Shewanella spp. from wastewater treatment plant-affected environment: isolation and characterization

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Abstract

Bacteria from the genus *Shewanella* are inhabitants of marine and freshwater ecosystems, recognized fish spoilage bacteria, but less known as fish disease agents. *Shewanella* spp. isolated

from fish living in waters close to effluents of a wastewater treatment plant (WWTP) were not previously characterized. We have tested *Shewanella* isolates from WWTP-affected waters and related fish. Genotypic characterization identified most strains as *S. baltica* and *S. oneidensis*. In order to investigate the sensibility and accuracy of their MALDI-TOF MS identification, they were grown on two culture media enriched by various NaCl concentrations, incubated at different temperatures and duration. We analyzed their antimicrobial susceptibility on a panel of antimicrobial drugs and capacity for biofilm production. With a view to demonstrate their capacity to produce fatty acids, we assessed the impact of different culture media on their lipid profile. We performed zebrafish embryotoxicity tests to simulate the environmental infection of the earliest life stages in *S. baltica*-contaminated waters. The best MALDI-TOF MS identification scores were for strains cultivated on TSA for 24 h at 22 °C and with supplementation of 1.5% NaCl. Less than 17% of isolates demonstrated antimicrobial resistance. Most isolates were weak biofilm producers. Strain-to-strain variation of MIC and MBC was low. The major fatty acids were C15:0, C16:0, C16:1, C17:1, and iC15:0. Exposure of *Danio rerio* to different *S. baltica* concentrations induced severe effects on zebrafish development: decreased heartbeat rate, locomotor activity, and melanin pigmentation. *S. baltica* passed through chorionic pores of zebrafish.

Keywords:

WWTP, *Shewanella*, ~~z~~Zebrafish, ~~i~~Identification, ~~f~~Fatty acid profile, Aa~~a~~ntimicrobial resistance, ~~b~~Biofilm, MALDI-TOF MS

Abbreviations~~:~~

AMC: aAmoxicillin

AW: Aartificial water

BCFA: bBranched chain fatty acid

CFU: Ceolony-forming unit

E: eErythromycin

EPA: Eeicosapentaenoic fatty acid

FAME: fFatty acid methyl ester

FFC: fFlorfenicol

MALDI-TOF MS: mMatrix-assisted laser desorption/ionization time of flight mass spectrometry

MBC: mMinimal bactericidal concentration

MBIC: mMinimal biofilm inhibitory concentration

MCA: MacConkey agar

MH: Mueller-Hinton agar

MIC: mMinimal inhibitory concentration

MUFA: mMonounsaturated fatty acid

NOR: nNorfloxacin

OA: oOxolinic acid

OD: oOptical density

ODc: oOptical density cut-off

OTC: oOxytetracycline

PUFA: pPolyunsaturated fatty acid

PVF: pPerivitelline fluid

SFA__: sSaturated fatty acid

SMX__: sSulfamethoxazole

TSA__: Tryptic soy agar

UB___: fFlumequine

WWTP_____: wWastewater treatment plant

ZET__: ZzebrafishZebrafish embryotoxicity test

1. Introduction

Bacteria from the genus *Shewanella* are Gram-negative, motile, rod-shaped bacteria, belonging to the family *Alteromonadaceae* (Bowman, 2005). They can be found in marine and brackish ecosystems, and can also be isolated from freshwater environments. *S. putrefaciens*, the most investigated species of the genus, is classified into several genomic groups, two of which were designated as new species — *S. baltica* and *S. algae* (Vogel et al, 1997; Ziemke et al, 1998). It is a recognized marine fish spoilage bacterium and important in the breakdown of organic matter (Buller, 2014; Pękala et al, 2015). It is less known as a fish disease agent, although it was associated with recent outbreaks in farmed fish, as well as ornamental fish worldwide (Kozińska and Pękala, 2004; Altun et al, 2014; Qin et al, 2014; Rusev et al, 2016; Esteve et al, 2017; Walczak et al, 2017; Sicuro et al, 2020).

Fish disease signs are frequently non-specific, such as lethargy, skin discoloration and skin darkening with lesions including ulcers, mouth erosion, exophthalmia, ascites, hemorrhage in kidney and spleen, necrosis in kidney, and bad odor (Esteve et al, 2017; Jung-Schroers et al, 2017; Pękala-Safińska, 2018). Mortality related to *Shewanella* spp. infection is up to 33% in farming conditions, but over 80% after laboratory infection, depending on fish species (Qin et al, 2012; Pękala et al, 2015). Ingestion of raw fish and contaminated water are risk factors for human infection, as bacteria from genus *Shewanella* are opportunistic human pathogens, and known to cause septicemia, soft tissue infections, otitis, and hepatobiliary infections, mostly in patients with underlying conditions (Vignier et al, 2013; Latif et al, 2019).

On the other hand, *Shewanella* isolated from guts of marine fish are able to colonize the intestines of freshwater fish (*Danio rerio*), and may be inoculated to farmed fish to prevent

harmful bacteria from colonizing their intestines (Jammal et al, 2017). *S. putrefaciens* was thus successfully used as fish probiotic (Cordero et al, 2016). Dietary administration of *S. putrefaciens* to *Sparus aurata* had beneficial effects as it depressed gene expression of pro-inflammatory cytokines (Chen et al, 2020). It also has a potential of producing omega-3 polyunsaturated fatty acids (PUFAs) (Zhang and Burgess, 2017; Satomi, 2014).

The identification of *Shewanella* spp. is possible by commercial phenotypic plate and tube methods, commercial biochemical methods, gene sequencing, and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Pękala et al, 2015; Jung-Schroers et al, 2017). However, it is known that the correct identification of bacteria by MALDI-TOF MS, granted the database coverage, is highly dependent on culture medium, temperature, and time of incubation (Kazazić et al, 2019a, 2019b). That has not been previously tested for *Shewanella* spp., nor their cellular fatty acid profiles related to various culture media. Similarly, although zebrafish (*D. rerio*) models for several pathogenic bacteria exist (Nag et al, 2020), the impact of *Shewanella* spp. on zebrafish during an early ontogenesis to date is unknown.

Therefore, with the aim to thoroughly characterize *Shewanella* spp. isolated from the WWTP-affected environment and examine its clinical relevance, we tested *Shewanella* isolates from WWTP-affected waters and related fish. In order to investigate the sensibility and accuracy of MALDI-TOF MS identification, the isolates were grown on two culture media enriched by various NaCl concentrations, incubated at different temperatures and incubation times. Furthermore, we analyzed their biofilm production capacity and antimicrobial susceptibility on a panel of antimicrobial drugs in order to evaluate susceptibility/resistance to antimicrobial agents, as the isolates were impacted by communal wastewaters. With a view to demonstrate the

capacity of our isolates to produce secondary metabolites, we assessed the impact of various culture media on their cellular fatty acid profiles. We performed zebrafish embryotoxicity tests on a wide range of bacterial concentrations to simulate the infection of the earliest life stages, all with a purpose to determine the potentially negative impact of *Shewanella*-contaminated waters on early life stages of non-target aquatic organisms.

2. Materials and mMethods

2.1. Bacterial strains

The strains in this work were the *Shewanella* spp. isolates, mainly *S. baltica* from freshwater fish (10 isolates), and their respective water body (2 isolates, *S. oneidensis*, as per 16S rRNA partial gene sequencing). A total of 12 strains were used in this study. They were initially cultured on Tryptic soy agar (TSA) which inherently contains 0.5% NaCl. Subsequently, they were regrown on the same growth medium, and on TSA additionally supplemented with 0.5, 1.0, 1.5, and 2.0% NaCl. They were also cultured on MacConkey agar (MCA), and on MCA additionally supplemented with 0.5, 1.0, 1.5, and 2.0% NaCl. All agars were Oxoid Ltd (England, UK). All plates were incubated at 22 °C and at 37 °C for 24 and 48 h ours before further analyses.

For zebrafish in vivo testing, *S. baltica* isolates were prepared as inocula in artificial water (AW; ISO, 1996), scraping the cells off unsupplemented TSA plates and suspending them in AW. The concentrations of bacteria were 7.5×10^7, 1.5×10^8, 9×10^8, and 2.1×10^9 CFU/mL,

as determined by Densimat (bioMerieux, Marcy l'Etoile, France), measuring the turbidity of a bacterial inoculum produced in an ampoule with AW.

