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Fish as a winter reservoir for *Vibrio* spp. in the southern Baltic Sea coast

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ABSTRACT

Vibrio spp. are ubiquitous in aquatic environments. In temperate regions, including the Baltic Sea, these bacteria, which include facultative pathogenic species, proliferate during warm water periods and may thus pose a risk to human health. However, while present at relatively high abundances in the summer months, *Vibrio* spp. are rarely detected during cold water periods. Several potential winter reservoirs, such as fish and sediment, have been discussed, but the seeding sources that give rise to *Vibrio* during the summer months are unknown.

In this study, we employed a combination of cultivation-independent genus-specific digital droplet PCR, Illumina 16S rRNA gene sequencing, culture-dependent determinations of colony-forming units (CFU) and genus-specific strain isolation to identify possible winter reservoirs of *Vibrio* spp. Using these techniques, we demonstrate that in the southern Baltic Sea especially fish can serve as a winter reservoir for distinct *Vibrio* assemblages, including facultative pathogenic *V. vulnificus*. Particularly the invasive species round goby (*Neogobius melanostomus*), with its potential to appear in high individual densities, has the capacity to carry a comparable *Vibrio* spp. cell number like water and sediment, while only accounting for 1/1000 of the spatial share in the overall coastal environment. Thus, by providing a winter reservoir for distinct *Vibrio* communities, coastal fish and, especially areas in which round goby densities are high, may be hot spots of pathogenic *Vibrio* species.

1. Introduction

The genus *Vibrio* consists of more than 130 gram-negative, motile, metabolically and genetically diverse species (Gomez-Gil et al., 2013). *Vibrio* spp. are found in water (Thompson et al., 2004), sediment (Givens et al., 2014), attached to particles (Oberbeckmann et al., 2011), and on the surface and intestines of higher organisms, such as crustaceans (Vandenberghe et al., 1999), bivalves (Givens et al., 2014), and fish (Noguchi et al., 1987). The lifestyles of these bacteria range from symbiotic to pathogenic and include species able to infect humans (Elyakov et al., 1991; Farmer Iii and Hickman-Brenner, 2006; Jayasree et al., 2006). Among the latter are *V. parahaemolyticus*, *V. cholerae, V. alginolyticus* and *V. vulnificus*. While ubiquitous distributed in aquatic environments including subpolar regions as Iceland (Haley et al., 2012), with their preference for warmer water temperatures, these species do not pose a major threat for humans in temperate regions (Baker-Austin et al., 2010; Baker-Austin et al., 2017; Hlady and Klontz, 1996; Huq et al., 2005), but this may change as global warming increases sea surface temperatures and therefore potentially the range of *Vibrio* spp. (Baker-Austin et al., 2013). This is especially true for the Baltic Sea, which is relatively shallow and is warming 5–6 times faster than the global average (EEA, 2019). In addition to temperature, the brackish water of the Baltic Sea, with a salinity of <25, supports the growth of *Vibrio* species such as *V. vulnificus* (Kaspar and Tamplin, 1993; Takemura et al., 2014), the main causative agent of vibriosis at the German Baltic Sea coast (Hauk and Duty, 2015). A preview of potential future scenario of increase in *Vibrio*-based abundance was obtained during the recent unusually warm summers in the Baltic Sea area, during which cases of *Vibrio* infections increased (Baker-Austin et al., 2016; Hauk and Duty, 2015).

Water temperatures in the Baltic Sea in summer rise above 15–20 °C, which is warm enough for the proliferation of *Vibrio* spp. to levels allowing its simple detection (Böer et al., 2012; Böer et al., 2013). However, in winter, *Vibrio* spp. are rarely detected in the cold waters of the Baltic (Böer et al., 2013; Oberbeckmann et al., 2011). This winter disappearance has been explained by the ability of *Vibrio* spp. cells to enter a dormant state, so-called VBNC (viable but not cultivatable), when the water temperature drops below 10 °C (Baffone et al., 2003;

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Weichart et al., 1992; Wolf and Oliver, 1992). VBNC cells are still alive and metabolically active but their transfer to cultivation medium results in their death (Bloomfield et al., 1998). This prevents the detection of *Vibrio* spp. cells by cultivation-based approaches, which remains the most common method of *Vibrio* spp. quantification (Kong et al., 2004; Wolf and Oliver, 1992).

The absence or extreme scarcity of *Vibrio* spp. cells during cold water periods in temperate regions raises the question whether refuges exist where these bacteria are more protected and survive in higher abundances until water temperatures again increase. While sediment is perhaps the most obvious possible refuge (Böer et al., 2013; Chase et al., 2015), others are possible, given the flexibility in lifestyle and habitat of *Vibrio* spp. For example, biofilms are robust towards environmental changes (Kubota et al., 2008; Wai et al., 1998) and *Vibrio* spp. are able to attach to particles and chitin (Kirschner et al., 2011; Matz et al., 2005; Shime-Hattori et al., 2006) such that seston might also offer a winter reservoir for members of this genus. A study at the subtropical U.S Gulf coast by DePaola et al. (1994) showed that fish could provide an advantageous winter habitat, albeit one that is influenced by the lifestyle of the fish species, as absolute *Vibrio* spp. abundances on bottom-feeding fish were up to five magnitudes higher than on filter-feeding and carnivorous species.

