Microbiological analysis of skin lesions of cod *(Gadus morhua)* from the southern part of the Baltic Sea

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Abstract

Introduction: Since the middle of the 1980s, severe skin disorders have been observed in Baltic cod (*Gadus morhua*) each year. Available data on the spectrum of bacteria isolated from the clinical cases being limited, and evaluation of the microbial background of fish skin lesions being useful, a bacteriological examination has been undertaken. **Material and Methods:** A total of 1,381 cod were caught during two voyages of the Baltica research vessel in the Polish exclusive economic zone of the southern Baltic Sea. After an examination which found lesions in 164 of the fish, a microbiological analysis was performed to isolate bacteria from them. The collected strains were phenotyped and genotyped, and their antimicrobial resistance was analysed by minimum inhibitory concentration (MIC) techniques. **Results:** Bacteriological examinations provided 850 isolates. The dominant microorganisms were mesophilic *Aeromonas* spp., *Pseudomonas* spp. and *Shewanella baltica*. Opportunistic bacteria potentially hazardous to human health were also isolated, *e.g. Alcaligenes faecalis*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia* and *Vibrio* sp. The MIC analysis determined the highest number of bacteria to resist sulphamethoxazole and amoxicillin and clavulanic acid. **Conclusion:** Most of the collected bacteria were opportunistic pathogens for fish, widespread in the aquatic environment, and potentially threatening to humans.

Keywords: Baltic cod (*Gadus morhua*), fish skin disorders, marine bacteria, Baltic Sea, antimicrobial resistance.

Introduction

The Baltic Sea fish population includes more than 100 marine and brackish water species. The low salinity and other specific environmental conditions are conducive to cod (*Gadus morhua*), herring (*Clupea harengus*) and sprat (*Sprattus sprattus*); these are the principal species for commercial fisheries, and together these three species constitute approximately 95% of the total catch (32). Favourable reproductive conditions, such as the inflow of well-oxygenated and more saline water from the North Sea, caused record catches of cod of around 650,000 tonnes in the early 1980s. Meanwhile, cod resource density has significantly decreased over the decades since to only 10%. This led to a recommendation to stop fishing the eastern cod stocks in 2020 (31, 32).

This phenomenon was attributed mainly to considerably higher fish mortality. Many hypotheses trying to explain the stock decline have arisen. The poor health condition of the fish and biological changes in the stock, including those related to changes in the ecosystem, *i.e.* poor oxygenation and low availability of prey fish, was pointed to as a cause. Moreover, the pollution of the Baltic Sea, its limited ability to self-purify due to its shallowness, and the limited inflow of water from the North Sea were indicated (21, 32).

The accumulation of contaminants in aquatic animals can cause severe stress, resulting in various pathophysiological disturbances in fish. Piscine health is determined by the sum of factors including the state of the immune system, the presence of different pathogens, and the conditions in the aquatic environment.

Disruption of the established balance can have serious consequences. One may be skin lesions and ulcers, which often penetrate the muscles and erode fins. In the last decade, Polish fishermen have observed severe health problems in Baltic cod (24). Similar symptoms were also noticed in fish from regions of the Baltic Sea outside the Polish exclusive economic zone (42, 58). In response, recommendations regarding monitoring Baltic cod diseases and parasites and identifying and grading the causative pathogens were formulated (30). They acknowledge the economic losses in the lower cod catch and intend skin lesion inspection to be implemented at all levels, from the fishery personnel to scientists and even the government.

Fish can be affected by various diseases and health disorders exhibiting different stages of development and intensity. Skin ulcers are the most commonly observed among Baltic Sea cod. Bacteria are known as aetiological factors, both primary and secondary, of these clinical signs, complicating previous mechanical irritations of the skin. The observed symptoms include haemorrhages on the fins and the skin's surface, skin lesions, and ulceration or erosion of the fins. Clinical signs of fish health disorders vary depending on the aetiological agent (24, 30, 42, 58). Previous reports indicated *Aeromonas* spp., *Flavobacterium* spp., *Pseudomonas* spp., the *Shewanella putrefaciens* group, and *Vibrio* spp. as the most common microorganisms isolated from ulcers on Baltic Sea fish (2, 17, 33). These bacteria were also isolated from skin ulcers of cod caught in the western Baltic Sea (6) and northwest of the German island of Rügen (8), but they were only characterised biochemically in the associated research. There are limited available data on the microflora spectrum currently isolated from the Baltic cod skin lesions from the southeastern part of the Baltic Sea. The genetic characterisation of bacteria from those sites and their antimicrobial analysis has not previously been performed. Genetic characterisation research encompasses investigation of bacterial resistance to antimicrobial drugs, because understanding the mechanisms of bacterial resistance is currently of crucial importance for animal and human health safety. These have not been investigated in wild fish pathogens, including the bacteria of cod from the Baltic Sea.

Therefore, the aim of our study was the analysis of the dynamics of skin lesions observed in Baltic cod in the context of the isolated bacteria which could be their aetiological factors. The results of the analysis were the species of microflora determined to be causing skin lesions in fish from the Polish exclusive economic zone (EEZ) of the southern Baltic Sea caught during two research surveys in 2016 and 2017.