2.2. Fish in the study

Most bacterial isolates in this study (*S. baltica*) were retrieved from freshwater fish living downstream from a canal receiving the effluent of a Croatian municipal wastewater treatment plant. Fish were mainly Prussian carp (*Carassius gibelio*) and European chub (*Leuciscus cephalus*), caught by recreational anglers and handed over as fresh catch (not alive) (Table 1). They were immediately manipulated by the licensed veterinarians in accordance with the national legislation. Swabs from the gills, liver, and kidney were streaked onto TSA.

Zebrafish *Danio rerio* wild-type WIK strains were obtained from the European Zebrafish Resource Center of the Karlsruhe Institute of Technology (KIT). Zebrafish maintenance and embryo production are described in detail in our previous study (Babić et al. 2017). Zebrafish housing and spawning were conducted in aquaria units approved by the Croatian Ministry of Agriculture. All tests on zebrafish were performed on the non-protected embryonal stages (up to 120 hpf), which do not require permission by animal welfare commissions. In handling all fish, relevant institutional ethical standards and international guidelines for the experimental use of aquatic animals were applied (EC Directives 86/609/EEC and 2010/63/EU).

2.3. Chemicals

Artificial water (AW) was prepared using chemicals all obtained from Sigma--Aldrich (Deisenhofen, Germany): calcium chloride dihydrate (CAS No. 10035-04-8; $CaCl_2x2H_2O$), magnesium sulfate heptahydrate (CAS No. 10034-99-8; $MgSO_4x7H_2O$), sodium bicarbonate (CAS No. 144-55-8; $NaHCO_3$), and potassium chloride (CAS No. 7447-40-7; KCl). For labelling bacterial cells, 5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein hydrochloride (CAS No. 21811-74-5; 5-DTAF) was used (Sigma--Aldrich). MALDI-TOF MS matrix and formic acid were from Bruker Daltonics, Bremen, Germany, and Fisher Chemical, Finland, respectively.

2.4. Biochemical tests and 16S rRNA gene sequence analysis

All isolates were analyzed by conventional plate and tube tests (Austin and Austin, 2016) and biochemical methods, namely API 20E panels (bioMerieux), with modified incubation temperature and duration (Topić Popović et al, 2007). The profile numbers were identified with the *apiweb* software (bioMerieux).

Amplification of the 16S rRNA of all isolates (as biological duplicates) was performed using universal primers (27F and 1492R, Lane, 1991), and the bacterial colonies were used directly as a PCR template (Colony PCR). A colony of each isolate was picked out with a sterile toothpick and suspended in 50 µL of sterile water, centrifuged for 5 min at 6 000 rpm, and 25 µL of clear supernatant was pipetted into a new tube. Amplification reactions were performed in 25 µL reaction volume using 1 µL of the supernatant and EmeraldAmp MAX PCR Master Mix (Takara). The PCR profile consisted of initial denaturation at 95 °C for 10 min, followed by 30

cycles of 95 °C for 45 s, 52 °C for 45 s, and 72 °C for 90 s, and final extension at 72 °C for 10 min. The PCR products were verified by 1% agarose gel electrophoresis and enzymatically purified with ExoSAP-IT Express PCR Product Cleanup (Affymetrix). The samples were sequenced with the same primers used for amplification at the Macrogen Europe B.V. (Amsterdam). Alignment and edition of sequences in both directions were carried out with the software Sequencher v.4.1.4. Sequences were compared with BLAST and were submitted to GenBank.

2.5. Matrix-assisted laser desorption/ionization time of flight mass spectrometry identification (MALDI-TOF MS)

Two biological replicates of each isolate were applied on a 96-spot polished stainless steel target plate by the on-target extraction method. The isolates were cultured on TSA and MCA, without and with the addition of NaCl (0.5, 1.0, 1.5, and 2.0%) after 24 and 48 h of incubation at 22 °C and 37 °C. Procedures were conducted in triplicates up to quintuplicates. In short, a pure colony was smeared on a target plate spot (Bruker Daltonics), followed by addition of 1 µL of 70% formic acid. After allowing to dry, each colony was overlaid with 1 µL of MALDI matrix (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid (Bruker Daltonics)). Mass spectra were acquired using a bench-top Bruker Microflex LT mass spectrometer equipped with the Bruker Biotyper 3.0 software (Bruker Daltonics), according to the manufacturer's instructions. The bacterial test standard *Escherichia coli* DH5 alpha spiked with two additional pure proteins (RNAse A and myoglobin) was used for calibration. Ions were

captured in the positive linear mode (mass range 2–20 kDa), and spectra were obtained by 240 laser shots in various regions of every spot. The cut-off logarithmic scores were as per the manufacturer: 0–1.699 unreliable, 1.700 to 1.999 probable genus identification, 2.000 to 2.299 probable species identification, and 2.300 to 3.000 highly probable species identification. Spectra were processed using the MALDI Biotyper Pre-processing Standard Method and the MALDI Biotyper MSP Identification Standard Method adjusted by the manufacturer (Bruker Daltonics, Germany).

2.6. Antimicrobial susceptibility and biofilm formation

Antimicrobial susceptibility of the isolates was determined by the Kirby–Bauer disc diffusion method on Mueller-Hinton (MH) agar (Oxoid) with the following chemotherapeutic agents (µg/disc): oxytetracycline, OTC (30), amoxicillin, AMC (30), oxolinic acid, OA (2), erythromycin, E (15), sulfamethoxazole, SMX (50), florfenicol, FFC (30), norfloxacin, NOR (10), and flumequine, UB (30). Inocula were prepared from overnight growth on TSA to 0.5 MacFarland by turbidity adjustment in sterile 5 mL 0.85% Suspension ~~m~~Medium (bioMerieux) and subsequently plated on MH agar using sterile cotton swabs. The diameter of the zones of inhibition was read and expressed by referring to the manufacturer's standard table and reported as susceptible, intermediate, or resistant (Table 1). The isolates were also tested using broth microdilution method for the assessment of minimal inhibitory concentration (MIC) in cation-adjusted MH broth by serial 2-fold dilutions in 96-well microtiter plates according to the CLSI guidelines (CLSI, 2015). MIC was determined as the lowest concentration of an antimicrobial

completely inhibiting growth of strains by visual assessment. Minimal bactericidal concentrations (MBC) were determined by culturing 20 µL of broth from wells exhibiting MIC and all wells with higher concentration of antimicrobials as available on TSA for 72 h~~ours~~ of incubation. MBC was determined as the lowest concentration killing 99.9% of bacteria (Balouiri et al. 2016). Broth microdilution tests were performed with a total volume of 100 µL per well and employed a positive growth control (drug-free wells), a negative sterility control (wells with culture media only), and a reference control strain *E. coli* NCTC 12241 according to Miller et al. (2003).

In addition, a subset of isolates were used for testing of the capacity for biofilm production according to Stepanović et al. (2007) with minor modifications. The cation-adjusted MH broth was supplemented with 1% (w/v) glucose with a total volume of 200 µL per well and similar to broth microdilution assays employed positive and negative controls. After incubation, each well was washed three times with 300 µL of sterile phosphate-buffered saline (PBS; pH 7.2), heat-fixed by exposure to hot air at 60 °C for 1 h ~~our~~ followed by staining with 2% crystal violet for 15 min at room temperature. The stain was aspirated with a pipette and excess stain rinsed off by placing the microtiter plate under running tap water and thereafter dried at room temperature. The dye bound to the cells was resolubilized with 150 µL of 95% ethanol per well and left covered at room temperature for 30 min before optical density (OD) of each well was measured at 570 nm using a spectrophotometer (Infinite 200 Pro, Tecan, Austria). The cut-off value (ODc) was defined as three standard deviations above the mean OD of the negative control (media only). Minimal biofilm inhibitory concentrations (MBIC) were determined as the lowest concentration of an antimicrobial with the average OD of a strain lower than ODc. For evaluation of the capacity for biofilm production, the strains were grown without antimicrobials

and categorized as follows: OD ≤ ODc = no biofilm producer, ODc < OD ≤ 2xODc = weak biofilm producer, 2xODc < OD ≤ 4xODc = moderate biofilm producer, and 4xODc < OD = strong biofilm producer. The antimicrobials used for broth microdilution and biofilm formation assays included, ampicillin, norfloxacin, flumequine, erythromycin, florfenicol, and oxytetracycline.

All antimicrobial susceptibility and biofilm formation tests were performed on two independent occasions each time in technical duplicates in aerobic atmosphere at 22 °C for 24 h of incubation unless otherwise stated. MIC, MBC_2 and MBIC indices were reported as subscripted 50% and 90% values indicating concentrations observed for 50% (n = 6) and 90% (n = 11) of the isolated strains.

