level. Twelve strains of *Staphylococcus* were coagulase positive (CoPS) and were identified as *S. aureus* (Table 1). Four of these strains were isolated from seawater, four from fish skin and four from fish gills samples. We observed that 100% of the CoPS were resistant to penicillin G. We identified three CoPS strains from fish gill samples that exhibited resistance to both erythromycin and fusidic acid, representing a resistance prevalence of 25%. In comparison, erythromycin resistance has been observed at a prevalence of approximately 30% in *S. aureus* strains isolated from fish imported from Egypt, India, Yemen, and Turkey (Ertas Onmaz et al., 2015; Obaidat et al., 2015). Fusidic acid resistance was also noted in 22% of *S. aureus* strains isolated from raw shrimp in China (Dai et al., 2023). Additionally, the induction of clindamycin resistance by erythromycin was observed in one CoPS strain (B3PA-RCPE17-62à71PEPE1), indicating a MLSB phenotype. This particular strain was isolated from a fish skin sample.

Two hundred and twenty six coagulase-negative *Staphylococcus* (CoNS) were isolated (Table 2) with 46 strains isolated from seawater, 63 strains from fish skin and 117 strains from fish gills. The predominantly identified CoNS *Staphylococcus* species were *S. pasteuri* (n = 89) and *S. warneri* (n = 85) followed by *S. epidermidis* (n = 19), *S. equorum* (n = 13), *S. haemolyticus* (n = 9), *S. saprophyticus* (n = 5), *S. hominis* (n = 4) and *S. cohnii* (n = 2). We noticed that 52.2% of these strains had phenotypic resistance to at least one antibiotic. All samples combined (fish skin, gills and seawater), the highest number of resistance was associated with erythromycin (40.7% of the CoNS), followed by fusidic acid (12.4% of the CoNS) and tetracycline (10.2% of the CoNS). Resistances were detected to tobramycin in 3.5%, to cefoxitin in 2.2% and to kanamycin in 0.4% of the CoNS. No phenotypic resistance to ten other antibiotics tested was observed for these strains and there was no