Cod (*Gadus morhua*) and herring (*Clupea harengus membras*) are carnivorous and facultative-filter-feeding species, respectively, indigenous to the Baltic Sea and both are migrating species of high economic value. As a winter reservoir for *Vibrio* spp., they could therefore transport and spread bacterial populations over a wider area or re-seed coastal *Vibrio* assemblages after longer cold water periods. In addition, following the introduction of the non-indigenous, bottom-feeding round goby (*Neogobius melanostomus*) in the Baltic Sea in the 1990s (Skóra and Stolarski, 1993), this species has spread over large coastal areas of the southern Baltic Sea, reaching densities of 20 to >100 individuals per m^2 in the Baltic Sea and freshwater habitats, respectively (Chotkowski and Marsden, 1999; EMI, 2017). Consequently, the round goby may be an important winter shelter, including for coastal *Vibrio* communities. However, despite these possible alternative reservoirs, the seeding banks of *Vibrio* spp. in the Southern Baltic Sea are practically unknown, although their identification would allow a better understanding of the spreading dynamics of *Vibrio*, including its pathogenic members, in temperate regions during warm water periods. Furthermore, it would enable predictions of coastal areas at probable risk, *i.e.*, those where *Vibrio* could quickly reach high concentrations as the water temperature rises.

In this study, to gain insights into the potential winter reservoirs of *Vibrio* spp. we examined the abundance and composition of this bacterial genus in water, sediment, and seston and in three fish species (cod, herring, round goby) during two late summer/autumn and winter periods, also taking into account the naturally occurring salinity gradient of the southern Baltic Sea coast. To exclude false-positive results introduced by members of the genus *Photobacterium,* we used optimized cultivation-dependent and -independent approaches, combining 16S rRNA Illumina sequencing and *in silico* in house-improved specific primers for *Vibrio* spp. with quantitative digital droplet PCR and classical strain isolation methods.

2. Material and methods

2.1. Sampling

Sampling map was created using the free software Ocean Data View version 5.1.5 (Schlitzer, 2021). Samples were collected at four registered bathing areas (Regional Office for Health and Social Affairs of Mecklenburg-Western Pomerania / LAGuS-MV) along the Baltic Sea coast in Mecklenburg-Western Pomerania: Warnemünde (W), LAGuS-MV number 236; Lubmin (L), LAGuS-MV number 750; Karlshagen (K), LAGuS-MV number 703; and Niendorf (N), LAGuS-MV number 278

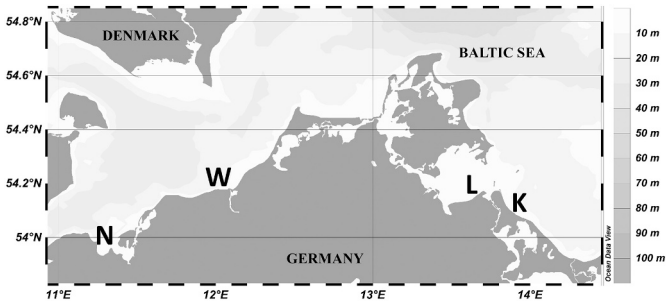

Fig. 1. Map of the German Baltic Sea coast and its bathymetry. Letters indicate the sampling stations (N: Niendorf, W: Warnemünde, L: Lubmin, K: Karlshagen).

(Fig. 1). Sampling at these sites was conducted in late summer, beginning of autumn 2015 (August 17–October 21, 2015), in winter 2015/16 (February 15–March 7, 2016) and winter 2016/17 (February 13–February 15, 2017). Additional samples were collected from site W during a hot period in summer, on August 7, 2018. Temperature and salinity were measured using a Hach® HQD40D portable meter with an INTELLICAL standard conductivity probe directly at the sampling stations. Water was collected from the beach at a depth of 1 m, 20 cm below the surface using acid-cleaned, autoclaved glass bottles and then stored on ice until further processing. Sediment was collected from the beach area at a water depth of 1 m using a sediment corer, and the upper 2 cm from each of six cores were separated and stored on ice until further processing. Seston was collected using a 10-µm plankton net and placed in 50-mL Falcon tubes that were stored on ice until further processing. Fish were caught by local fisherman (herring, cod) or using fish traps (round goby) in the area of Warnemünde. The fish were rinsed with sterile filtered seawater and their gills, guts, and skin mucus were collected aseptically. For all fish samples, except those collected in 2018, tissue from all individuals of one species were pooled. In summer/autumn 2015, the sample sizes were as follows: 17 gobys (8–15 cm), 5 herring (10–20 cm) and 1 cod (60 cm). In winter 2015/16 the sample sizes were: 2 goby (17 and 25 cm), 11 herring (28–30 cm) and 3 cod (45–60 cm). In summer 2018, each fish was analyzed separately. From one cod, the gut, gill, and skin mucus were collected separately. For herring (n = 12, 17–24 cm) and goby (n = 6, 8.5–16.5 cm), the gills, mucus, and gut of half of the individuals were collected separately and for the other half they were mixed.

2.2. Sample preparation

In six replicates between 300 and 1000 mL of water was filtered on a 0.22-µm GV filter, 47 mm Ø (Merck Millipore GVWP04700). The filters were shock frozen in liquid nitrogen and stored at −80 °C until DNA extraction. The rest of the water was used for cultivation. Sediment from all cores of one sampling station was pooled and portions of 10 g were shock frozen in liquid nitrogen and stored at −80 °C until DNA extraction. The rest of the sediment was used for cultivation. For seston, the net haul samples were centrifuged at 3000*g* at 4 °C for 10 min and the water was removed by pipetting. The samples were divided and either three replicates were shock frozen and stored at −80 °C or three replicates were used for cultivation. One portion of the combined fish tissue was shock frozen in liquid nitrogen and stored at −80 °C until DNA extraction and the other was used for cultivation.