Material and Methods

Fish sampling and clinical examination. The Baltic cod were collected during two surveys by the Baltica research vessel which took place in April 2016 and January 2017. The explored region was the Polish EEZ of the southern Baltic Sea in the International Council for the Exploration of the Sea Subdivisions 25 and 26. Fish caught in the following five fishing grounds were examined: the Gdańsk Bay, Słupsk Furrow, zone near Kołobrzeg-Darłowo and Bornholm Basin north and south parts (Fig. 1). After each haul, cod were measured for body length and their health was examined. The presence of parasites and externally visible disease symptoms, particularly skin lesions and ulceration, was ascertained. The visual disorders observed on the skin were classified according to Bucker *et al.* (12) and biological effects quality assurance in monitoring programmes (BEQUALM) guidelines (30): epidermal necrosis, skin erosion, necrotic lesions, ulcer healing, and cicatrix.
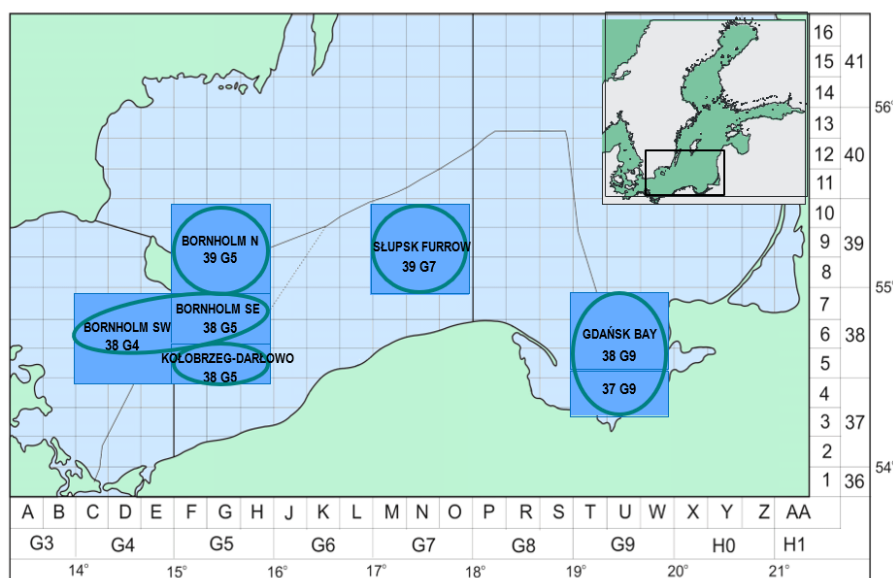
Fig. 1. Areas where Baltic cod were sampled in 2016 and 2017 within the Polish exclusive economic zone of the southern Baltic Sea (by Podolska M. and Trella K.)

Bacteriological studies. Each fish that exhibited external disorders manifested on its skin was collected for bacteriological examination. Scrapings from the skin lesions/ulceration were taken separately and inoculated onto appropriate media: nutrient agar supplemented with 5% horse blood (BA) (BioMaxima, Lublin, Poland), trypticase soy agar (TSA) (bioMérieux, Marcy-l'Étoile, France), *Cytophaga* agar (CA) (Oxoid, Basingstoke, UK) and thiosulphate citrate bile salts sucrose (TCBS) agar (Merck, Darmstadt, Germany). Depending on the medium used, the samples were incubated at one of two different thermal and time variants: at 22°C ± 2°C for 72–96 h (in BA, TSA and TCBS) or at 15°C ± 1°C for seven days (in CA) (13). Then the growth of bacteria was estimated, and the dominant types of bacterial colonies were reisolated on BA and CA and incubated at 22°C for 24–48 h (BA) or at 15°C for three days (CA). For further studies, pure cultures were frozen at −80°C in cryobank vials (Nunc, Roskilde, Denmark). The same methodology was used to perform bacteriological analysis of samples from 30 healthy fish from each fishing area.

After Gram staining and morphological assessment of pure bacterial isolates, their biochemical identification was performed using appropriate analytical profile index (API) tests (API 20 E, API 20 NE, API Staph and API Strep) and the VITEK 2 system (bioMérieux), according to the manufacturer's instructions. The temperature of API test incubation was modified to 22°C ± 2°C.

Molecular analysis of the 16S rRNA gene. The 16S rRNA gene was sequenced as described previously (45, 48). The total genomic DNA was isolated from pure bacterial cultures with a DNeasy Blood & Tissue Kit following the producer's protocol (Qiagen, Hilden, Germany). The quality and concentration of DNA were analysed using a Multiskan GO Microplate Spectrophotometer (Thermo Scientific, Vantaa, Finland). The extracted DNA was used for 16S rRNA gene sequence analysis. Amplification of the V1–V9 regions of the 16S rRNA gene was performed using Eubac27F (5′-AGAGTTTGATC(C/A)TGGCTCAG-3′) and Eubac1492 (5′-TACGG(C/T) TACCTTGTTACGACTT-3′) universal primers as described previously (45, 46, 48). The purified PCR products were sequenced using a 3730xl DNA Analyzer (Life Technologies, Singapore) by Genomed S.A. (Warsaw, Poland) and their sequence data were processed with Molecular Evolutionary Genetics Analysis software (MEGA version 7.0). Phylogenetic analysis of the 16S rRNA gene was performed by the maximum-likelihood method with the Tamura–Nei model using 1,000 bootstrap replicates (55). A tree was created based on the nucleotide sequences of the 16S rRNA gene, which were compared with sequences available in GenBank. For the *Shewanella* group, the reference sequences were ATCC BAA-1090 (AJ457093) for *S. denitrificans*, NCTC 10735 (NR025267) for *S. baltica*, ATCC 700550 (NR074798) for *S. oneidensis*, JCM 16212 (NR116732)

for *S. xiamenensis*, ATCC 8071(X82133) for *S. putrefaciens*, NBRC (AB681314) for *S. morhuae* and CECT:7627, pdp11 (CP015194) for *Shewanella* sp., and the outgroup was ATCC 15381 (NR040842), a *Moritella marina* sequence. For the *Pseudomonas* group, they were R3_6_4 (MN197571) for *Ps. laurentiana*, ATCC 17472 (AB016428) for *Ps. putida*, CIP 105469 (NR024928) for *Ps. gessardii*, DSM 50071 (NR026078) for *Ps. aeruginosa*, LMG:24016 (NR042607) for *Ps. guineae*, B13 (NR117756) for *Ps. knackmussii*, ATCC 13525 (NR114476) for *Ps. fluorescens*, KMM 3042 (NR041592) for *Ps. marincola*, NCIMB 1949 (NR029319) for *Ps. anguilliseptica*, HY-14 (NR116388) for *Ps. caeni*, ATCC 49968 (NR024704) for *Ps. lundensis* and KACC 10847 (NR025227) for *Ps. umsongensis*, and the outgroup was JCM 13198 (LC507964), a *Burkholderia cepacia* sequence.