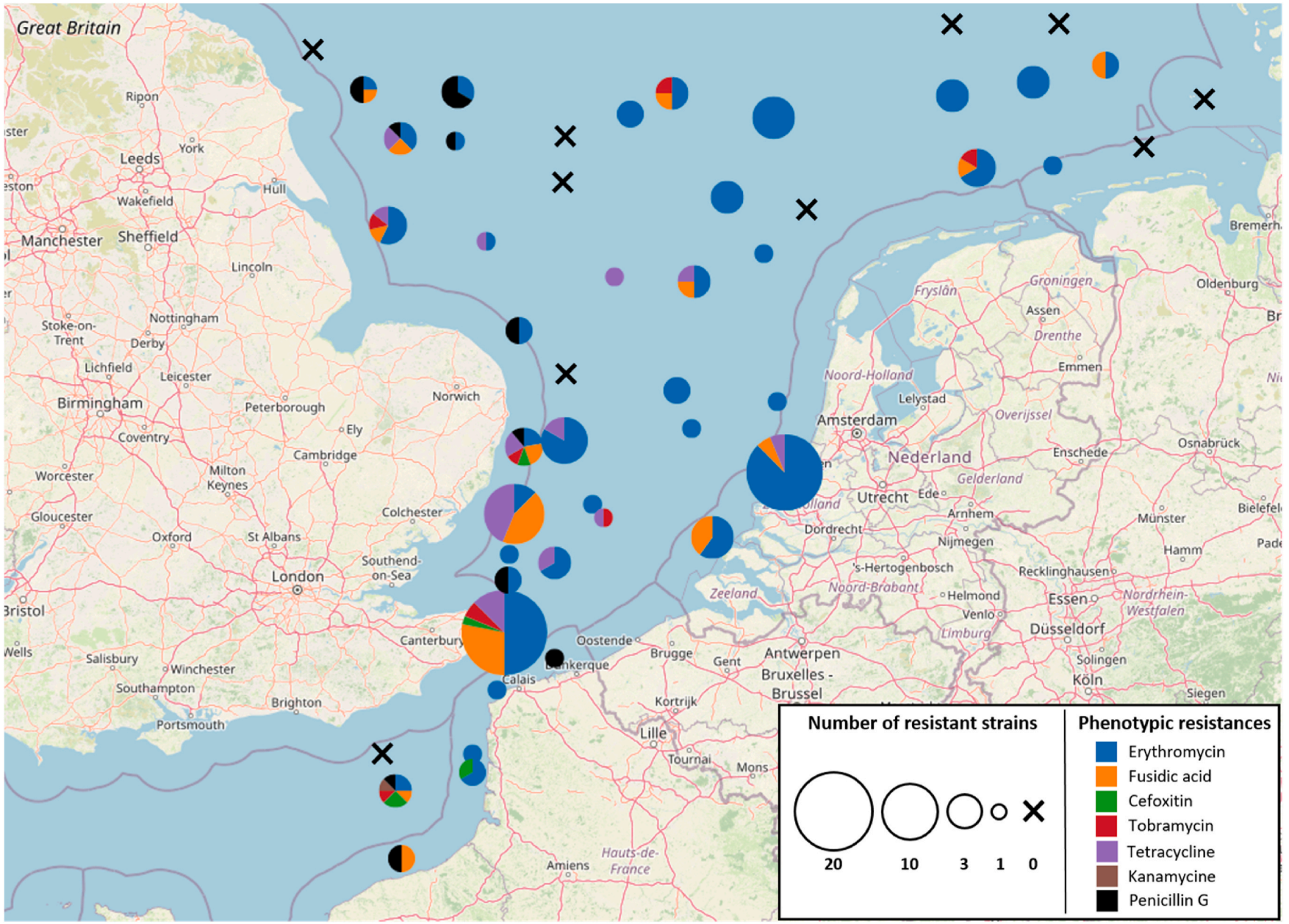

Fig. 2. Geographical distribution of antimicrobial resistant *Staphylococcus* strains (CoNS and CoPS).

specific resistance for the type of samples analyzed. We identified 7 MDR strains among the CoNS. Two strains isolated from seawater had three phenotypic resistances, one (*S. hominis* B3PA-RCPE-6-FE-AL1) to fusidic acid, cefoxitin, tobramycin and the other (*S. pasteuri* B3PA-RCPE-24FEPE1) to erythromycin, fusidic acid, tobramycin. Another CoNS strain (*S. warneri* B3PA-RCPE-17-72à80OUPE(2)) with all three resistances to erythromycin, fusidic acid and tetracycline was isolated from fish gills. We also observed resistance to 4 antibiotics (erythromycin, fusidic acid, tobramycin and tetraycline) for 2 strains of *S. hominis* (B3PA-RCPE-17-418OUPE(3) and B3PA-RCPE-17-424OUPE (1)) isolated from fish gills and one strain of *S. warneri* (B3PA-RCPE-14FEPE1) isolated from seawater. Finally, one strain of *S. haemolyticus* B3PA-RCPE2FEMRSA1 resistant to 5 antibiotics (erythromycin, fusidic acid, cefoxitin, tobramycin and kanamycin) was also isolated from seawater. In order to assess the genetic basis of these phenotypic resistances, we sequenced the total genome of the seven MDR strains and identified the resistance genes present in their genomes with 100% identity (Table 3). Ten antibiotic resistance genes confirming phenotypique resistance results were identified in the MDR *Staphylococcus* strains. These genes were consistent with the observed phenotypic resistances: the *fusC* (4 strains) and *fusB* (3 strains) genes coding for fusidic acid resistance, *mecA* (2 strains) for cefoxitin resistance, *aadD* (5 strains) for tobramycin resistance, *mphC* (3 strains), *msrA* (3 strains) and *ermC* (1 strain) for erythromycin resistance, *tet(K)* (4 strains) for tetracycline resistance and *aph(3′)-III* (1 strain) for kanamycin resistance. Moreover other resistance genes were also identified: *blaZ* gene encoding penicillin resistance through the production of a β-lactamase in all the MDR strains, *bleO* gene (bleomycin resistance) in 6 MDR strains and the *fosD* gene (fosfomycin resistance) in the *S. warneri* B3PA-RCPE-14FEPE1 strain isolated from seawater. Other resistance genes were also identified in the *S. haemolyticus* B3PA-RCPE2FEMRSA1 strain isolated from seawater such as the *vgaA* gene coding for resistance to streptogramin A and relative compounds as well as the *qacA* gene involved in resistance to quaternary ammoniums. This latest strain carried 9 antimicrobial genes. And, the induction of resistance to clindamycin by erythomycin was observed for three CoNS strain (*S. haemolyticus* B3PA-RCPE2FEMRSA1 isolated in seawater sample at the V0003 station, *S. epidermidis* B3PA-RCPE17-72à80OUTSA(2) isolated in seawater sample at the V00039 station and *S. warneri* B3PA-RCPE17-134à144PECH(1)) isolated in seawater sample at the V0060 station), which is a marker of a phenotype MLS_B. For the MDR *S. haemolyticus* B3PA-RCPE2FEMRSA1 strain, we identified the *ermC* gene.