2.3. Cultivation-based Vibrio spp. quantification

The summer/autumn 2015 and winter 2015/16 samples were used for the determination of *Vibrio* spp. colony-forming units (CFU). All sampling materials were plated directly, with either quintuplet (water) or triplicate (all other materials) samples. For the direct plating of water,

0.1 mL was plated on TCBS (TCBS-agar, Merck, 110,263) and CHROMagar™ (CA) Vibrio (CHROMagar, VB910) and then incubated for 24 h at 37 °C. For the sediment, 10 g of sediment was vigorously mixed with 90 mL of 1× alkaline peptone water (APW), pH 8.5 (1% [*w/v*] peptone [Polypeptone™ peptone, BD, 211910], 1% [w/v] NaCl [CELLPURE® ≥99.8%, Carl ROTH®, HN00.1]) after which 0.1 mL were directly plated on CA and TCBS plates and incubated as described for the water samples. For seston, net haul samples (0.1–0.25 g fresh weight) were vigorously mixed with 100 mL of 1 × APW and used for direct plating as described for water. Fish tissue samples were prepared as described for the sediment samples. Growing colonies were counted after 24 h and were presumed as *Vibrio* spp. The number of presumable *Vibrio* colonies were divided by the amount of applied sampling material to calculated colony forming units (CFU) for *Vibrio* spp. To reduce the occurrence of false-positive *Vibrio* colonies occasionally growing on TCBS and CA, species identity of isolates was determined by MALDI-ToF-MS (for more details see below).

2.4. Enrichment of Vibrio spp. at low in situ cell numbers

In addition to direct cultivation and isolation on media as described above, *Vibrio* isolates were obtained from the summer/autumn 2015 and winter 2015/16 samples cultured in APW. All enrichment steps were carried out in triplicate in 500-mL acid-cleaned glass bottles containing 90 mL of sample water and 10 mL of 10 × APW and incubated for 24 h at 37 °C. Enriched APW was ten-fold serial diluted five times to a final concentration of 10^{-5} and every dilution was plated on CA and TCBS plates. Sediment was prepared as described above for the CFU determination. The APW sediment mixtures were incubated and plated as described above for water. Seston APW samples from the CFU determination were incubated and plated as described for the water and sediment samples, and the fish tissue samples as described for the sediment samples.

2.5. Identification of Vibrio isolates by MALDI-ToF-MS

From each of the direct cultivation and enrichment samples, up to 100 colonies were picked, streaked on marine broth (MB) containing 1.5% agar [*w/v*] (CP73.1, 2263, respectively, Carl ROTH®), and maintained at 37 °C until pure strains were obtained. MALDI-ToF-MS was used to identify the strains according to the method of Erler et al. (2015). Briefly, a portion of a single colony was transferred into a 2-mL Eppendorf tube filled with 0.9 mL MB medium, with duplicate samples prepared. After an overnight incubation at 37 °C, the tubes were centrifuged at 3000*g* for 5 min, the MB medium was removed and the cell pellet was resuspended in 300 μL of HPLC-grade water (Water HiPerSolv CHROMANORM®, VWR, 83645.290) by vortexing vigorously. The cell suspension was mixed with 900 μL of pure ethanol (molecular grade, AppliChem) and stored at −20 °C for several months until analyzed. For MALDI-ToF, the proteins were extracted using the fast extracting method described by Mellmann et al. (2008). For all steps, only Eppendorf tips and tubes were used, to avoid potential problems caused by plasticizer. One μL of protein extract was spotted on a polished steel target plate (MSP 96, Micro Scout Target, 224,989, Bruker) with two replicates per sample, air-dried, and then covered with 1 μL of saturated matrix solution (50% acetonitrile, 47.5% MALDI-water, 2.5% trifluoroacetic acid, α-cyano-4-hydroxycinnamic acid till saturated) [Sigma-Aldrich T6508-M; Bruker, 255,344). The samples were prepared in duplicate. As a standard, Bacterial Test Standard (BTS, Bruker, 8,290,190) was prepared on two spots. Mass spectra were created using a microflex LT/SH system (Bruker Daltonics Inc., Bremen) with the Bruker Flex Control software and analyzed using the MALDI-Biotyper RTC 3.1 software. Species identification was carried out by comparing the generated spectra with those in the Bruker Daltonics (BDAL) and Vibrio-Base (Alfred Wegener Institute Helgoland/AWI-Helgoland) databases. The analyses resulted in scores ranging from 1 to 3, as a measure of the reliability of the species identification. Scores between 2 and 3 allowed identification up to the species level, scores between 2 and 1.7 allowed genus-level identification, and scores ≤1.7 indicated no comparable match with a database entry. Measurements resulting in values <2 were repeated. For corrected CFU values from the direct plating, only isolates identified as true *Vibrio* spp. were considered in the CFU calculations.

2.6. DNA extraction from environmental and fish samples

Environmental DNA was extracted using the QIAgen DNeasy Power soil kit for water, sediment and seston samples. For fish tissues DNA was extracted by a combination of the QIAgen blood and tissue kit for cell disruption and the QIAgen DNeasy Power soil kit for purification with the same DNA-Binding matrix as in the water, sediment and seston samples. All final elution steps were performed twice using 50–100 μL of DEPC-treated water. Sediment and seston were processed as described in the kit protocol and using 250 mg of starting material. Water DNA filters cut into small pieces (≈2 × 2 mm) served as the starting material for the water samples and were processed according to the kit instructions. For the fish tissues, 25 mg were first treated using the QIAgen blood and tissue kit disrupting the cells by an overnight incubation in proteinase K, as described in the kit protocol. Two hundred μL of buffer was then removed from the bead beating tube of the QIAgen Power soil kit and replaced with the disrupted fish cell suspension. The sample was then processed as described in the DNeasy Power soil kit protocol. The DNA extracts were stored at −20 °C until further analysis.

2.7. ddPCR quantification of Vibrio

The primers used in this study are described in S1 of the Supporting Information. Quantitative PCR based on digital droplet PCR (ddPCR) was performed using the Bio Rad EvaGreen system. Each PCR contained 11 μL of 2× EvaGreen, 100 nmol L^{-1} of each primer, and 1 μL of sample DNA (0.1–10 ng μL^{-1}) in a final reaction volume of 22 μL. The droplets were generated using a QX100 droplet generator according to the manufacturer's instructions. The emulsified samples were transferred into a 96-well plate and sealed with a pierceable foil hot seal (BioRad, 181–4040). The PCR was performed using a Bio Rad C1000 Touch™ thermal cycler. Following the PCR, the plate was transferred to the QX100 droplet reader and analyzed using the Quantasoft 1.74.09.17 software. Each run included at least one positive and one negative control, containing 0.1 ng of DNA of *V. vulnificus* (DSM 10143) and DEPC-treated water, as replacement for the sample DNA, respectively.