Analysis of minimal inhibitory concentration (MIC) value. Broth microdilution methods provided in the Clinical and Laboratory Standards Institute VET04-A2 guide were used to determine the MICs of antimicrobials against the collected bacterial isolates (16). The selection of bacteria isolates for the MIC analysis was based on the biochemical properties of the microorganisms and represented each of the obtained biochemical profiles. Mueller–Hinton broth (Oxoid, Basingstoke, UK) was used to prepare inoculum, and the incubation condition was set at 22°C for 24 h. No MIC plate designated for bacteria isolated from aquatic animals being on the market, a user-defined POLARGEN Sensititre plate (Trek Diagnostic Systems, East Grinstead, UK) was used. The concentrations of the following antimicrobials representing different prescribing advice categories were selected for analysis: two fluoroquinolones (enrofloxacin at 0.03–16 µg/mL and ciprofloxacin at 0.03–4 µg/mL) and a quinolone (flumequine at 0.25–64 µg/mL) from category B ("Restrict"); an aminopenicillin in combination with a beta-lactamase (amoxicillin and clavulanic acid at 0.12/0.06–16/8 µg/mL) and a phenicol (florfenicol at 0.015–2 µg/mL) from category C ("Caution"); and a tetracycline (doxycycline at 0.5–64 µg/mL), oxytetracycline at 1–128 µg/mL and a sulfonamide (sulphamethoxazole at 8–1024 µg/mL) from category D ("Prudence") (20). The results of MIC tests described as epidemiological cut-off values establish an isolate as a wild type (WT) or a non-wild type (NWT) (53). A wild-type isolate is defined as one without phenotypically detectable resistance mechanisms, in contrast to an NWT.

Statistical analysis. Based on the examination of single fish for the presence of pathological changes, the prevalence of each disease in a sample was calculated according to the formula $p = x/n$, where p is the prevalence, x is the number of fish affected and n is the number of fish examined. The prevalence was expressed as a percentage ($p ≤ 100\%$).
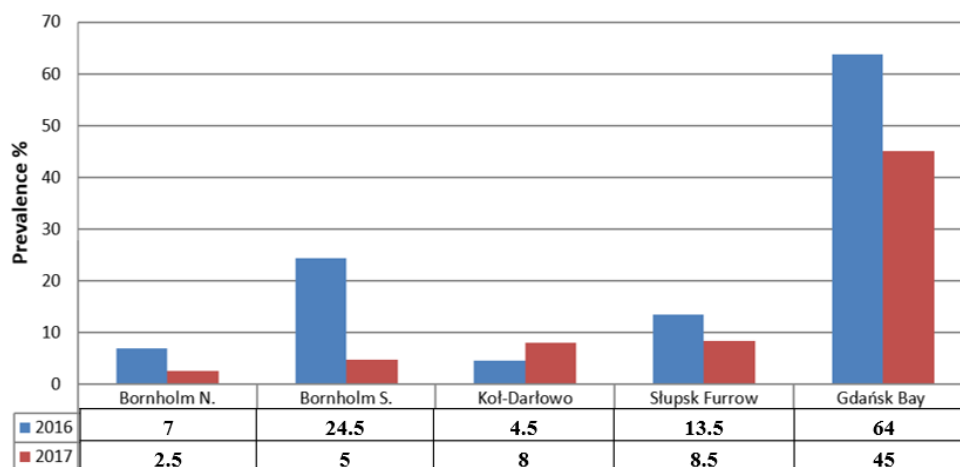
Results

Clinical examinations of fish. During two research surveys, 1,381 Baltic cod were caught and examined (667 in 2016 and 714 in 2017) in the following fishing grounds: the Gdańsk Bay, Słupsk Furrow, zone near Kołobrzeg-Darłowo, and Bornholm Basin north and south parts. Overall, 164 fish (p = 11.2%) exhibited pathological changes manifested by skin ulceration. The prevalence of diseased fish was p = 15% (98 individuals) in 2016 and 9% (66 individuals) in 2017. Of all the cod fishing grounds, the Gdańsk Bay was the source of the fish with the highest prevalence of skin disorders (p = 64% and p = 45%, respectively in 2016 and 2017) (Figs 2 and 3). The acute stage, characterised by red and open or almost open inflammatory skin lesions (Fig. 4), was observed among 8.5% of the diseased Baltic cod collected from this fishing ground (Fig. 3). The highest prevalence (11.2%) of the skin changes characteristic of the healing process (Fig. 3) was also observed among the cod from the Gdańsk Bay. This group included both scar formation and melanin deposits at the periphery of the lesion (Fig. 5) as well as complete closure of the lesion (Fig. 6). Necrosis and excessive cell debris representing the chronic stage of the disorders (Fig. 7) occurred in diseased cod from the Gdańsk Bay with a prevalence equal to only 4% (Fig. 3). Skin disorders in the cod appeared to be the least prevalent in the northern part of the Bornholm Basin

area. They were estimated at p = 7% and p = 2.5% in 2016 and 2017, accordingly (Fig. 2). Comprehensive data on the prevalence of skin disorders in the Baltic cod broken down by fishing area is presented in Figs 2 and 3.

Among the Baltic cod caught in the Gdańsk Bay fishing ground, 1% exhibited deep necrotic lesions defined by their regular round shape (Fig. 8). Lesions at the other extreme of severity, which were flat, irregular, shallow and superficial, were observed only in fish from the Bornholm Basin area (Fig. 9).

More than 1,200 fish were clinically healthy. Parasitological examinations of the lesions on these fish did not reveal the presence of parasites.

Bacteriological examination. Approximately 850 bacterial isolates originating from Baltic cod skin disorders were collected and identified during our study. Based on the analysis of their biochemical properties with the API and VITEK system tests, they were classified to genus and species levels. Among the collected strains, groups of bacteria such as the *Shewanella putrefaciens* group (30% of collected bacteria) and mesophilic *Aeromonas* spp., and *Pseudomonas* spp. (both 25%) were identified (Supplementary Table 1). Various numerical profiles representing different biochemical properties were noted within one bacterial taxon, providing species- or genus-level identification. The obtained results are presented in Supplementary Table 1.