2.7. Zebrafish embryotoxicity test (ZET)

In order to simulate an early life exposure to contaminated waters, zebrafish embryotoxicity test (ZET) was performed following OECD (203), with slight modifications. To identify whether the embryonic or larval stage of zebrafish development is more sensitive to *S. baltica* exposure, the experimental design was adjusted as follows.

2.7.1. Embryonic infection procedure

Fertilized eggs (up to 64-cell blastomeres) were transferred into a 24-well plate (NEST Scientific, USA) containing 2 mL of pre-warmed (27 ± 0.5 °C) test sample. AW was used as a dilution medium and negative control. Ten embryos per well were exposed in five replicas, amounting to a total of 50 embryos per each *S. baltica* concentration (7.5×10^7, 1.5×10^8, 9×10^8, and 2.1×10^9 CFU/mL) and a negative control. Plates were incubated (27.0 ± 0.5 °C) under static conditions in Innova 42 incubator shaker (New Brunswick). Lethal and sublethal effects on embryos/larvae were recorded daily during 120 h of exposure (Fig. 1a) using Leica DMIL LED inverted microscope, equipped with Leica EC3 digital camera. Furthermore, cardiotoxicity was determined (from 48 to 120 hpf) by measuring heartbeats per 15 s~~ee~~. The rate of pigmentation formation and hatching rate were assessed in specimens from 48 to 120 hpf using a score ranging from 0 to 3 (0 – no pigmentation; 1 - decreased body and eyes pigmentation; 2 – decreased body pigmentation and normal eyes pigmentation; 3 – fully formed pigmentation), and the percent of hatched larvae, respectively.

2.7.2. Larval infection procedure

Fertilized eggs were incubated (27.0 ± 0.5 °C) in AW for 72 h. At 72 hpf, healthy larvae were exposed in a static exposure regime (n = 10 larvae per well) towards three *S. baltica* concentrations (7.5×10^7, 1.5×10^8, and 9×10^8 CFU/mL), while the non-challenged controls were exposed solely to AW. The test was carried out in five replicas of 10 larvae, amounting to a total of 50 larvae per each sample. Lethal and sublethal effects were recorded at 72, 96, and 120

hpf (Fig. 1b). During three days of exposure to tested bacterial concentrations, heartbeats and hatching rates, as well as pigmentation formation, were recorded.

2.7.3. Labeling of S. baltica

The presence of *S. baltica* inside the embryos/larvae was observed using fluorescent substrate 5-DTAF. The method was adapted from Rojas et al. (2015). Briefly, bacterial cells were recovered in tryptic soy broth (Oxoid) at 22 °C and agitated in orbital shaker at 100 rpm over 24 h. The suspension was subsequently centrifuged at 4200 rpm for 16 min at 22 °C and the pellet resuspended in 4 × 10 mL of AW, adjusting the density to 3.5 – 4.2 MacFarland (McF). 5-DTAF was dissolved up to 0.5 mg/mL in sterile PBS, and filtered (0.22 μm, pH 7.4). An aliquot of 0.5 mL of 5-DTAF solution was added to the 9.5 mL bacterial suspension and agitated in shaker (90 rpm), in darkness for 1 h. Another centrifugation was performed as above, and pellet was resuspended in AW, repeating the process until suspension became unstained. The bacterial density was adjusted to 7.5×10^7, 1.5×10^8, 9×10^8, and 2.1×10^9 CFU/mL.

The identical embryonic infection procedure was performed as previously described (see 2.7.1.) with the exception of immersing embryos ($n = 40$) in a mixture of each concentration of 5-DTAF-labeled *S. baltica*. From 48 to 120 hpf, specimens ($n = 7$) were rinsed three times with AW and anesthetized with 150 mg/L MS-222. Fluorescence was observed using a fluorescence microscope (Olympus® BX51 light binocular microscope equipped with the Microsoft® AnalySIS Soft Imaging System Software) with a green fluorescent filter.

2.7.4. Statistical analysis

Statistical differences between zebrafish treatment groups and negative control (AW) were evaluated by one-way analysis of variance (ANOVA) implemented in GraphPad Prism 6.01. (GraphPad Software Inc., USA). When the assumption for normality was violated, the Kruskal-Wallis test was performed. The results are expressed as means ± SD, and $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*) were used as a cut-off value of statistical significance. The results of the heartbeat rate are presented as box-plots. A line within the box represents the median value, while the boundaries of box-plot indicate 25th and 75th percentiles. Whiskers above and below the box indicate 10th and 90th percentiles.

2.8. Lipid extraction and preparation of fatty acid methyl esters

Bacterial cells were weighed (approx. 50 mg) and washed in PBS. An internal standard (methyl nonadecanoate, C-19:0) was added to each sample, and extraction of total lipids was performed according to Christie and Han (2010). Fatty acid methyl esters (FAMEs) were prepared by sulfuric acid catalyzsed trans-esterification (Christie and Han, 2010), and the corresponding FAMEs were extracted with n-hexane and analyzed by gas chromatography (GC). GC analyses of total fatty acids were performed by Varian 450-GC equipped with a flame ionization detector. A Stabilwax column (eCrossbond eCarbowax polyethylene glycol, 60 m × 0.25 mm) was used as a stationary phase at a programmed temperature with helium as the carrier gas. The heating was

carried out at a temperature of 150 °C for 1 min followed by an increase of 5 °C/min up to 250 °C. Methyl esters were identified by comparison with the retention times of authentic samples.

3. Results

3.1. 16S rRNA gene sequencing of strains

NCBI-BLAST search results indicated that our samples belong to two *Shewanella* species, with next to negligible differences. All the isolates from fish were identified as *S. baltica* (*n* = 10) with the similarity to the GenBank closest hit (*S. baltica*, accession number LR134321) ranged from 99.58% to 100%. The two isolates from water downstream of the WWTP effluent were identified as *S. oneidensis* (99.79% similarity to the *S. oneidensis*, acc. number JQ795830).

3.2. Biochemical profile

All the strains were tested by API 20E panels in order to investigate their variations in biochemical patterns. Their biochemical identification profiles gave three typical numerical scores (Table 1), which all indicated to *S. putrefaciens* with an 89.0–99.9% identification reliability. Since *S. putrefaciens* is the only *Shewanella* spp. in the *apiweb* database, its identification is limited regarding the precise species identification within the genus. Our isolates were all positive for cytochrome oxidase, H_2S production, and variable for ornithine decarboxylase, citrate utilization, and gelatinase production. They were negative for ß-

galactosidase, arginine dihydrolase, lysine decarboxylase, urease, tryptophan deaminase, indole production, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin, and arabinose fermentation, as well as for cytochrome oxidase.

Table 1– Identification results for 12 strains from freshwater fish (sample number 1–10) and water (sample number 11–12) downstream of a canal receiving the effluent of a municipal wastewater treatment plant by the biochemical API 20E panels, identified as *S. putrefaciens*. Resistance to antimicrobials by disc diffusion method was tested for oxytetracycline, (OTC), amoxicillin, (AMC), oxolinic acid, (OA), erythromycin, (E), sulfamethoxazole, (SMX), flurfenicol, (FFC), norfloxacin, (NOR), or flumequine, (UB).

Sample number	Source	Tissue	ID result	ID numerical profile	ID %	Clinical signs	Resistant to
1 I9SK	Prussian carp	Gills	*S. putrefaciens*	0400004	89.0	None	None
2 I9SK1	Prussian carp	Gills	*S. putrefaciens*	0400004	89.0	None	None
3 I10SK	Prussian carp	Gills	*S. putrefaciens*	0702004	99.9	None	None
4 I10SK1	Prussian carp	Gills	*S. putrefaciens*	0502004	99.0	None	None

5 I29J	Prussian carp	Liver	*S. putrefaciens*	0400004	89.0	Opercular hemorrhages	~~n~~None
6 I29J1	Prussian carp	Liver	*S. putrefaciens*	0702004	99.9	Opercular hemorrhages	~~n~~None
7 I29B	European chub	Kidney	*S. putrefaciens*	0400004	89.0	Opercular hemorrhages	~~n~~None
8 I29B1	European chub	Kidney	*S. putrefaciens*	0400004	89.0	Opercular hemorrhages	~~n~~None
9 III17B	Prussian carp	Kidney	*S. putrefaciens*	0502004	99.0	None	SMX, AMC
10 III17B1	Prussian carp	Kidney	*S. putrefaciens*	0502004	99.0	none	SMX, AMC
11 I9.4	WWTP water	n.a.	*S. putrefaciens*	0400004	89.0	n.a.	~~n~~None
12 I9.41	WWTP water	n.a.	*S. putrefaciens*	0400004	89.0	n.a.	~~n~~None

n.a. ~~=~~not applicable.