The geographical distribution of all antimicrobial resistant CoPS and CoNS strains was investigated (Fig. 2). Regarding the number of isolated strains, a significantly higher number of AMR *Staphylococcus* strains were located at the sampling stations near the Thames, Humber and Rhine estuaries. Erythromycin-resistant strains were widely distributed in the English Channel and the North Sea extending from coastal to offshore stations without distinction. Similar observations have been made for fusidic acid resistance in these seas. Penicillin G and cefoxitin-

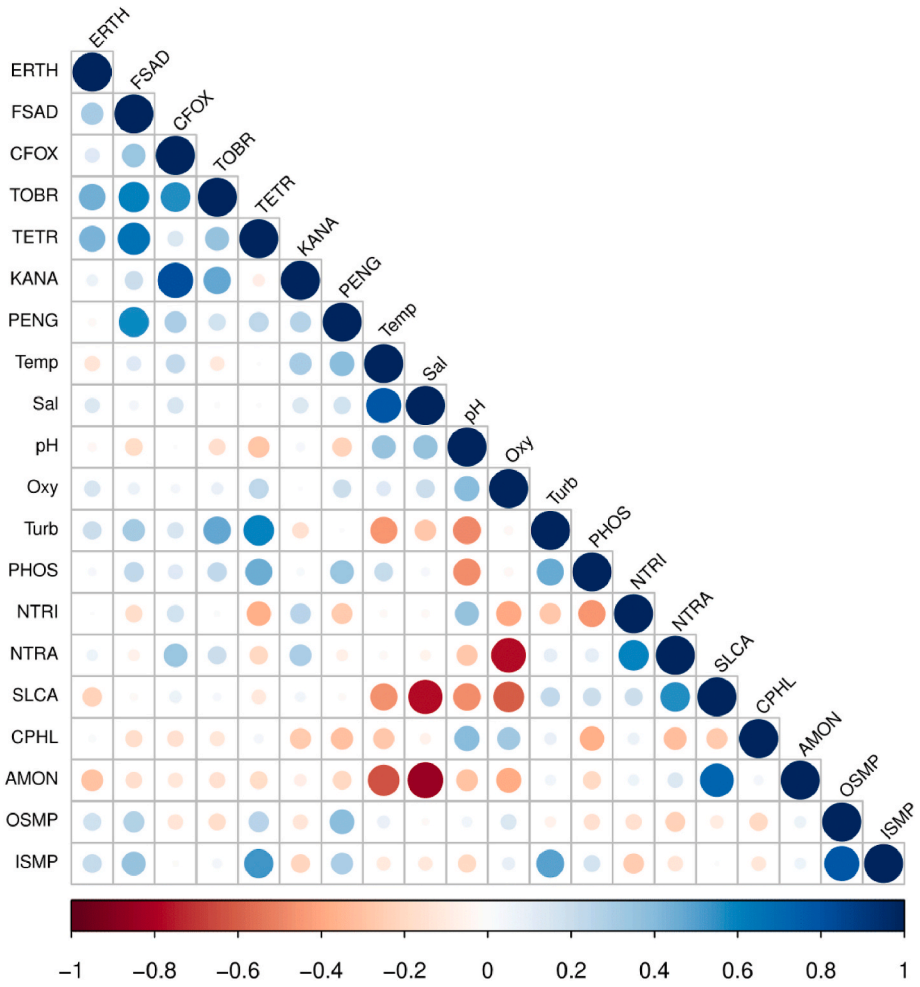

Fig. 3. Pearson correlation matrix between the phenotypic resistances of coagulase-negative and coagulase-positive *Staphylococcus* strains and the environmental parameter values. ERTH: erythromycin; FSAD: fusidic acid; CFOX: cefoxitin; TOBR: tobramycin; TETR: tetracycline; KANA: kanamycin; PENG: penicillin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

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

In order to evaluate the potential relationships between the occurrence of the phenotypic resistance of *Staphylococcus* and the environmental parameters, we performed a correlation analysis (Fig. 3). For all the nature of samples (seawater and fish), we observed that the number of tobramycin-resistant strains was moderately and positively correlated with the number of strains with a resistance to erythromycin, fusidic acid and cefoxitin. The number of tetracycline-resistant strains was positively correlated with the number of strains resistant to erythromycin and fusidic acid as well as the turbidity, the phosphate concentration and the inorganic suspended matter (ISMP). Regarding the number of kanamycin-resistant strains, we observed a positive correlation with the number of cefoxitin and gentamycin-resistant strains. Finally, a positive correlation was also observed between the number of strains resistant to penicillin G and fusidic acid.

4. Discussion

In this study, we investigated the occurrence and geographical distribution of AMR *Staphylococcus* in whiting (*Merlangius merlangus*), a widely harvested and consumed wild fish, as well as in seawater samples from the English Channel and the North Sea. These areas experience significant anthropogenic pressures, both directly and through river discharges (Tappin and Millward, 2015; Schöneich-Argent et al., 2020; Bourdonnais et al., 2022; Bourdonnais et al., 2023; Mauffret et al., 2023). Initially, we isolated and identified 238 *Staphylococcus* strains before assessing their resistance to 16 antibiotics crucial for human health, using the disk diffusion method following the EUCAST guidelines. Methicillin-resistant *Staphylococcus* represents a major concern, as these strains are typically resistant not only to all β-lactams but also to a wide range of other antibiotics. The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) varies significantly across institutions and geographical regions. In Europe, a North-South gradient is generally observed, with MRSA strains being less common in Scandinavian hospitals (<2%) compared to Mediterranean ones (>40%) (Stefani and Varaldo, 2003). Our study conducted in northern waters seems to support this trend, as no MRSA was detected. Specifically, the cefoxitin phenotypic resistance was not observed in the 12 *S. aureus* (CoPS) isolates in our samples, and all isolates were resistant to penicillin G, which aligns with previous observations in beach sand and bathing seawater samples Akanbi et al. (2017). It has been reported by Daurel and Leclercq (2008) that over 80% of *S. aureus* produce a penicillinase enzyme. We observed the MLS_B phenotype in one CoPS strain and three CoNS strains. In the MDR CoNS strains, we detected the presence of the *ermC* gene, which confirmed the underlying phenotypic mechanism in the S. *haemolyticus* B3PA-RCPE2FEMRSA1 strain. The *ermC* gene that we identified in the MDR *S. haemolyticus* strain has previously been detected in marine sediments collected in the Arctic region, but its presence has been associated with human activities (Tan et al., 2018).