The two primer systems used in this study targeted either the bacterial 16S rRNA gene (Com-f, –Com-r (Schwieger and Tebbe, 1998)), or the *Vibrio* 16S rRNA gene (567f, 680r (Thompson et al., 2004)). The optimal annealing temperature was determined by performing a gradient PCR with the temperature ranging from 3 °C above to 3 °C below the described annealing temperature of the primers. Additionally, both 30 or 40 cycles and 2-step or 3-step protocols were tested. DNA from *V. vulnificus* (DSM 10143) served as the template, and DEPC-treated water as the negative control. The final PCR conditions were as follows: For the Com-primer-system, an initial annealing step of 5 min at 95 °C was followed by 40 cycles of 1 min at 95 °C, 1 min at 50 °C, and 1.5 min at 72 °C. For dye stabilization, a 5 min step at 4 °C was followed by 5 min at 90 °C. The *Vibrio* primer set was used under the following conditions: 95 °C for 5 min followed by 40 cycles at 95 °C for 1 min, 61.6 °C for 1 min, and 72 °C for 1 min. The final steps consisted of 5 min at 4 °C and 5 min at 90 °C. The ddPCR results are reported as bacterial 16S rRNA gene copies or preliminary *Vibrio* 16S rRNA gene copies per g or mL of sampling material. However, for the *Vibrio* primer set, due to the known cross-reaction of this primer set with *Photobacterium* spp. (Thompson et al., 2004), a correction factor, obtained as described below, was included to avoid an overestimation of *Vibrio* spp. counts.

2.8. Generation of a correction factor for Vibrio spp. quantification based on 16S rRNA gene Illumina sequencing

Illumina sequencing was conducted for all summer/autumn 2015 W samples to determine the ratio of *Vibrio* and *Photobacterium* 16S rRNA gene sequences, which are amplified by the same primer system. Illumina 16S sequencing was conducted according to Bennke et al. (2018) using the bacterial 16S Com-primers (Herlemann et al., 2011). A cluster density of 934 ± 80 K mm^{-2} was achieved for sequencing and a Q-score ≥ 30 of 67%. The run generated 3.5 million reads for the samples. FASTQ files were converted from *bcl files and used for further sequence data processing. *Vibrio* spp. and *Photobacterium* spp. in the Illumina dataset were quantified using MOTHUR version 1.39.5 (Schloss et al., 2009) and the SILVA database version 132. Sequences were processed with the MOTHUR pipeline largely following the MiSeq SOP guidelines (Kozich et al., 2013; MiSeqSOP – Mothur, 2018). For the correction factor calculations, the settings were set at zero wobbles to prevent sequence changes by the program. Quality-filtered sequences were identified using the Wang approach (Wang et al., 2007) and SILVA SSURef version 132 as a reference database (Parks et al., 2018, Quast et al., 2013, Yilmaz et al., 2013). The bootstrap was set to ≥85%. Operational taxonomic units (OTUs) were created based on 97% sequence similarity. OTUs containing three or fewer reads in the complete dataset were removed. Sequences classified as Eukaryota, mitochondria, chloroplasts, or unknown were also removed. All sequences identified as belonging to *Photobacterium* were tested for potential hybridization with the *Vibrio* 16S primer by comparing the sequences with the primer sequence, allowing up to one mismatch at each of the 3`-site primer endings. The number of hybridizing *Photobacterium* sequences, the total number of *Photobacterium* sequences, and the total number of *Vibrio* sequences were used to calculate a correction factor for each tested material. *Vibrio* 16S rRNA gene counts from the ddPCR quantification were multiplied by the correction factor of the accordant material. Finally, *Vibrio* 16S genes copy numbers were normalized to "1" by dividing them by 10.1, which is the mean number of 16S genes per *Vibrio* spp. genome [rrndB D database, accessed 1.08.2018, (Stoddard et al., 2014)].

2.9. Multivariate analysis of overall bacterial and Vibrio communities and statistical analysis

Absolute and relative abundance of *Vibrio* spp. resulting from ddPCR were used for statistical analysis of the different sampling materials and time points to test for potential significant differences. A Kruskal-Wallis-test was used followed by a pairwise comparison with a conover iman test from the package conover.test v.1.1.5 (Dinno, 2017) for significant results. A Benjamine-Hochberg-correction was applied for *p*-values.

The relative abundance of OTUs identified as bacteria were used for comparisons of habitat-specific communities. Concerning the comparison of *Vibrio* assemblages, only OTUs identified as members of this genus were included in the analysis. For analysis of community structure a non-metric multidimensional scaling (nMDS, 100 tries) was applied. These were based on a Bray-Curtis-Dissimilarity of the square root transformed relative abundances of the whole bacterial OTUs and the Hellinger transformed abundances of *Vibrio*-OTUs. The nMDS plots were created with the packages vegan (Oksanen et al., 2019; R-Core-Team, 2018) and ggplot2 (Wickham, 2016). The ordination yielding low stress values was based on 100 attempts.

The bacterial nMDS-Plot was tested for significant differences between the visual group of fish and water-sediment-seston by ‚permutational multivariate analysis of variance'(PERMANOVA (Anderson, 2001)). Additional PERMDISP-routine (‚analysis of multivariate homogeneity of group dispersions'(Anderson, 2006)) was used to test if significance differences resulted from dispersion effects with the R-package funfuns (Trachsel, 2020). The groups were visualized by a 95% confidence interval in the nMDS-Ordination. For the *Vibrio* communities single materials were tested for significant differences by the program Primer7 with the add-on PERMANOVA+. A PERMANOVA was applied with a Monte-Carlo-based permutation test, followed by a PERMDISP-analysis.