3.3. MALDI-TOF MS identification

As opposed to 16S rRNA, which identified two samples as *S. oneidensis*, MALDI-TOF MS measurements identified all of the tested strains as *S. baltica.* The type of the culture medium affected MALDI-TOF MS identification results. Overall, the TSA medium enabled a correct identification against the Biotyper database as probable genus identification or probable species identification in 84.53% of all measurements, irrespective of NaCl supplementation, time of incubation, and incubation temperature (Table 2). The probable species identification on TSA (cut-off scores over 2.000) overall were 34.55%, while probable genus identification (cut-off scores 1.700–1.999) were 49.97%. Concurrently, the MCA medium allowed a correct identification in 56.59% of all tests (Table 3). The probable species identification on MCA (scores over 2.000) overall were as low as 15.53%, while probable genus identification (scores 1.700–1.999) were 41.06%, and unreliable were 43.41%, for the conditions presented in Table 3.

When subdividing these results according to cultivation conditions regarding time and temperature, the best total identification scores were found for strains cultivated on TSA for 24 h at 22 °C (2.059 ± 0.122) for 75% of tested strains. However, when analyzing the impact of added NaCl on these strains, the 1.5% NaCl stands out as the optimum concentration (2.114 ± 0.076) for 91.67% of strains under these conditions. These results are followed by 1% and 0.5% NaCl added to the medium (2.093 ± 0.097 and 2.092 ± 0.101, respectively), both for 83.33% of strains under respective conditions, and pointing to the probable species identification on TSA for 24 h at 22 °C.

The strains cultured on MCA received poorer identification results than on TSA. When incubated for 48 h at 37 °C, they returned unreliable identification results, which were thus excluded from Table 3. The number of unreliable scores was high also when strains were

cultured for 24 h at 37 °C (1.381 ± 0.193). Their best identification scores were after 48 h at 22 °C (1.954 ± 0.192), for 43.33% of tested strains. The optimum concentration of supplemented NaCl under these conditions was 2% NaCl (2.041 ± 0.123) where 66.66% of strains gave probable species identification. This result was followed by 1.5% NaCl (1.984 ± 0.184), with probable genus identification of 50% of strains.

Table 2 –Identification results for 12 *Shewanella* strains from freshwater fish and water downstream of a canal receiving the effluent of the WWTP, by MALDI-TOF MS (as log scores). Strains were cultured on Tryptone soy agar (TSA) containing 0, 0.5, 1.0, 1.5, 2.0% of added NaCl, incubated at 22 °C and 37 °C, and identified at two time-points (24 h and 48 h). The on-target extraction method was applied in all instances, and strains were assayed in two biological replicates in minimum five measurements each. A total of 1,200 measurements were performed. The scores presented are the best scores obtained for each measured sample, identified as *S. baltica*. Highlighted fields represent probable and highly probable species level identification scores.

Sample number:			1	2	3	4	5	6	7	8	9	10	11	12
Hours	Temperature	TSA NaCl (%)												
24 h	22 °C	0	1.857	2.060	1.976	1.651	2.102	2.022	1.917	1.890	1.810	1.868	1.745	2.130
		0.5	2.090	2.038	2.120	2.226	2.070	2.100	2.150	1.975	2.080	2.270	1.910	2.090
		1.0	2.240	2.147	2.220	2.020	2.100	2.180	2.080	2.113	1.981	2.000	1.900	2.120
		1.5	2.160	2.230	2.110	2.230	2.081	2.168	2.063	2.130	1.960	2.080	2.110	2.050
		2.0	1.942	2.071	2.016	2.120	2.168	2.092	2.112	2.050	1.980	2.101	2.180	2.080
	37 °C	0	2.098	2.108	1.928	1.938	2.052	2.088	1.962	1.921	1.922	1.898	1.627	1.640

		0.5	2.139	1.913	1.828	1.879	1.987	1.968	1.931	1.973	1.823	2.015	1.807	1.670
		1.0	2.145	1.926	1.965	1.942	1.991	2.054	1.796	2.151	1.978	1.927	1.707	1.716
		1.5	2.071	2.104	2.038	1.921	2.051	2.065	2.024	1.878	1.889	1.852	1.688	1.718
		2.0	1.825	1.985	1.983	1.896	1.977	1.551	1.803	2.044	1.910	1.723	1.777	1.761
48 h	22 °C	0	1.853	1.959	1.881	1.804	1.909	1.965	1.959	2.052	1.914	1.905	1.759	1.661
		0.5	1.814	1.943	1.846	1.932	2.010	1.969	2.052	1.575	1.923	1.903	1.802	1.723
		1.0	1.876	2.054	1.917	2.073	1.988	1.853	1.990	1.982	1.967	1.995	1.746	1.693
		1.5	2.089	2.024	2.014	2.070	2.000	1.934	2.136	2.012	2.051	2.010	1.894	2.017
		2.0	1.992	2.144	1.915	1.874	2.056	1.986	2.031	2.048	1.938	1.857	1.978	1.673
	37 °C	0	1.798	1.674	1.570	1.833	1.810	1.927	1.857	1.662	1.763	1.728	1.681	1.654
		0.5	1.693	1.687	1.670	1.792	1.930	1.970	1.840	1.840	1485	1.477	1.547	1.481
		1.0	1.758	1.729	1.578	1.725	1.923	1.879	1.636	1.894	1.886	1.593	1.709	1.873
		1.5	1.795	1.839	1.381	1.622	2.062	1.630	1.514	1.579	1.774	1.775	1.762	1.886
		2.0	2.033	1.951	1.605	1.679	1.669	1.668	1.669	1.244	2.014	1.462	1.746	1.678

Table 3 –MALDI-TOF MS identification results (as log scores) for 12 *Shewanella* strains cultured on MacConkey agar (MCA) containing 0, 0.5, 1.0, 1.5, 2.0% of added NaCl, incubated at 22 °C and 37 °C, and identified at two time-points (24 h and 48 h). The on-target extraction method was applied, and strains were assayed in two biological replicates in minimum five measurements each. A total of 1,200 measurements were performed. The scores presented are the best scores obtained for each measured sample, identified as *S. baltica*. Highlighted fields represent probable and highly probable species level identification scores. Scores of isolates grown on MCA and incubated for 48 h at 37 °C were not reliable (NR; log score < 1.7-), and therefore are not presented.

Sample number:			1	2	3	4	5	6	7	8	9	10	11	12
Hours	Temperature	MCA NaCl (%)												
24 h	22 °C	0	1.895	1.918	1.849	1.953	1.845	1.776	2.054	1.876	1.807	1.819	1.927	1.882
		0.5	1.852	1.948	1.845	1.954	1.857	1.964	1.878	1.923	1.897	1.657	1.911	1.891
		1.0	1.618	1.712	1.476	1.476	1.776	1.881	2.011	1.953	1.698	1.887	1.788	1.905
		1.5	1.728	1.619	1.830	1.893	1.649	1.807	1.735	1.552	1.607	1.577	1.813	1.658
		2.0	1.929	1.797	1.886	1.917	1.716	1.984	1.684	1.526	1.953	1.589	1.374	1.790
	37 °C	0	1.419	1.349	1.728	1.614	1.274	1.341	1.270	1.154	1.681	1.320	1.384	1.468

		0.5	1.779	1.751	1.712	1.732	1.646	1.782	1.466	1.549	1.623	1.468	1.280	1.530
		1.0	1.245	1.161	1.238	1.186	1.276	1.251	1.368	1.336	1.221	1.265	1.310	1.476
		1.5	1.696	NR	NR	NR	1.266	1.212	1.315	1.271	1.240	1.159	1.068	1.291
		2.0	1.666	1.240	1.121	1.350	1.320	1.263	1.276	1.170	1.210	1.311	1.248	1.383
		0	1.893	2.061	1.933	1.957	1.849	1.934	1.943	1.996	1.993	1.854	1.967	2.017
		0.5	2.123	2.172	2.029	2.099	1.881	2.052	2.066	1.964	1.809	1.632	1.637	1.670
48 h	22 °C	1.0	2.241	2.156	1.384	1.357	1.877	2.100	2.082	2.118	1.818	1.959	1.638	1.671
		1.5	1.519	2.218	2.156	2.044	2.080	2.098	1.945	2.086	1.928	1.880	1.927	1.922
		2.0	2.138	1.836	2.234	2.167	2.060	2.066	2.086	2.090	1.876	2.089	1.928	1.923

3.4. Antimicrobial susceptibility

Only 16.66% of isolates (deriving from Prussian carp kidney) demonstrated antimicrobial resistance, to sulfamethoxazole and to amoxicillin, when tested by disc diffusion method (Table 1). These fish had no clinical disease signs, although were caught downstream from a canal receiving the effluent of a municipal WWTP. The results of antimicrobial susceptibility using broth microdilution method are presented in Table 4. Only one isolate was observed as a moderate biofilm producer, while the remaining were weak biofilm producers, and their MBIC values are also presented in Table 4.