Furthermore, only 2.2% of the CoNS strains (2 *S. cohnii*, 1 *S. haemolyticus*, 1 *S. hominis* isolated from seawater samples, and 1 *S. saprophyticus* isolated from fish gills) showed resistance to cefoxitin, indicating methicillin resistance. These values are lower than those reported in other studies. The prevalence of MRSA in fish and fishery products varied between 1% and 60%, depending on the sample type and origin (Vaiyapuri et al., 2019) and 1.0% of wild freshwater fish samples in Germany were found to be positive for MRSA (EFSA and ECDC, 2023). In the case of the MDR *S. haemolyticus* B3PA-RCPE2FEMRSA1 and *S. hominis* B3PA-RCPE-6-FE-AL1 strains isolated from seawater samples, we identified the *mecA* gene, which is responsible for methicillin resistance and can be carried by the mobile chromosomal cassette SCC*mec*, as described by Gomez et al. (2017). *S. hamemolyticus* is associated with nosocomial infections such as bacteremia and infective endocarditis. Methicillin-resistant *Staphylococcus* strains have previously been isolated from mackerel, salmon, tuna, flounder, and yellowtail sashimi (Hammad et al., 2012). We also identified one *S. saprophyticus* strain showing phenotypic resistance to cefoxitin. This species is commonly implicated in certain types of CoNS clinical infections, such as skin and urinary tract infections (Silva et al., 2020). Similar to previous studies that identified the *mecA* and *aph (3′)-III* genes in marine *S. aureus* strains and river waters (Levin-Edens et al., 2012; Paulus et al., 2020), we observed that the *S. haemolyticus* B3PA-RCPE2FEMRSA1 strain also carried these two antimicrobial resistance genes. However, we did not observe phenotypic penicillin resistance in any of the 7 MDR CoNS strains, but we identified the presence of the *blaZ* gene for further whole-genome sequencing analysis. Similarly, Pimenta et al. (2023) demonstrated that a species of *S. haemolyticus* isolated from a clinical sample harbored the *blaZ* genes and *femA* plasmid but showed a negative result for phenotypic resistance to cefoxitin and penicillin G. Two hypotheses could explain this result: the first is that *blaZ* might not be expressed due to genetic mutations in the regulatory regions of the gene. Indeed, the *blaZ* gene is regulated by two other genes, the *blaRl* antirepressor and the *blal* repressor. Following exposure to beta-lactams, *blaR1*, a transmembrane sensor-transducer, undergoes autocatalytic cleavage, promoting the cleavage of the repressor gene, *blal*, thereby allowing transcription of *blaZ*. A mutation in these regions could affect the expression of *blaZ* and thus the expression of phenotypic resistance (El Feghaly et al., 2012; Pimenta et al., 2023). Another hypothesis could be that penicillin resistance might not be consistently detected using commonly employed susceptibility testing methods. In support of this, El Feghaly et al. (2012) demonstrated that 9.5% of clinically susceptible *S. aureus* isolates to penicillin G carried the *blaZ* gene. They proposed that enhancing interpretation criteria for the disc diffusion zone size from 29 to 35 mm could improve the sensitivity of phenotypic penicillin resistance testing. Furthermore, our investigation revealed the presence of the *bleO* gene in 6 out of the 7 MDR CoNS strains, indicating resistance to bleomycin. Numerous studies have shown that over 80% of CoPS strains resistant to aminoglycosides also exhibit resistance to bleomycin.