	Bornholm N.	Bornholm S.	Koł-Darłowo	Słupsk Furrow	Gdańsk Bay
2016	7	24.5	4.5	13.5	64
2017	2.5	5	8	8.5	45

Fig. 2. Prevalence of Baltic cod ulceration by fishing ground and International Council for the Exploration of the Sea Sub-divisions
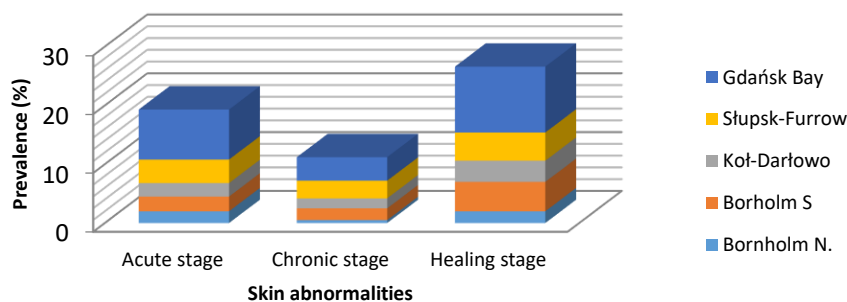
Fig. 3. Stages of Baltic cod ulceration in 2016 and 2017 by fishing ground

Fig. 4. Skin ulceration observed in a Baltic cod – acute and first-stage inflammatory process with visible flat erosions of the skin

Fig. 5. Skin ulceration observed in a Baltic cod – beginning of the healing process with scar formation and melanin deposits

Fig. 6. Skin ulceration observed in a Baltic cod –final stage of the course of ulceration with healing and star-shaped closure

Fig. 7. Skin ulceration observed in Baltic cod – chronic stage of the course of ulceration. a, b – advanced disease with tissue swelling, developed inflammation and tissue lysis; c, d – advanced disease process with tissue necrosis


Fig. 8. Skin ulceration observed in a Baltic cod – chronic stage of the course of ulceration: advanced disease process with developed inflammation, tissue lysis and deep necrotic lesions