2.10. Vibrio vulnificus virulence gene detection

To identify human pathogenic *Vibrio vulnificus* clinical strains, *V. vulnificus* colonies identified by MALDI-ToF-MS were tested for the presence of putative key virulence genes as follows: 10 mL of MB medium was inoculated with a single colony and then incubated at 37 °C for 24–37 h. DNA was extracted from 2 mL of the cell culture using the QIAamp® DNA mini and blood mini kits according to the protocol for gram-negative bacteria. The MALDI-ToF-MS-based identification was verified in a *V. vulnificus*-specific PCR targeting the *vvh* gene, using the primers described by Warner and Oliver (2008) and Panicker et al. (2004). Three virulence-gene-specific primer systems targeting the potential main virulence genes were also included (*rtxA1* (Chung et al., 2010), *HP1* (Han et al., 2009), and *viuB* (Jones et al., 2008; Panicker et al., 2004) [S1]. Five additional *Vibrio* spp. strains served as controls. A representative selection of PCR products was sequenced by LGC Genomics (Berlin) and prepared in accordance with LGC Genomics Sanger sequencing requirements. The sequence results were compared with the NCBI database to ensure correct amplification of the targeted genes.

2.11. Dry weight calculation

Dry weights of sediment, seston, and fish tissue were determined by weighing heat-dried samples on a Mettler AM100 balance (Mettler Toledo) as follows: The lids of 2-mL Eppendorf tubes were punctured using a glowing hot needle (Ø 0.9 mm), after which 20–2000 mg of the sample was transferred into the tubes, dried for 72 h at 90 °C followed by an additional 24 h to ensure completeness, and then weighed. Empty Eppendorf tubes served as the negative control. The dry weight was calculated based on the loss of weight of the samples.

2.12. Projected Vibrio spp. distribution in a coastal segment during winter

The theoretical load of *Vibrio* spp. and its spatial share at each habitat within a coastal segment were estimated using *Vibrio* spp. ddPCR cell counts from the first winter sampling period and is based on the following assumptions: (1) The average weight and length of a goby is 40 g and 140 mm, respectively (Hempel and Thiel, 2013; Piria et al., 2011; Skabeikis and Lesutienė, 2015). (2) The average density of the fish tissue is 1.026 g cm^{-3} (Davidovits, 2018). (3) The average density of the first 2 cm of sediment is 2.65 g cm^{-3} (Zobkov and Esiukova, 2017). (4) The seston load is 10 mg/L (Nausch et al., 2002) and the average density is 2.65 g cm^{-3}. (5) The water depth where gobies for this study were caught is 4 m. (6) Goby densities of 2, 20 and 100 individuals m^{-2} are sufficient to calculate the theoretically maximum possible *Vibrio* spp. load in goby tissue, based on goby numbers in previously investigated aquatic habitats (Brandner et al., 2013; Chotkowski and Marsden, 1999; EMI, 2017).

3. Results

3.1. Environmental conditions

Salinity at the four stations decreased from west to east, with the salinity of the W and N samples ranging from 11.6‰ to 15.3‰ and that of the L and K samples from 6.0‰ to 8.3‰ (Table 1). The exception was the water of the W samples from the summer of 2018, which had a salinity of 7.4‰. The water temperature in winter was at least 10 °C lower than in summer/autumn, ranging from 1 °C to 5.9 °C and from 10.6 °C to 16.8 °C, respectively. Again, the summer 2018 samples were the exception, as the temperature range was >20 °C.

Table 1
Overview on the sampling sites, sampling times, salinity, and temperature.

	Niendorf (N)	Warnemünde (W)	Lubmin (L)	Karlshagen (K)
	Lat: 53.930299°	Lat: 54.181291°	Lat: 54.137104°	Lat: 54.122105°
	Long: 11.270197°	Long: 12.078845°	Long: 13.611340°	Long: 13.842652°
Summer/ Autumn 2015	Date: 21.10.2015 Temp.: 10.6 °C Salinity:14.6‰	Date: 17.8.2015 Temp.: 16.8 °C Salinity:11.6‰	Date: 22.09.2015 Temp.: 15.8 °C Salinity:8.3‰	Date: 22.09.2015 Temp.: 15.0 °C Salinity:8.0‰
Winter 2015/ 16	Date: 7.03.2016 Temp.: 4.6 °C Salinity:12.0‰	Date: 15.02.2016 Temp.: 3.7 °C Salinity: 13.1‰	Date: 22.02.2016 Temp.: 5.9 °C Salinity: 6.4‰	Date: 29.02.2016 Temp.: 3.7 °C Salinity:6.0‰
Winter 2016/ 17	Date: 13.02.2017 Temp.: 1.0 °C Salinity: 13.1‰	Date: 13.02.2017 Temp.: 3.2 °C Salinity:15.3‰	Date: 15.02.2017 Temp.: 1.5 °C Salinity: 6.0‰	Date: 15.02.2017 Temp.: 1.0 °C Salinity: 6.5‰
Summer 2018	–	Date: 07.08.2018 Temp.: 21.7 °C Salinity:7.4‰	–	–

3.2. *Vibrio* spp. abundances

Independent of the methodological approach, absolute *Vibrio* spp. cell numbers in fish in summer/autumn and winter were significant higher, with at least ten times the *Vibrio* cell abundance (Figs. 2, 3), than in the surrounding water, sediment and seston (<0.05). Moreover, relative *Vibrio* abundances were at least three times higher in the fish tissue than in the other sampled materials (Figs. 2, S2, S3). In general, a gradient in *Vibrio* abundances could be detected from water, to sediment to seston up to the fish. While the fish in general was characterized by a higher relative and absolute *Vibrio* abundance compared to water, seston and sediment, this trend was particularly pronounced in goby. In the summer, goby-associated *Vibrio* spp. cell abundances were at least 10 times higher compared to herring and cod (Fig. 3). Furthermore, relative abundances of *Vibrio* spp. were at least one third higher in goby than in the other two species in winter and summer (Fig. 2, S3). Direct cultivation-based quantification could only quantify *Vibrio* spp. in the summer/autumn 2015 W samples, but with *Vibrio*-specific ddPCR *Vibrio* 16S rRNA genes were detected and quantified in all samples, and the correction factor was applied.