Regarding the CoNS isolates, we identified resistance to erythromycin (40.7%), fusidic acid (12.4%), tetracycline (10.2%), tobramycin (3.5%), cefoxitin (2.2%) and kanamycin (0.4%) which were lower than those reported in clinical settings (Archer and Climo, 1994; Schmitz et al., 1999; Schreckenberger et al., 2004; Koksal et al., 2009). It is widely accepted that higher levels of antibiotic resistance in clinical isolates are due to constant exposure to antibiotics compared to the marine environment. The open marine environment allows for dilution and dispersion of antibiotic residues and antimicrobial resistance (Stefani and Varaldo, 2003). However, the environment can also contribute to the development of antibiotic resistance in microorganisms due to human/animal therapeutics, wastewater, agriculture, and industrial use of antibiotics (Larsson and Flach, 2021). Bourdonnais et al. (2022) highlighted the presence of the *sul1* and *intI1* genes in seawater from the English Channel and the North Sea with concentrations of up to 3.5 log gene copies/L of seawater. Marine environment is thus a reservoir of

antimicrobial resistance, notably as it is subjected to anthropogenic discharges. Therefore, the wide distribution of AMR *Staphylococcus* in the marine environment can be considered as a source of concern. Two hundred and thirty eight strains of *Staphylococcus* were isolated from the samples collected in the English Channel and the North Sea, and 54.6% showed at least one antibiotic resistance with 3% of multi-resistant strains. We also revealed that the number of resistant *Staphylococcus* and the diversity of resistance associated with them varied between the different sampling stations in the English Channel and the North Sea. The highest prevalence of antibiotic resistance was found along the English coast. Furthermore, the majority of MDR *Staphylococcus* was isolated in these areas (6/7 strains). Several hypotheses can be put forward to explain this occurrence. The first would be the anthropogenic impact associated to river discharges from the Thames and the Humber effluents. These two rivers are the largest sources of freshwater that England discharges into the North Sea. Previous observations have shown the presence of numerous antimicrobial residues (erythromycin, clarithromycin and azithromycin) downstream of the Thames source (White et al., 2019). In addition, Xu et al. (2019) quantified the antimicrobial resistance genes (ARGs) bla_{TEM}, *tetA, sul1, sul2, ermB, tetQ, tet (X), drfA1, drfA2* and *intI1* in these waters with abundances ranging from 10^5 to 10^8 copies/L. We did not identify these genes in the genomic DNA of the MDR *Staphylococcus* strains isolated from the fish and seawater samples collected near these two areas. To our knowledge, this was the first time that the *mphC, aadD*, *fusC* and *fusB* genes were identified in the marine environment. We identified the *tetK* gene in MDR CoNS 4 strains isolated in fish gills. In Chile, the *tetK* resistance gene was identified in *Pseudoalteromonas*, *Shewanella*, *Cobetia* and *Vibrio* strains isolated from sediment and water samples from an aquaculture site but also from the marine environment (Tomova et al., 2015). The prevalence of resistance to fusidic acid varies between 4% and 30% depending on the country (Briet, 2018). Resistance to this antibiotic is acquired either by mutation of the *fusA* target or by acquisition of a *fusB* plasmid determinant (Briet, 2018). In our study, the MDR *S. hominis* and *S. haemolyticus* strains carried the *fusC* gene and the MDR *S. warneri* and *S. pasteuri* strains carried the *fusB* gene that conferred the resistance at fusidic acid confirming by phenotypic and genotypic resistance. For one *S. warneri* strain, the *fusD* gene was described in more by genotypic method. In addition, we have identified the *qacA* gene in a *S. haemolyticus* B3PA-RCPE2FEMRSA1strain isolated from the English Channel seawater. This gene codes for a subunit of the QacA multidrug efflux pump conferring resistance to antimicrobial compounds such as quaternary ammoniums. This gene has been identified in several MDR *S. haemolyticus* strains isolated from dairy cows, a human with septicemia and also in a persistent strain from a veterinary clinic floor despite disinfection (Anthonisen et al., 2002). The *qacA* gene was located on a plasmid with the *blaZ* gene, coding for a β-lactamase and identified in the 7 MDR *Staphylococcus* strains in our study. Disinfectant resistant bacteria surviving disinfection thus represent a threat to the food industry and to the public health. This *S. haemolyticus* B3PA-RCPE2FEMRSA1strain, isolated in seawater sample, carried 9 ARGs (*ermC* (ERTH), *fusC* (FSAD), *mecA* (CFOX), *aadD* (TOBR), *aph (3')-III* (KANA), *blaZ* (penicillin), *bleO* (bleomycin), *vga(A)* (streptogramin A), *qacA* (quaternary ammoniums)) with resistance to antibiotics but also to disinfectant in food industry. The transfer of AMR genes between CoNS and *S. aureus* has been reported that CoNS may act as a AMR gene reservoir for CoPS due to their ability to exchange genetic material inter/intra species (Rossi et al., 2020).