Table 4 - Minimal concentrations (µg/mL) of antimicrobials for observed effects of inhibition of growth (MIC), bactericidal effect (MBC), and inhibition of biofilm formation (MBIC) in 50% (n = 6) and 90% (n = 11) of 12 *Shewanella* isolates.

Antimicrobial agents	MIC $_{50 / 90}$	MBC $_{50 / 90}$	MBIC* $_{50 / 90}$
Ampicillin	4 / 16	32 / 64	0.5 / 2
Norfloxacin	1 / 1	1 / 2	0.5 / 1
Flumequine	0.06 / 0.06	0.125 / 0.25	0.03 / 0.06
Erythromycin	4 / 16	16 / 32	4 / 32
Florfenicol	2 / 4	8 / 16	4 / 8
Oxytetracycline	1 / 2	16 / 16	0.5 / 2

*-The capacity for biofilm production and effects of antimicrobials for inhibition of biofilm formation was performed on culture media supplemented with 1% (w/v) glucose.

3.5. Zebrafish embryotoxicity test (ZET)

Exposure of *D. rerio* to different *S. baltica* concentrations induced severe effects on zebrafish development. Mortality and developmental abnormalities depended on bacterial concentration but also on developmental stage of the zebrafish at the time of the exposure. During embryonic exposure (Fig. 2a), high acute toxicity was observed already at 24 hpf on 9×10^8 and 2.1×10^9 CFU/mL, as only 40 embryos (14%) survived. All of the survived embryos showed strong developmental deviations (Fig. 3p-t). Prolonged infection (96–120 h) with 1.5×10^8 CFU/mL of *S. baltica* induced the death of more than 90% of specimens. No significant mortality was observed during exposure to 7.5×10^7 CFU/mL, but severe developmental abnormalities were recorded such as pericardial edema (Fig. 3h, i), yolk sac edema (Fig. 3g, h), and scoliosis (Fig. 3i, j). Even more pronounced toxicity was observed during the larval exposure experiment (Fig. 2b), since among all treatment groups, no specimen survived 24 h exposure. Coagulation and cardiac arrest were the most frequent lethal endpoints observed. Mortality and abnormality rates among the control group on AW were less than 5%.

Based on the high mortality of *D. rerio* upon challenge with *S. baltica*, additional sublethal effects were observed (Fig. 4a). During the entire embryonal exposure duration, concentrations of 7.5×10^7 and 1.5×10^8 of *S. baltica* significantly decreased heartbeat rate, reaching its maximum at 120 h (13 specimens or 45% of decrease, respectively). Reduced cardiac rate of zebrafish was also manifested through a decrease in locomotor activity (reduction and/or absence of spontaneous tail coiling, spontaneous tail contraction, and spontaneous

swimming behavior). Additionally, *S. baltica* did not affect hatching of specimens (data not shown), but significantly decreased melanin pigmentation formation at 48- and 72 h of development (Fig. 4b). The lowest tested concentration (7.5×10^7) of *S. baltica* reduced pigmentation formation for 56%, while the absence of pigmentation was noted in more than 98% of specimens exposed to bacterial concentrations up to 1.5×10^8. On the contrary, bacterial infections had no significant impact on pigmentation formation during later stages of zebrafish development (96 and 120 hpf) (Fig. 3).

Fluorescence imaging of embryos/larvae up to 120 h of exposure to 5-DTAF-labeled *S. baltica* enabled visualization of bacterial distribution within the live specimens (Fig. 5). During the early embryonic development, *S. baltica* mainly deposited in the yolk sac and trunk. At 72 hpf, *S. baltica* entered the cardiovascular system and transferred within the whole body, which was revealed through the brightness of the dorsal aorta. Moreover, significant *S. baltica* accumulation was noted in the eyeball and swim bladder. No fluorescence was observed in the control specimens.

3.6. Cellular fatty acid profiles

From the fatty acid profiles of all *Shewanella* isolates as shown in Table 5, saturated (SFAs) and monounsaturated fatty acids (MUFAs) represented the greatest proportion of total fatty acids. Bacteria grown on TSA media contained 32.39% SFAs, 47.19% MUFAs, 18.18% branched chain fatty acids (BCFA), and 2.16% eicosapentaenoic fatty acid (EPA), the only polyunsaturated fatty acid observed. Following profiles were obtained for the MCA medium:

36.36% SFAs, 36.86% MUFAs, 25.94% branched, and 1.83% of EPA. Among these, C15:0, C16:0, C16:1, C17:1, and iC15:0 were the most abundant fatty acids.

Table 5 –Relative percentage of fatty acids as methyl esters (FAME) expressed as % in total lipid content in cell homogenate grown on different culture media (TSA and MCA). Data is presented as mean value ± SEM.

Fatty acids	TSA (rel % FAME)	MCA (rel % FAME)
C14:0	3.72 ± 0.35	4.85 ± 0.16
C15:0	9.44 ± 0.77	10.55 ± 0.40
C16:0	14.92 ± 0.66	16.00 ± 0.19
C17:0	1.92 ± 0.20	1.99 ± 0.22
C18:0	2.39 ± 0.90	2.97 ± 1.41
Σ Saturated	32.39	36.36
iC13:0	2.80 ± 0.41	4.66 ± 0.11
iC15:0	15.38 ± 1.34	21.28 ± 0.53

Σ Branched	18.18	25.94
C16:1	31.45 ± 0.85	22.28 ± 0.30
C17:1	11.38 ± 1.09	10.04 ± 0.05
C18:1ω9	2.09 ± 0.28	2.97 ± 0.55
C18:1ω7	2.27 ± 0.26	1.57 ± 0.07
Σ Monounsaturated	47.19	36.86
C20:5	2.16 ± 0.45	1.83 ± 0.33
Σ Polyunsaturated	2.16	1.83
TOTAL	100%	100%

4. Discussion

Discharges from WWTPs directly and indirectly affect both terrestrial and aquatic ecosystems (Babić et al, 2016). Treated wastewater discharged from the WWTP in this work modified the environmental parameters and xenobiotic concentrations of the receiving surface waters, while potential bacterial pathogens from fish and respective waters were observed in relatively low numbers, as described previously in Topić Popović et al. (2015). *S. baltica* was retrieved from the gills, liver, and kidney of fish living in WWTP-affected waters, having none to slight clinical signs.

Biochemical and cellular fatty acid profiles

The biochemical profiles obtained by API 20E rapid identification system mostly correspond to the already described properties of the bacterium (Paździor et al, 2019; Austin and Austin, 2016; Pękala et al, 2015). In comparison with charts in Austin and Austin (2016), some of our strains had divergent reactions for ornithine decarboxylase, citrate utilization, and gelatinase production. The isolates described by Pękala et al. (2015) had even greater number of variable phenotypical characteristics. Nevertheless, 16S rRNA gene sequencing in this work identified two species: *S. baltica* (83%) from fish tissues, and *S. oneidensis* (17%) from WWTP-affected water.

There were a total of 12 fatty acids consistently determined in *Shewanella* grown on two different culture media. We identified low-melting-point fatty acids of the genus *Shewanella* including BCFA (i13:0, i15:0), SFA (C16:0), MUFA (16:1), and PUFA (20:5, EPA) which is in agreement with previously reported findings (Venkateswaran et al, 1999). Slightly different percentage of individual fatty acids identified in bacteria grown on two different culture media can be attributed to various nutrient compositions of agars, which leads to a conclusion that various culture media affect the lipid profile of *Shewanella* species.