Fig. 9. Skin ulceration observed in a Baltic cod – flat, irregular, superficial skin lesions without tissue inflammation
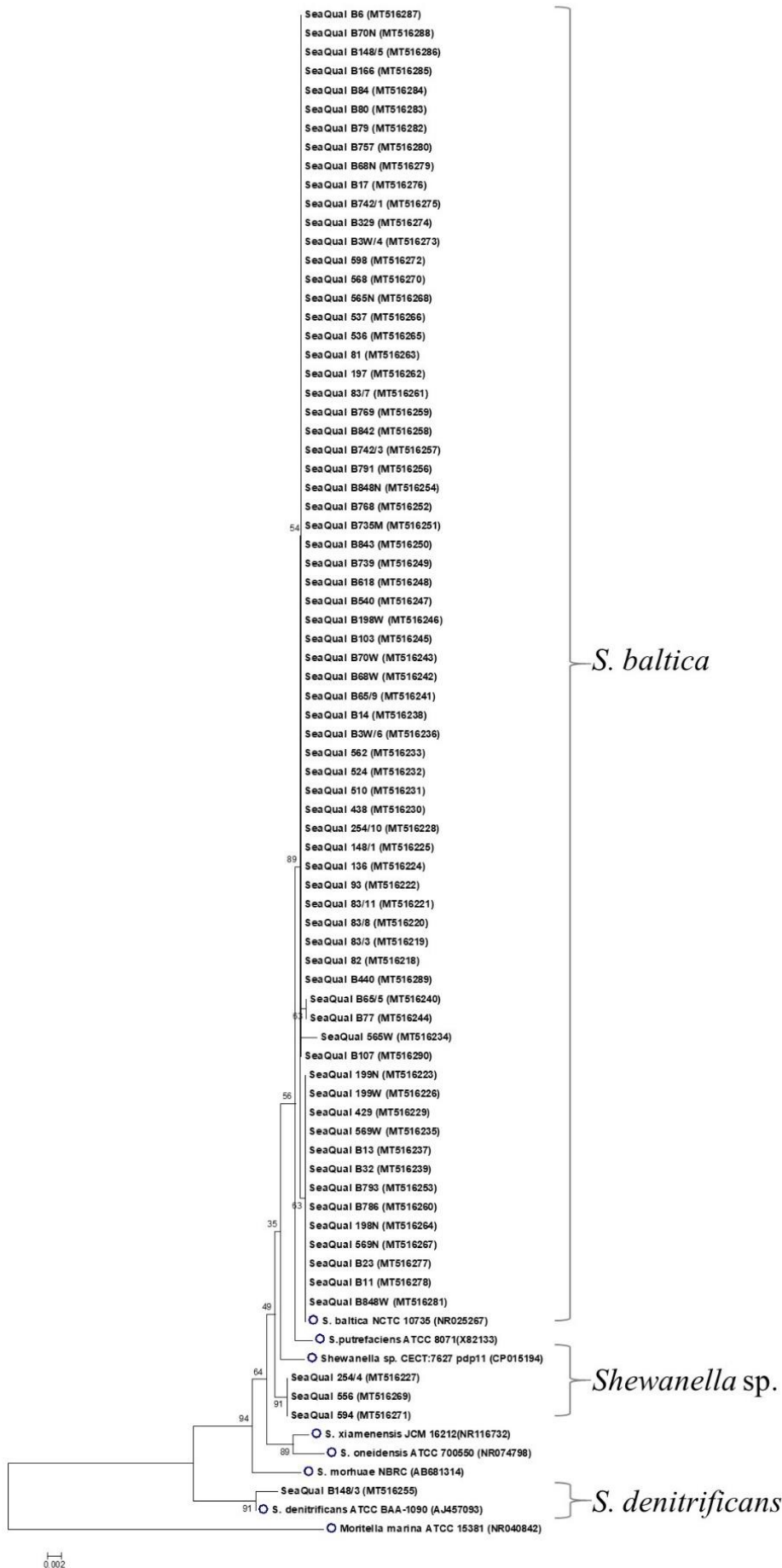
Fig. 10. Analyses of the 16S rRNA gene of *Shewanella* isolated from Baltic cod and GenBank sequences. The maximum-likelihood phylogenetic tree was constructed with the Tamura–Nei model using 1,000 bootstrap replicates. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches. O – reference sequence
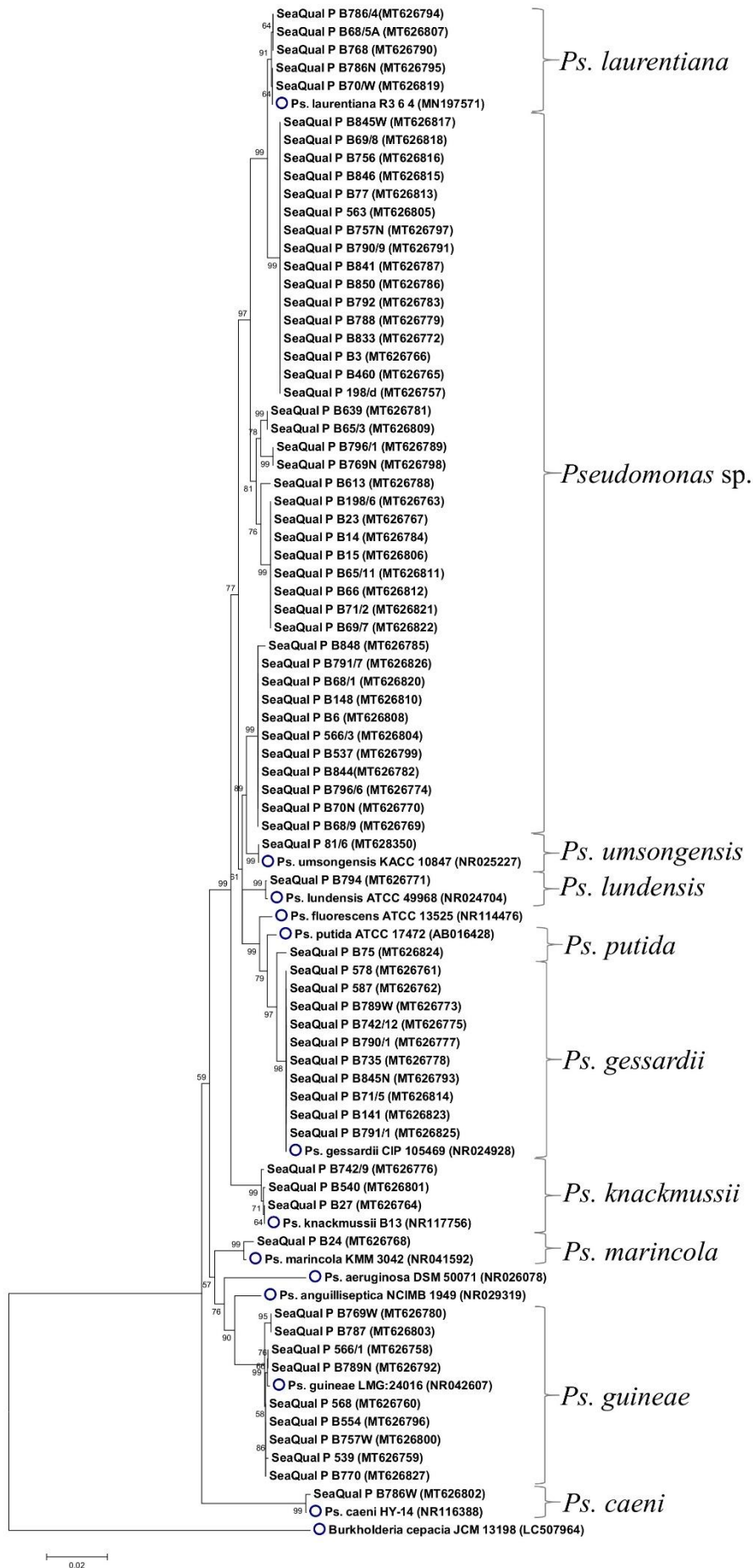
Fig. 11. Analyses of the 16S rRNA gene of *Pseudomonas* isolated from Baltic cod and GenBank sequences. The maximum-likelihood phylogenetic tree was constructed with the Tamura–Nei model using 1,000 bootstrap replicates. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches. O – reference sequence
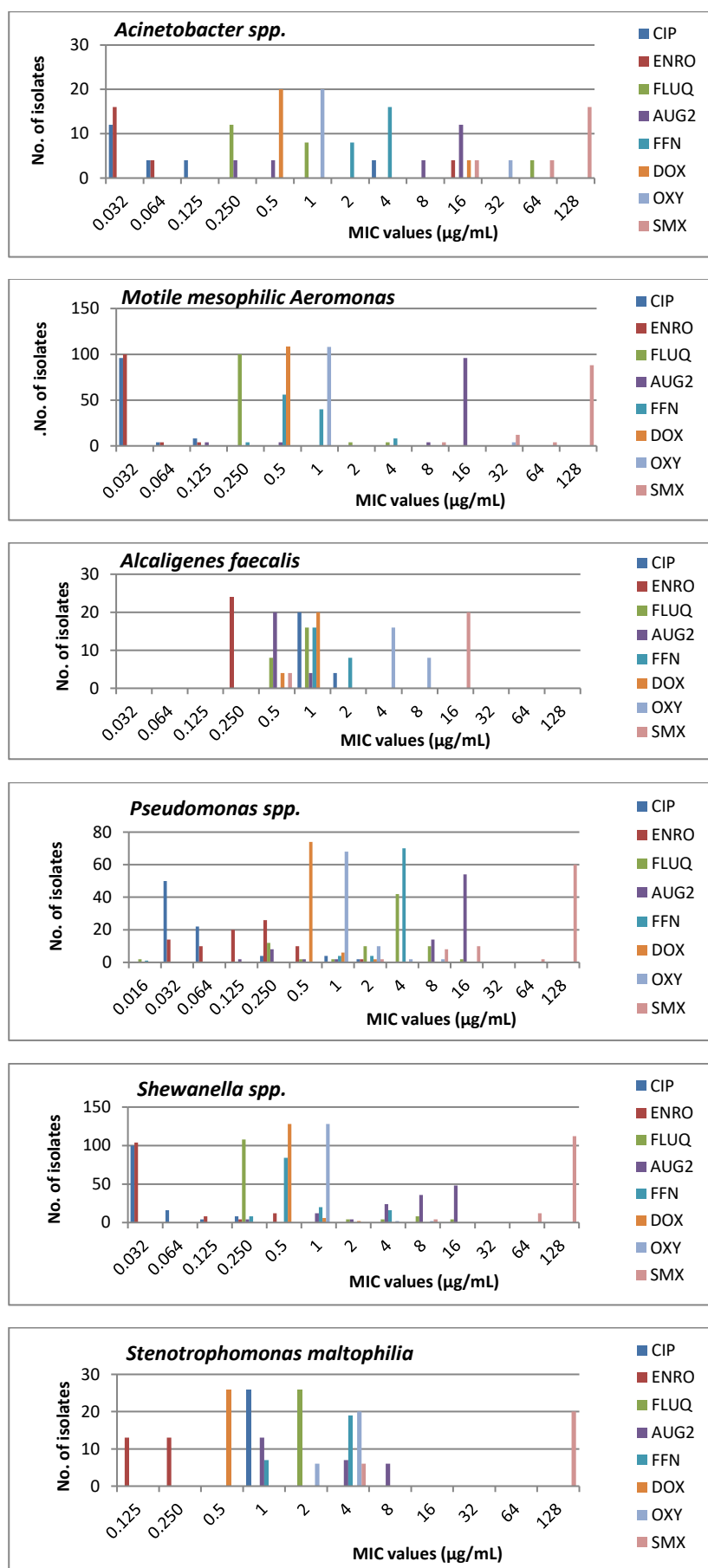
Fig. 12. Minimal inhibitory concentration (MIC) values determined for bacteria collected from the ulcers of Baltic cod. CIP – ciprofloxacin; ENRO – enrofloxacin; FLUQ – flumequine; AUG2 – amoxicillin and clavulanic acid; FFN – florfenicol; DOX – doxycycline; OXY – oxytetracycline; SMX – sulphamethoxazole