Illumina 16S rRNA gene sequencing of the water samples revealed that 84% (± 2.1%) of the detected relative 16S rRNA gene copy numbers in goby and cod were those of *Photobacterium* spp. (data not shown) compared to 4.8% (±1.7%) in herring and 0.02% (±0.02%) in the water, sediment, and seston samples. The *in silico* test showed that ~90% of the *Photobacterium* sequences were identical to or only had a one base pair mismatch with the *Vibrio*-specific primer, accounting for the amplification results with the primer pair. To eliminate false-positive results, for each sampling material a correction factor ranging from 4.47 $\times 10^{-1}$ to 2.07 $\times 10^{-4}$ was calculated (Supplementary Information S4).

The ddPCR-based absolute *Vibrio* cell numbers showed an increasing abundance from water to sediment, to seston up to fish (Fig. 3). *Vibrio* cell abundances in the summer/autumn 2015 samples ranged from 1 $\times 10^{1}$ cells mL^{-1} for the N water samples to 3.11 $\times 10^{6}$ cells g^{-1} in the round goby. Analyses of the gut, gill and skin mucus of the fish identified the gut as the main carrier of *Vibrio* spp. cells in fish tissue (S5). In all of the goby samples and four out of the five herring samples the concentration of *Vibrio* spp. cells in the gut exceeded that in the other two compartments by nearly ten-fold. Cellular abundances ranged from 1.3 $\times 10^{3}$ to 3.43 $\times 10^{6}$ *Vibrio* spp. cells (g dry weight)$^{-1}$. The exception was

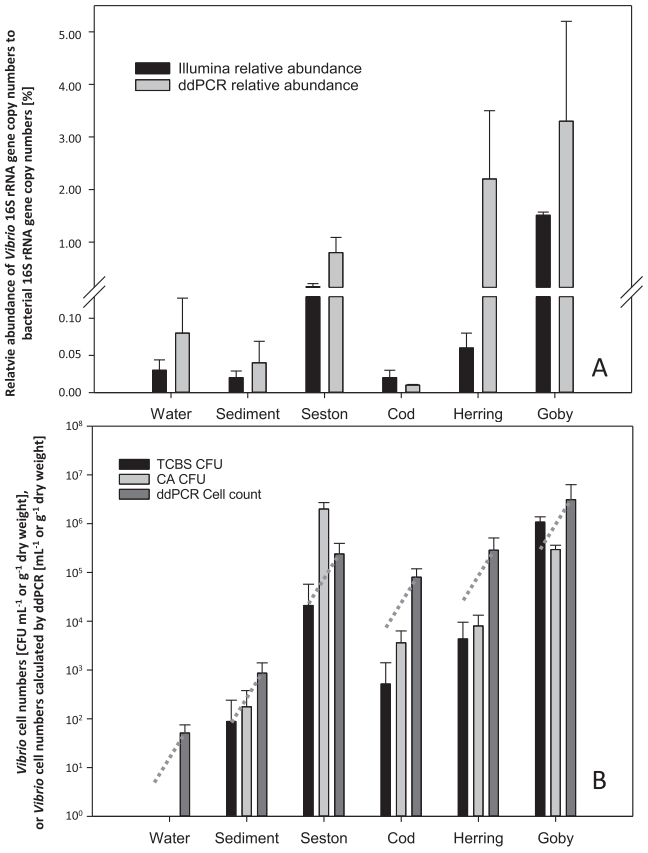

Fig. 2. Comparison of the different quantification methods for the Warnemünde summer 2015 samples. **A:** Relative abundance of *Vibrio* 16S rRNA gene copy numbers *vs.* overall bacterial 16S rRNA gene copy numbers based on ddPCR and Illumina data, respectively. Note the break in the y-axis between 0.129 and 0.14. **B:** *Vibrio* CFU on TCBS and CA plates, based on the corrected CFU (elimination of false-positives), and absolute *Vibrio* cell numbers based on 16S rRNA gene ddPCR analyses.
- - - -: a one order of magnitude difference compared to the ddPCR results.

a single cod, in which 4.21 $\times 10^{4}$ *Vibrio* cells g dry weight)$^{-1}$ were detected in the skin mucus and thus exceeded the abundances in the gills and guts by nearly three orders of magnitude (Supplementary Information S5).

For the two winter periods, a *Vibrio* concentration gradient between water, sediment, seston, and fish was still evident but there was a general decrease in absolute *Vibrio* cell numbers (Fig. 3). The decrease in the *Vibrio* cell concentration between the first summer/autumn and winter periods differed depending on the sampling material. The smallest decrease was in the sediment, where average *Vibrio* spp. cell abundances between summer/autumn and winter decreased by less than an order of magnitude. The highest concentrations of *Vibrio* spp. cells in summer/autumn were in goby and seston, which had the largest decreases between summer/autumn and winter, about two orders of magnitude in both. As the temperature increased, so did the cell numbers in all sources (Fig. 4). For the two summer periods, significant increases in *Vibrio* cell numbers of 20-fold and 580-fold were determined in sediment and water, respectively (Supplementary Information S6). By contrast, *Vibrio* cell numbers in the three fish species were unaffected by further increases in temperature. A comparison of the ddPCR and Illumina MISeq results showed that the relative *Vibrio* cell concentrations were, except for herring, always similar, without significant differences and within the same order of magnitude (Fig. 2, S2).