MALDI-TOF MS identification

MALDI-TOF MS identified all the tested strains as *S. baltica*. In order to investigate the sensibility and accuracy of MALDI-TOF MS identification, we grew the isolates on two culture media enriched by various NaCl concentrations, and incubated them at different temperatures

and incubation times. A general-purpose medium was used (TSA), as well as MCA medium listed in Austin and Austin (2016) as one of the media of choice for *Shewanella* growth. The overall accuracy of MALDI-TOF MS identification of bacteria from TSA significantly outperformed identification from MCA. Valentine et al. (2005) described that bacteria form characteristic signature ions from the constitutively expressed proteins notwithstanding the various media. However, the media we used might have induced diverse metabolic behavior of cultivated isolates and hence overmatched the housekeeping functions of such proteins. For example, phosphate levels and the presence/absence of iron in medium are often cited as affecting secondary metabolite production (Corbett, 1985). Similarly, the impact of media type on MALDI-TOF MS identification accuracy was previously shown for *Photobacterium damselae* (Kazazić et al, 2019b) and *Staphylococcus aureus* (Walker et al, 2002). The impact of temperature on identification sensibility and accuracy was even more adverse, particularly for MCA, as 37 °C yielded a prevailing number of not reliable identification scores for both 24 h and 48 h time-points. However, for TSA 37 °C incubation gave a 91.60% and 55.00% of scores pointing to probable genus or species identification for both time-points, respectively. That can be explained with the strain growth on a particular medium, as indeed, our strains did not grow optimally on 37 °C on MCA, hence their poor identification. Although *Shewanella* do grow between 4 and 37 °C but not at 40 °C, growth is not supposed to occur in the absence of sodium chloride according to Austin and Austin (2016). Therefore, we enriched our media with four concentrations of NaCl, but also successfully grew the isolates on the unamended media. Salinity of media had the impact on the success of correct identification, and for both media, it was a crucial factor for 48 h time-point at 22 °C of incubation. Indeed, the concentration of 1.5% NaCl enhanced the identification on TSA, while on MCA 2% NaCl supplementation increased

MALDI-TOF MS identification accuracy. There are no comparable research results on the impact of NaCl supplementation on MALDI-TOF MS identification of *S. baltica*, which as described by Satomi (2014), does not require Na^+ for growth. We have previously shown that halophilic *Vibrio* (*Listonella*) *anguillarum* (Kazazić et al, 2019a) also demonstrated the enhancement of identification score using the 1.5% NaCl-supplemented TSA.

In summary, the environmental bacterial isolates should be tested regarding their culture conditions and NaCl media enrichment for enhancing their MALDI-TOF MS identification. Additionally, for identification of environmental isolates, it is advisable to compare MALDI-TOF MS data with results obtained from phenotypic and genotypic identification methods, as differentiation between environmental and clinical isolates of the same bacterial species might be significantly diverse, particularly in enzyme activities (Topić Popović et al, 2017). In environmental samples, including the WWTP-related environments, some easily isolated bacteria constitute a minor fraction of the total bacterial community. Therefore, grouping of environmental isolates from the unknown environments is essential in biodiversity studies (Dieckmann et al., 2005). In that sense, MALDI-TOF MS is a powerful tool for a high-throughput dereplication of environmental samples, in recognizing identical bacteria at a specific taxonomic level and their grouping (Ichiki et al., 2008; Ghyselinck et al., 2011).

Antimicrobial susceptibility

The most common classes of antibiotics used worldwide include beta-lactams (penicillin, cephalosporins, monobactams, carbapenems), macrolides, tetracyclines, quinolones, aminoglycosides, sulfphonamides, glycopeptides, and oxazolidinones (Noor et al, 2021). The

major sink for these and other antimicrobials are WWTPs (Babić et al, 2018) because the discharge from industry and excretion by humans and animals eventually end up into WWTPs via sewer networks (Wang et al, 2020). The most frequently detected classes of antibiotics in WWTPs include macrolides, sulfonamides, trimethoprim, quinolones, and tetracyclines. Anjali et al. (2019) summarized the occurrence of antibiotics in the wastewater of several countries, where ciprofloxacin, sulfamethoxazole, and trimethoprim were the most commonly found antibiotics.

Although *Shewanella* are mostly susceptible to a range of antimicrobial drugs, but resistant to cephalosporins, penicillin, and colistin (Cimmino et al, 2016), when tested by disc diffusion method, they were resistant to AMC and SMX. The resistance to AMC in two strains in this study was expected, as it is a beta-lactam, semisynthetic derivative of penicillin. The resistance to SMX could reflect previous exposure of fish to antimicrobial drugs in wastewater-related waters deriving from the WWTP. It is known that WWTPs are probable hotspots for antibiotic resistance dissemination to the environment, as the presence of antibiotic residues might facilitate antibiotic resistance genes (Pazda et al, 2020). On the other hand, antimicrobial susceptibility can be variable within and between species of *Shewanella* genus as reported for *S. putrefaciens* and *S. algae* (Holt et al, 2005). In particular, only studies utilizing one strain of *S. baltica* were identified where that strain was either a part of a larger panel of bacterial indicators and included therein as an important seafood spoilage organism or the strain was one of the isolates in studies of diverse aims. Those studies covered antibacterial activity of natural products (Yilmaz et al, 2018; Kačániová et al, 2018) and probiotic potential of autochthonous gut microbiota of yellowtail amber-jack (Ramírez et al, 2019) in the former, and isolates from samples of a river (Zheng et al, 2011), seafood (Romero et al, 2017), and a gold mine (Uhrynowski et al, 2019) in the latter group. While the antimicrobial agents varied between those

studies, a common pattern is that *S. baltica* strains are by and large susceptible to most antimicrobials tested. The resistance was reported to vancomycin (Yilmaz et al, 2018) and (various) sulfonamides (Zheng et al, 2011; Uhrynowski et al, 2019; Romero et al, 2017) with only Ramírez et al. (2019) reporting susceptibility to sulfamethoxazole. Therefore, the results of this study agree with these reports, as resistance to only two antimicrobials in 2 out of 12 strains was observed. This study also shows that strain-to-strain variation of MIC and MBC in tested strains is rather low since values for 50% and 90% of strains were the same or within one 2-fold dilution for both indices.

Biofilm producing

A rather unexpected finding~~s~~ of this study was~~ere~~ that MBIC values were either lower or equal to MIC values~~,~~ and that majority of tested *Shewanella* strains were weak biofilm producers. Unfortunately, the literature review returned no study that evaluated the MBIC by antimicrobials in *S. baltica* or *S. oneidensis*. That is, biofilm studies of *S. baltica* were mostly directed at investigations of conditions, mechanisms, and regulations of biofilm formation either as a monospecies or mixed species biofilm studies in food science and technology due to being one of the most important psychrotrophic spoilage organisms in seafood (Zhang et al, 2020; Zhu et al, 2019). Therefore, antimicrobials were not expected to have been evaluated against spoilage of food items and those studies evaluated treatment with quorum sensing inhibitors (Zhao et al, 2016), while some studies evaluated natural products such as green tea polyphenols (Zhu et al, 2015). *Shewanella* spp. are known key players in biofilms of submerged surfaces (Martín-Rodríguez et al, 2014), and weak producers identified in this study could be due to a vast

majority of strains isolated from fish rather than water column or fouled submerged surfaces and perhaps more importantly, fish subjects being all apparently healthy. Human medicine studies of diseases affected by biofilms such as cystic fibrosis have shown the isolated *Streptococcus pneumoniae* strains have higher biofilm formation capacity and their MBIC are higher than MIC compared to strains from blood- cultures that have lower biofilm formation capacity and MBIC lower than MIC for various antimicrobials (García-Castillo et al, 2007). In addition to antimicrobials, lower MBIC than MIC values can be obtained using various natural products against multi-drug resistant *Klebsiella pneumoniae* (Magesh et al, 2013) or vitamin C against *Streptococcus mutans* (Eydou et al, 2020). Studies in human medicine have shown the correlation of biofilm formation capacity with multidrug resistance to vary between bacterial species and antimicrobial agents (Ramos-Vivas et al, 2019; Cepas et al, 2019). Whether these associations hold true for *S. baltica* strains between healthy and diseased fish and/or between fish and environmental (e.g., water column and submerged surfaces) isolates that would point to different (sub)populations and niche associations remains to be further investigated.