Table 1. Groups of bacteria isolated from skin ulcers of Baltic cod in correlation with the fishing area

Fishing area	Bacteria
Gdańsk Bay	*Acinetobacter* spp., *Aeromonas* spp., *Alcaligenes faecalis*, *Chryseobacterium indologenes*, *Enterococcus faecalis*, *Microbacterium* sp., *Pseudomonas* spp., *Shewanella putrefaciens* group, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, *Vibrio* sp.
Słupsk Furrow	*Acinetobacter* spp., *Aeromonas* spp., *Chryseobacterium indologenes*, *Delftia* spp., *Micrococcus* sp., *Pseudomonas* spp., *Shewanella putrefaciens* group
Kołobrzeg-Darłowo	*Acinetobacter* spp., *Aeromonas* spp., *Chryseobacterium indologenes*, *Pseudomonas* spp., *Serratia* spp., *Shewanella putrefaciens* group, *Stenotrophomonas maltophilia*
Bornholm North	*Acinetobacter* spp., *Aeromonas* sp., *Pseudomonas* spp., *Shewanella putrefaciens* group, *Stenotrophomonas maltophilia*
Bornholm South	*Acinetobacter* spp., *Citrobacter freundii*, *Pseudomonas* spp., *Shewanella putrefaciens* group, *Stenotrophomonas maltophilia*

The bacterial species identified from skin ulcers differed by the fishing ground where the cod was caught (Table 1). The microorganisms predominating in the Polish EEZ represented different species: *Acinetobacter* sp., *Aeromonas* spp. (motile mesophilic strains), *Chryseobacterium indologenes*, *Pseudomonas* spp., and the *Shewanella putrefaciens* group. Bacteria such as *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia* and *Vibrio* sp. were also identified in the Gdańsk Bay. In the Bornholm fishing ground, *Acinetobacter* sp., *Pseudomonas* sp., the *Shewanella putrefaciens* group and *Stenotrophomonas maltophilia* were detected (Table 1). No bacteria were found in the samples taken from healthy fish.

Molecular analysis of the 16S rRNA gene. Phylogenetic analysis based on the 16S rRNA was performed on the PCR products of 145 bacterial strains representing the two most numerous groups of microorganisms, biochemically identified as the *Shewanella putrefaciens* group (73 isolates) and *Pseudomonas* spp. (72 isolates). A dataset was generated with 1,260 positions for the *S. putrefaciens* group, in which three species were distinguished: *S. baltica* (n = 69), *S. hafniensis* (n = 2), and *S. denitrificans* (n = 1). One isolate (SeaQual_254/4) was located in a separate clade close to *Shewanella* sp. CECT:7627, pdp11, but could not be classified to the species level (Fig. 10) (45). Their sequences were deposited in the GenBank database under accession numbers MT516218–MT516290.

Molecular analysis of the PCR products of 72 isolates belonging to the *Pseudomonas* genus revealed the following species in the 1,163-position dataset: *Ps. gessardii* (n = 10), *Ps. guineae* (n = 9), *Ps. laurentiana* (n = 5), *Ps. knackmussii* (n = 3), *Ps. putida* (n = 1), *Ps. lundensis* (n = 1), *Ps. marincola* (n = 1), *Ps. umsongensis* (n = 1) and *Ps. caeni* (n = 1). The attempt to classify the remaining 40 *Pseudomonas* isolates into particular species was unsuccessful (Fig. 11). These sequences were also entered into GenBank, being assigned the numbers MT626757–MT626827 and MT628350.