A cultivation-dependent quantification of *Vibrio* cell numbers was successful only for the W samples (Figs. 2, S2). In the samples from all of the other sites the direct plating approach yielded either no colonies or

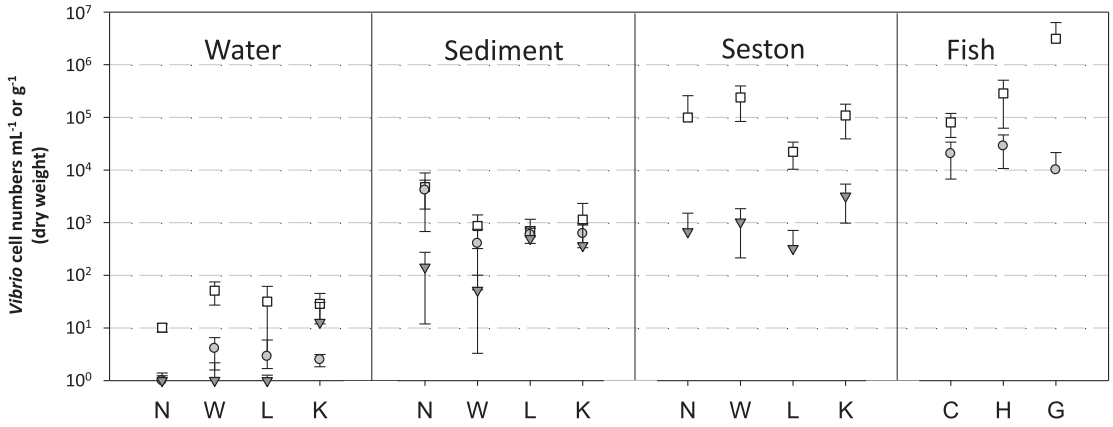

Fig. 3. *Vibrio* spp. corrected 16S rRNA gene ddPCR quantification. The results are expressed as absolute *Vibrio* spp. cell numbers, either as cells m$-^{-1}$ or cells (g dry weight) $^{-1}$ in summer and winter.
N: Niendorf, W: Warnemünde, L: Lubmin, K: Karlshagen, D: cod, H: herring, G: goby.
□: summer 2015, ○: winter 2015/16, ▽: winter 2016/17.

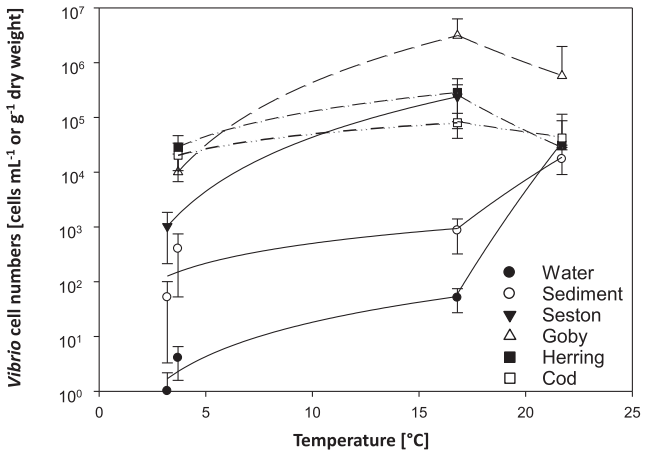

Fig. 4. *Vibrio* cell abundances in water, sediment, seston, goby, herring, and cod as a function of temperature as determined at the sampling station in Warnemünde. Cell numbers were calculated from the ddPCR results and are expressed as cells mL^{-1} or cells (g dry weight)$^{-1}$.

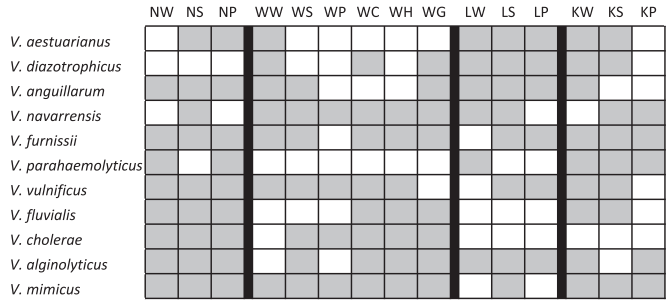

Fig. 5. *Vibrio* species composition in the different sampling materials collected in summer 2015 from the different stations, based on MALDI-ToF-MS species identification.
Gray indicates no detection of the species, and white indicates species detection. Top row: the first letter indicates the sampling station (N: Niendorf, W: Warnemünde, L: Lubmin, K: Karlshagen) and the second letter the sampling material (W: water, S: sediment, P: seston, C: cod, H: herring, G: goby).

only a single colony. CFU numbers ranged from below the detection limit (water) to 1.08 × 10^6 and 2.00 × 10^6 *Vibrio* CFU (g dry weight)$^{-1}$ in goby and seston, respectively. These results were mostly lower than those obtained based on ddPCR (Fig. 2), with difference of approximately one order of magnitude for sediment, seston, and goby and two orders of magnitude for herring and cod.