Exposure of zebrafish to S. baltica

Zebrafish *D. rerio* is increasingly being utilized as a model for studying the pathogenesis and immune responses of fish to infection with numerous bacterial and viral pathogens (Sullivan and Kim, 2008). Various studies have been conducted, mostly to reveal the impact of pathogens that cause human infectious diseases, i.e., *Aeromonas*, *Staphylococcus*, *Streptococcus*, and *Mycobacterium* species (Neely et al. 2002, Sullivan and Kim, 2008, van der Sar et al. 2004). Experimental infections of zebrafish were mostly introduced by microinjection in the caudal

vein, in the pericardial cavity, or the yolk sac (van der Sar et al. 2004). However, the natural infection route is far different from such experimental infections. Therefore, experimental designs involving pathogens of aquacultured fish species should be modified. In natural conditions, pathogens enter fish organisms through gastrointestinal tract, gills, and damaged surface, or in the case of early developmental stages (embryos) through the body surface (van der Sar et al. 2004). Thus, with the purpose to reveal the environmentally relevant impact of *S. baltica* pathogen on zebrafish during early ontogenesis, embryos and larvae were incubated in media containing the bacteria (co-incubation). Although bacterial infection with *Shewanella* spp. has been reported in numerous fish species including *Siganus rivulatus*, *Dicentrarchus labrax*, and *Misgurnus anguillicaudatus* (Qin et al. 2012, Paździor 2016), to date no study examined the zebrafish as a host for *S. baltica*. The in vivo experiments conducted within this study for the first time proved the toxic potential of *S. baltica* on zebrafish development in a time- and dose-dependent manner. Even sub-optimal growth temperature (27.0 ± 0.5 °C) set within this study did not prevent *S. baltica* to cause lethal infection of juveniles (1.5–21.0 × 10^8 CFU/mL) and developmental abnormalities at the lowest tested concentration (7.5 × 10^7 CFU/mL). Among sublethal endpoints that we observed during embryonic set up, heartbeat rate appeared to be the most sensitive factor, since statistically significant reduction of heartbeats was observed among all survived specimens. While a significant decrease in pigmentation formation was noted during the first 72 h of development, it was to a certain degree associated with the observed growth deviation.

Upon larval exposure set-up, no specimen survived 24-h exposure to tested concentrations, indicating that zebrafish larvae were more sensitive to *S. baltica* infection than embryos. During juvenile stages, the chorion serves as an effective barrier and thus protects the

embryo by reducing the pathogen penetration during the pre-hatching period (de la Paz et al. 2020). Moreover, the embryo itself is immersed within the perivitelline fluid (PVF), an extraembryonic fluid that fills the space between embryo and chorion (De la Paz et al. 2020). Proteomic analysis of PVF revealed the presence of numerous sugar-binding proteins (i.e., lectins) that can exert a defensive role against bacterial infection, thus protecting the zebrafish embryo before the development of the immune system. De la Paz et al. (2020) proved a strong agglutination capacity of zebrafish PVF on *E. coli* and *Salmonella typhimurium*, but also fish pathogen *Edwardsiella tarda*. Considering those findings, we can conclude that mechanic (chorion) and chemical (PVF) barriers reduced the ability of *S. baltica* to reach and penetrate the embryo, thus diminishing (but not preventing) their negative impact on a specimen during embryonic exposure.

The results of in vivo fluorescence-based assay for detection and localization of *S. baltica* within zebrafish demonstrated pathogen interaction with the chorion and penetration within the organism. Increased mortality at higher bacterial concentrations (9.0×10^8 and 2.1×10^9 CFU/mL) was associated with an increased bacterial aggregation on the chorion surface and consequently proliferation in embryos infected with *S. baltica*. Co-incubation with lower pathogen concentrations (7.5×10^7 and 1.5×10^8 CFU/mL) resulted in a systemic infection in bloodstream of trunk and yolk sac, and notable accumulation in eye regions. Such preferential accumulation of *S. baltica* reveals skin-absorption as the main route for bacterial entrance into zebrafish. All the above findings corroborate zebrafish as a valuable model organism that can be effectively used to study *S. baltica* infection in order to timely predict negative effects of the pathogen on aquatic biota.

5. Conclusions

Based on the findings of this study, a larger sample size study could be designed to confirm the soundness of our approach of characterization of *Shewanella* spp., as its weakness lies in the small number of *Shewanella* retrieved from fish and water affected by the WWTP. It remains to be elucidated would *S. baltica* isolated from fish with evident clinical disease symptoms have some variations in any of its traits, such as pathogenicity, biochemical, chemical, or antimicrobial susceptibility properties. All analyses in this work were conducted in technical and/or biological duplicates up to quintuplicates, thus compensating for a small sample size and allowing reproducibility.

The purpose of the study was to introduce a novel approach of using a number of complementing methods and techniques in order to comprehensively characterize the bacterium: testing the sensibility and accuracy of MALDI-TOF MS mass signals for various cultivation and identification protocols; analyzing *Shewanella* antimicrobial susceptibility and resistance to antimicrobials, its fatty acid profile relative to culture media; and simulating environmental infection of the earliest life stages of zebrafish in *Shewanella*-contaminated waters.

We proved a strong efficacy of using MALDI-TOF MS identification for clinical isolates of *S. baltica* when cultivated on TSA for 24 h at 22 °C and with supplementation of 1.5% NaCl. We established that the exposure of zebrafish to different *S. baltica* concentrations induced severe effects on zebrafish development in form of the decreased heartbeat rate, locomotor activity, and melanin pigmentation formation. We also demonstrated that *S. baltica* could pass through the zebrafish chorion and enter into the embryos. The tested strains were generally susceptible to antimicrobials as resistance was observed only to amoxicillin and

sulfamethoxazole. In addition, we established that total cellular fatty acid analysis could be utilized as a method for rapid identification of bacterial species in laboratory cultures. Therefore, for performing the characterization of an environmental bacterium having a pathological potential such as *S. baltica*, we suggest an array of described methods to clarify the interactions between the bacterium and affected biota, as well as to identify the most appropriate approaches to understand its physiology and relationship with the vulnerable WWTP-affected environment.

~~Statements and Declarations~~

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Author~~s~~ c~~C~~ontribution~~s~~

NTP: conceptualization, writing original draft, writing – review and editing, data curation, formal analysis, investigation, and methodology. SK: writing – review and editing, data curation, formal analysis, investigation, and methodology. BB, SB, KB, MB, ITB: data curation, formal analysis, investigation, and methodology. MJ, ISP: data curation, formal analysis, and investigation. SlK: formal analysis and investigation. RČR: writing – review and editing, funding acquisition, and investigation.

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Availability of data and materials

Data will be available on reasonable request.

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Ethics approval and consent to participate

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Consent for publication

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Competing interests

The authors declare no competing interests.

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~~FIGURES~~

Fig. 1 Experimental design for *Danio rerio* embryonic (a) and larval (b) exposure

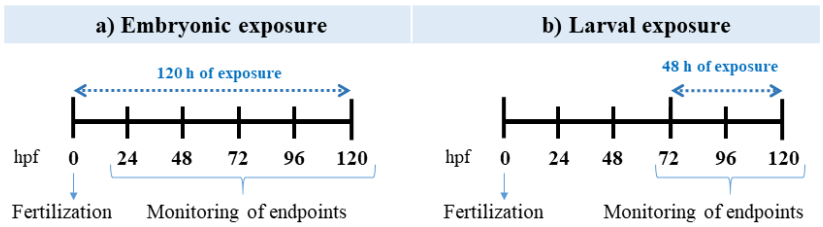


Fig. 2 Stacked bar graph showing the distribution of normal, affected, and dead zebrafish specimens among three *S. baltica* concentrations and negative controls (AW). Letter (**a**) denotes embryonic exposure set up, while (**b**) denotes larval exposure set up. Values represent mean ± SD (n = 40)