Minimum inhibitory concentration analysis. The phenotypical characteristics of the isolated bacteria which were investigated included their antimicrobial resistance. The MICs are epidemiological cut-off values categorising isolates as possessing (NWT) or not possessing (WT) mechanisms that reduce their susceptibility to drugs. They were determined for the chosen groups of bacteria and are presented in Fig. 12. Wild-type and NWT isolates were distinguished in each tested group of microorganisms. The highest number of NWT strains (≥8) was found with resistance against sulphamethoxazole and amoxicillin and clavulanic acid in all groups of studied isolates. The diversity of WT and NWT distribution among individual bacterial groups was evident in the collected isolates of *Acinetobacter* spp., *Pseudomonas* spp. and *Shewanella* spp. (Fig. 12).

Discussion

Numerous studies regarding fish health disorders, including Baltic cod skin ulceration, have been conducted since the 1980s (27, 28, 29). During this period, various trends in the extent of the diseases in cod were recognised (29, 40). For example, the highest prevalence of fish ulceration (40%) was recorded in 1982 in the Bornholm area (28), while in 1990, only 5% of fish from there were observed to be diseased (36). At the beginning of the 2000s, the prevalence of Baltic cod skin ulceration increased in the southeastern part of the Baltic Sea (23, 24), and in this location, mainly in the Gdańsk Bay as one of the species' primary spawning areas, increased episodes of skin ulceration in Baltic cod have been recorded regularly since the beginning of the 21st century (7, 23, 24). Our studies confirmed these data, indicating the Gdańsk Bay as an area with a higher number of diseased cod than any of the other four research fishing grounds.

The clinical signs observed in cod were described as an externally visible fish disease (EVFD), a term defining pathological changes commonly found in wild marine fish and used as indicators in environmental monitoring programmes (41). These pathological changes may have an infectious (*e.g.* viral, bacterial or parasitic) or non-infectious (various causes) aetiology. The occurrence of EVFD or changes in their prevalence are considered generic, non-specific indicators of habitat quality and environmental stress, reflecting the well-being of fish and the status of their specific and non-specific immune systems' functioning (41). Considering

what EVFD designation implies and the results of the conducted studies, the Gdańsk Bay should be regarded as a stressful area for fish with a low welfare value.

During our study of Baltic cod, various stages of development were detected in the visible skin disorders (Figs 4–9). As mentioned above, the observed pathological signs of this kind may have an infectious aetiology. The pertinent literature offers limited data from comprehensive microbiological studies of such skin lesions in Baltic cod. Mallergaard and Bagge (42) described the isolation of *Aeromonas* spp., *Pseudomonas* spp. and *Vibrio anguillarum* from ulcers of three Baltic cod caught around the island of Bornholm. Our research also revealed the presence of *Aeromonas* spp. and *Pseudomonas* spp. in ulcers of cod from this fishing ground. However, accompanying bacteria such as *Acinetobacter* spp., *Shewanella putrefaciens* group and *Stenotrophomonas maltophilia* were also detected. Irrespective of the fishing ground, the following microorganisms were most commonly isolated from cod skin lesions: motile mesophilic *Aeromonas* spp., *Pseudomonas* spp. and *Shewanella putrefaciens* group. For this reason, we consider that these three groups of bacteria are of significant importance in the aetiology of skin lesions in cod, as they are a lesion aetiology in different species of freshwater fish, where they cause severe economic losses in aquaculture (37, 39, 44, 48). The pathogenicity of mesophilic and motile *Aeromonas* species to various cultured and ornamental fish has been previously described (19, 37, 57). This group of bacteria mainly causes pathological lesions observed on the body surface, where dermatitis, skin lesions, and ulcers penetrating subcutaneous muscles were noted (15, 37, 39). Similar clinical signs manifested in fish as necrotic skin lesions or ulcers were detected in infections caused by the *Shewanella putrefaciens* group (46, 47, 48). Although bacteria of *Pseudomonas* spp. are considered opportunistic pathogens, their pathogenicity to various fish species like the Japanese eel (*Anguilla japonica*), rainbow trout (*Oncorhynchus mykiss*) and tilapia (*Oreochromis mossambicus*) has also been reported (3, 18, 50). Darkening of the skin, anaemia of the gills and fin damage manifested infections in fish caused by these bacteria. Abnormal mortalities were also observed (44). It is necessary to also point out that *Pseudomonas* spp. and *Shewanella putrefaciens* are opportunistic human pathogens and have been reported to harm human health, causing bacteraemia and skin and soft tissue infections (14). Those bacteria are also involved in the spoilage process of seafood products (43).

Infections caused by motile mesophilic *Aeromonas* in fish vary in severity depending on the geno- and phenospecies and serogroup of the bacteria causing them (37, 39). The extent of the information for *Aeromonas* shows the data available in the literature regarding fish infections caused by *Pseudomonas* spp. and *Shewanella* spp. to be limited. Sequencing of the 16S rRNA gene of the collected isolates of *Pseudomonas* spp. and *Shewanella* spp. was performed to expand the knowledge in this field.

Based on the 16S rRNA gene sequences of bacteria belonging to the *Shewanella putrefaciens* group isolated from Baltic cod skin ulcers, three species were distinguished – *S. baltica*, *S. hafniensis* and *S. denitrificans* (45). It should be emphasised that the *S. baltica* species was dominant in this group (69 isolates out of 73 tested). The analysis of the available literature showed that *S. baltica*, biochemically identified as *S. putrefaciens* (34, 35), also dominated during the storage of iced fish in the Baltic Sea (56). This species was isolated from skin ulceration in carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), shrimp (*Neocaridina* spp.) and big-belly seahorse (*Hippocampus abdominal*), where mortality was observed (35). In the available literature, there is only one report regarding isolating *S. hafniensis* in cod from the Baltic Sea (52). *Shewanella denitrificans*, on the other hand, had not previously been isolated from any fish species but from the water column of a basin with anoxic deep water in the central Baltic Sea (Gotland Deep) (10). Therefore, our isolation of *S. denitrificans* from Baltic cod is the first in the literature. One isolate of *Shewanella*, which was not classified to species level, showed high similarity to the non-pathogenic, probiotic pdp11 strain isolated from gilt-head sea bream (*Sparus aurata*) (51).