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

A high abundance of AMR *Staphylococcus* (especially to erythromycin) was also observed near the mouths of the Rhine and the Meuse, two large rivers flowing into the North Sea. This is in agreement with the observations of Blaak et al. (2012) who found several *Enterococcus faecium* strains resistant to erythromycin, tetracycline and ampicillin in the Meuse, Rhine and New Meuse rivers. These *E. faecium* strains may have been responsible for horizontal transfer of ARGs to *Staphylococcus* strains. More recently, Paulus et al. (2020) have demonstrated the presence of resistance genes to erythromycin (*ermB* and *ermF*), sulfonamide (*sul1*), tetracycline (*tetM*), aminoglycosides (*aph(III)*) and carbapenem (bla_{OXA}) along the entire length of the Rhine in Switzerland, Germany and the Netherlands due to potential pollution by pharmaceutical industries in the Lower Rhine. The impact of these river effluents on the pollution of the North Sea is cumulative with that of the city and harbor of Rotterdam, the largest harbor in Europe and therefore the source of much anthropogenic pollution. However, there is no information on the contamination of effluents from port activities with ARGs or antibiotic residues.

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

A further consideration is the impact of environmental factors that may favor the spread and persistence of AMR *Staphylococcus*. Thus, in this study we looked at environmental factors related to phenotypic resistance of *Staphylococcus*. We showed that resistance to tobramycin and tetracycline was strongly correlated with turbidity and suspended matter. Both descriptors are proxies of organic matter and of aggregation of microorganisms. Similarly, Bourdonnais et al. (2022) observed that turbidity values were positively correlated with the abundance of the *sul1* and *intI1* genes, and with the abundance of the bacterial population in the North Sea and the English Channel seawaters. Chen et al. (2020) showed that the occurrence of ARGs in an estuary in China was correlated with turbidity with a higher prevalence of the ARGs in areas with high turbidity. Although not carried by the same strains, co-occurrence of penicillin G and cefoxitin resistance was observed in several strains isolated from samples along the English coast. A plausible explanation lies in the fact that Staphylococcus strains are exposed to similar environmental conditions or medical treatments along the English coast, potentially creating a common selective pressure that favors the development of resistance mechanisms to both penicillin G and cefoxitin. This situation may result from the frequent use of these antibiotics in the region. Indeed, the study by Curtis et al. (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 β-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 4–8 mg/L) and penicillin (MIC 0.25–0.5 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).

5. Conclusion

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

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CRediT authorship contribution statement

Thomas Brauge: Writing – original draft, Visualization, Validation, Supervision, Conceptualization. **Erwan Bourdonnais:** Writing – review & editing, Investigation, Formal analysis. **Sylvain Trigueros:** Investigation, Formal analysis, Data curation. **Pierre Cresson:** Writing – review & editing, Resources, Methodology, Conceptualization. **Sabine Debuiche:** Investigation, Formal analysis. **Sophie A. Granier:** Methodology. **Graziella Midelet:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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