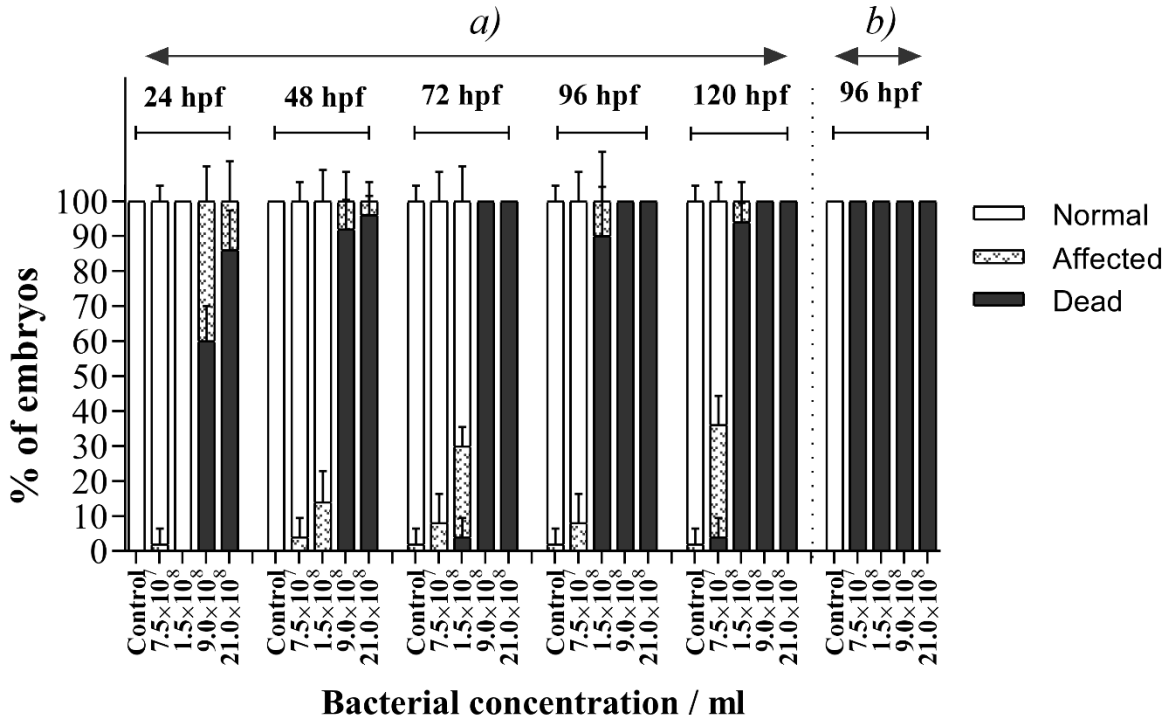


Fig. 3 Developmental abnormalities observed in zebrafish *D. rerio* during embryonic exposure to *S. baltica:* **a**), **b**), **c**), **d**), **e**) normally developed control embryo at 24, 48, 72, 96, and 120 hpf, respectively; **f**) normally developed embryo; **g**) yolk sac edema (arrow); **h**) pericardial edema (dashed arrow), yolk sac edema (arrow), delay or anomaly in the absorption of the yolk sac; **i**) underdeveloped head, pericardial edema (dashed arrow), scoliosis (arrow); **j**) scoliosis (arrow); **k**) underdeveloped head region*;* **l**) yolk sac edema (arrow); **m**) blood accumulation at yolk sac (arrow); **n**) scoliosis (arrow); **o**) non-hatched specimen with pericardial edema (dashed arrow), blood accumulation at yolk sac (arrow); **p**) developmental deviation, tail not detached from yolk sac; (**r**) embryo deformation, edema (arrow); **s**) and **t**) developmental deviation, tail not detached from yolk sac, no somite formation. Absence (**l**) and delay (**m**) in pigmentation formation

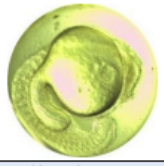
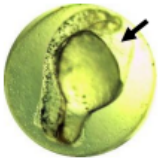
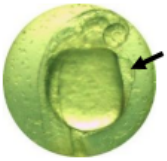
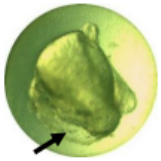
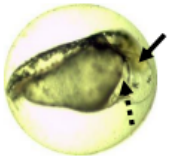
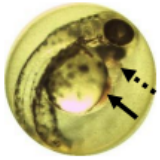
	Bacterial concentration/ml			
Control	$7.5x10^7$	$1.5x10^8$	$9.0x10^8$	$21.0x10^8$
24 hpf				
a 	*f* 	*k* 	p 	s 
48 hpf				
b 	*g* 	*l* 	r 	t 
72 hpf				
c 	*h* 	*m* 		
96 hpf				
d 	*i* 	*n* 		
120 hpf				
e 	*j* 	*o* 		

Fig. 4 Heartbeat rate (**a**) and pigmentation formation (**b**) of zebrafish during embryonic exposure to *S. baltica*. Values represent mean ± SD ($n = 20$; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). Asterisk marks a significant difference between treatment and negative control (AW) values. Due to developmental deviation that occurred during embryonal exposure at higher *S. baltica* concentrations, the heartbeat rate could be observed in up to 1.5×10^8 CFU/mL

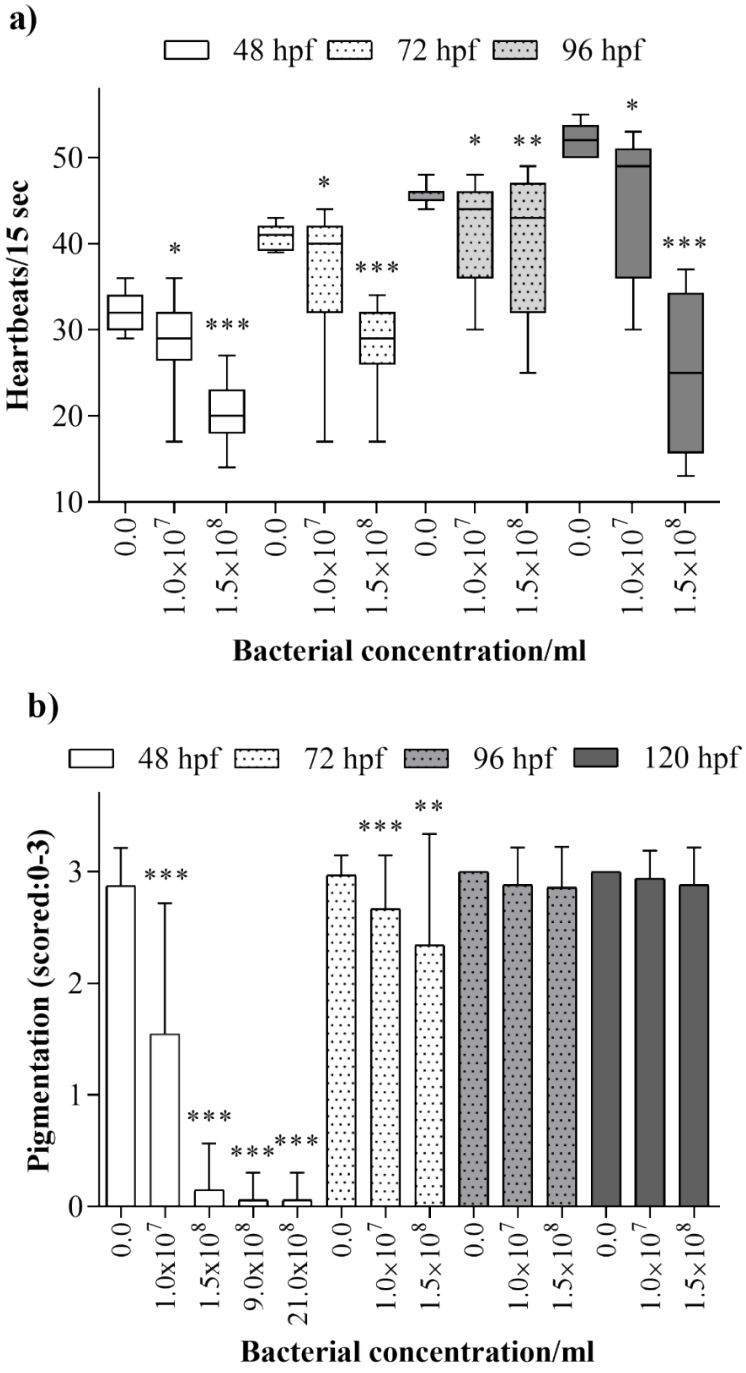

a)
48 hpf
72 hpf
96 hpf
Heartbeats/15 sec
10
20
30
40
50
0.0
1.0×10^7
1.5×10^8
Bacterial concentration/ml
b)
48 hpf
72 hpf
96 hpf
120 hpf
Pigmentation (scored:0-3)
0
1
2
3
0.0
1.0x10^7
1.5x10^8
9.0x10^8
21.0x10^8
1.0×10^7
1.5×10^8
Bacterial concentration/ml

Fig. 5 Fluorescence images of zebrafish embryos after exposure to *S. baltica* labeled with fluorescent substrate 5-DTAF