Diversity among the *Pseudomonas* species isolated from Baltic cod skin ulcers was shown by sequencing 16S rRNA genes. The following nine *Pseudomonas* species were identified: *Ps. gessardii*, *Ps. guineae*, *Ps. laurentiana*, *Ps. knackmussii*, *Ps. putida*, *Ps. lundensis*, *Ps. marincola*, *Ps. umsongensis* and *Ps. caeni*. However, most isolates categorised in this group were not classified to the species level. Multiple *Pseudomonas* species have been reported in various fish species. *Pseudomonas aeruginosa*, *Ps. anguilliseptica*, *Ps. chlororaphis*, *Ps. fluorescens*, *Ps. oleovorans*, *Ps. plecoglossicida*, *Ps. putida* and *Ps. tructae* are responsible for infections in ayu (*Plecoglossus altivelis altivelis*), rainbow trout, yellow tail (*Seriola quinqueradiata*), tilapia, and Japanese and European eels (*Anguilla japonica* and *Anguilla anguilla*) (9, 13, 44). Only one species, *Ps. putida*, a significant pathogen in rainbow trout fisheries causing disease outbreaks with skin ulceration and fish mortality up to 35% (3), was isolated during our study. The roles of many fish-associated bacteria need to be clarified because fish carry a diversity of bacterial taxa, which often reflects the microflora composition of the surrounding water (5). This conclusion is undoubtedly true for the *Pseudomonas* bacteria isolated from Baltic cod skin ulcers. As shown in our research, in addition to the *Ps. putida* described elsewhere as pathogenic, the other eight species that were isolated from fish should be included in the group of *Pseudomonas* bacteria pathogenic to fish. Their isolation from cod has been correlated with the occurrence of skin ulceration in these fish.

The research has shown that which bacteria could be isolated from Baltic cod skin ulcers depended on the fishing area. Regardless of the region, *Acinetobacter* spp. and *Aeromonas* spp. were isolated in addition to the

bacteria of the two genera described above. A wider diversity of microorganism species was also found in cod caught in the Gdańsk Bay: *Alcaligenes faecalis*, *Chryseobacterium indologenes*, *Enterococcus faecalis*, *Microbacterium* spp., *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia* and *Vibrio* spp. Among these, *Acinetobacter* spp. and *Stenotrophomonas maltophilia* are known to be associated with health disorders in fish, the first in rainbow trout and carp and the second in African and channel catfish (*Heterobranchus bidorsalis* and *Ictalurus punctatus*) (1, 22, 38, 49).

Epidemiological cut-off values estimated by MIC aim to categorise isolates based on whether they possess mechanisms that reduce their susceptibility to antimicrobials (53). However, interpreting the obtained results is difficult because the available data with their interpretative criteria are limited (54). Considering the global threat of the spread of bacterial resistance to antimicrobials, the presence of *Stenotrophomonas maltophilia* and *Acinetobacter* spp. in fish ulcers is significant. These bacteria are currently classified as multidrug-resistant microorganisms, they have inherent resistance to a wide range of antibacterial agents, and they are mainly associated with human respiratory diseases (11). The low MICs of quinolones against *Stenotrophomonas maltophilia* and *Acinetobacter* spp. determined in our study are noteworthy in light of the classification of this antimicrobial group to category B ("Restrict"), which is a group critically important to human medicine, and one of which the use in animals should be restricted to mitigate the risk to public health (20). The same trends applied to other isolates, including *Pseudomonas* spp. The MIC values obtained in our study for *Aeromonas* strains are highly significant in the context of the role of these bacteria as resistance indicators in aquatic environments (25). Isolates belonging to the *Aeromonas* genus often show acquired resistance determinants. Therefore, they have been used as indicators to survey water quality and wastewater pollution. Wild aquatic species such as cod must also not be ignored in this context. They constitute potential antimicrobial resistance reservoirs in natural environments, as they are actively involved in spreading resistant bacteria and determinants of resistance in various habitats (25).

The occurrence of health disorders in fish may relate to the pollution of the Baltic Sea. Most parts of this area were classified as "disturbed by hazardous substances", the substances being heavy metals and persistent organic pollutants but also dumped chemical munitions, including chemical warfare agents from the Second World War (26, 27, 29). Those substances can irritate the skin through direct contact, causing damage and erosion. The evidence of the effect of such hazardous substances could be noted in the Baltic cod caught around the Bornholm area, where around 34,000 tonnes of chemical munitions, including approximately 12,000 tonnes of chemical warfare agents, were dumped east of

Bornholm and near Gotland in 1947 (27). Our studies indicated atypical skin lesions in fish caught near the Bornholm area (Fig. 9), which differed from those found in cod caught in other regions of the Baltic Sea (Figs 4–7). These disorders were not characteristic of the clinical picture of the typical ulcers caused by bacteria. The skin lesions were shallow and did not penetrate deep into the tissues in these cases. There was no zone of inflammation and necrosis characteristic of the ongoing process resulting from the infection caused by the bacteria. Therefore, the theory regarding the impact of exposure to chemical substances in the Baltic Sea on fish health remains unproven. The mechanism of the effect of the chemicals and other contaminants on fish skin is insufficiently studied. It is known that sustained exposure to even low concentrations of anthropogenic pollutants can injure the epithelium of fish and cause immunosuppression (4). On the other hand, other unusual skin lesions in regular circular shapes have been observed in some Baltic cod caught in the Gdańsk Bay (Fig. 8). Such clinical signs, similar in shape to the mouth of a lamprey, in combination with the location where the fish bearing these injuries were caught, may suggest that the fish were bitten by European river lamprey (*Lampetra fluviatilis*) rather than suffering a disease mediated by bacteria (data not published).

Conclusion

It was shown that different bacteria species could be isolated from Baltic cod skin ulcers. Most of them were indicated to be opportunistic fish pathogens, which are known to be widespread in the aquatic environment and potentially harmful to humans.

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