3.3. *Vibrio* species composition determined by MALDI-ToF-MS

Identification of the *Vibrio* isolates revealed spatial- and material-dependent patterns in the species distribution. *V. vulnificus*, a potential pathogen in humans, was detected in three of the 15 summer/autumn samples, including in round goby. In summer 2015 and winter 2015/16, 1188 and 1324 potential *Vibrio* isolates were collected, respectively. The MALDI-ToF-MS analyses showed that 772 of the summer/autumn isolates (64% of all isolates) and 155 of the winter isolates (11.5%) belonged to *Vibrio*. In summer/autumn 2015, *Vibrio* strains were obtained from all of the environmental samples, and 11 different *Vibrio* species were identified (Fig. 5). While all species were isolated from particles, only five different *Vibrio* species were determined in the fish, which thus had the lowest *Vibrio* diversity. *V. cholerae* and *V. mimicus* were restricted to samples from the eastern Baltic Sea while *V. parahaemolyticus* was more common in the western Baltic Sea. *V. vulnificus* was found in three of the summer/autumn 2015 samples: in the L water samples, K particle samples, and goby W samples. In the winter samples, only four species were identified: *V. parahaemolyticus*, *V. diazotorophicus*, *V. anguillarum*, and *V. aesturianus*, restricted to four of the 15 samples from three sites: water (W), sediment (K), and water and sediment (N).

3.4. *Vibrio vulnificus* virulence genes

The PCR results indicated that none of the *V. vulnificus* strains were of clinical relevance. All three virulence genes could be detected in different strains, but a maximum of two were detected within the same isolate (Supplementary Information S7). PCR using the primers for the *rtxA1* gene yielded positive results in the control strains. All of the *V. vulnificus* strains were positive for the species-specific *vvh* gene.

3.5. Bacterial and Vibrio communities in fish and environmental materials

The nMDS analysis showed a material-dependent separation of the bacterial and *Vibrio* communities. Within the bacterial community, based on a 95% confidence interval, only two significant groups (<0.05) were detected (Fig. 6A). The first group consisted of sediment, seston, and water, and the second the three fish species. For the *Vibrio* assemblages, round goby and cod had a distinct *Vibrio* assemblage and formed their own distinct while the herring samples were spread and overlapped with the sediment-seston-water group (Fig. 6B).

3.6. Vibrio share distribution in a coastal segment during winter

According to the projected *Vibrio* spp. cell loads for the different habitats in winter, water and sediment, together comprising the largest portion of the coastal material, each accounted for >30% of the *Vibrio* spp. load. Seston comprised only 1/100,000 of the water volume and the overall *Vibrio* spp. cell load was accordingly negligible. However, despite making up only 1/1000 of the water volume, the round goby hosted a considerable or even equal number of *Vibrio* spp. cells as found in water and sediment (Fig. 7).

4. Discussion

The identification of the winter habitats of *Vibrio* spp. and its facultative pathogenic members in temperate coastal regions represents an important step in understanding *Vibrio* dynamics during the onset of warm water periods. Moreover, it can also allow better predictions of potential risk areas from which *Vibrio* spp. may be able to reach high or even harmful concentrations once water temperatures again become favourable.

4.1. Fish as a winter reservoir and potential impact on coastal Vibrio communities

The compositions of the bacterial communities associated with herring, cod, and round goby were, in general, similar and the fish-specific cluster was significantly different from the assemblages found in the surrounding water, sediment, and seston. Moreover, the three fish species harbored significant numbers of *Vibrio* cells also during winter. Also, the *Vibrio* community composition was specific for cod and round goby, indicative of the distinct impact of fish on *Vibrio* assemblages. But, unlike cod, the round goby is a recently introduced species in the Baltic, originating from the Black Sea, and was first detected in the Gulf of Gdańsk in the 1990s (Skóra and Stolarski, 1993). Since then, its numbers have significantly increased as the fish has spread over major parts of the Baltic Sea (Ojaveer, 2006), reaching abundances of up to 30 to 100 individuals m^{-2} in coastal and other aquatic areas (Brandner et al., 2013; Chotkowski and Marsden, 1999; EMI, 2017; Sapota, 2004). Considering these densities and the high *Vibrio* spp. load of fish in general, especially the invasive round goby may newly and significantly contribute to coastal *Vibrio* populations, including species-specific *Vibrio* assemblages and facultative pathogenic members, as the round goby was one of the few sampled materials, and the only fish, in which the potential human pathogen *V. vulnificus* was detected. A similar scenario was already demonstrated by Tamplin and Capers (1992), who found that oysters can release up to 10^6 *Vibrio* cells per hour in to the water, potentially influencing surrounding bacterial assemblages. In the Baltic Sea, the lifestyle of the goby as a territorial non-migrating species would ensure that overwintering *Vibrio* populations can remain in the coastal zone. Furthermore, in areas where the goby is overly represented, it might alter natural occurring *Vibrio* populations. Thus, areas with high round goby abundances may be starting points for blooms of pathogenic *Vibrio* in summer.

4.2. Vibrio spp. in fish and influence of lifestyle

Fish intestines have long been discussed as an important habitat for *Vibrio* that facilitates the spread of these bacteria, including species pathogenic for humans (Givens et al., 2014). Kaneko and Colwell (1973) hypothesized that *V. parahaemolyticus* in the Chesapeake Bay overwinters in shellfish or scavenger bottom-feeding fish, such as gobies, given the constant contact of these organisms with the sediment, which may also harbor *Vibrio* species during periods of colder water. In a cultivation-based study in the subtropical gulf coast of the USA, *V. vulnificus* reached significantly higher (up to five magnitudes) concentrations in bottom-feeding fish than in surrounding seawater, sediment, oyster tissue, or tissue from carnivorous or filter-feeding fish (DePaola et al., 1994). This is similar to our results in the samples from the first summer/autumn, when *Vibrio* spp. cell concentrations in the bottom-feeding round goby were significantly higher than in the surrounding water and sediment and at least one order of magnitude higher than in carnivorous cod or filter-feeding herring. In fact, the differences in *Vibrio* spp. cell concentrations between the different fish species may be even larger, as the concentrations in cod and herring were probably overestimated in the ddPCR assay, since in both fish *Vibrio* cell numbers determined by ddPCR were much higher than those determined by CFU quantification. This difference between molecular and cultivation-based approaches can be explained by the fact that only a portion of the *Vibrio* cells and species in a sample will grow on the selected medium (Bolinches et al., 1988; Nakashima et al., 2007) but also because dead

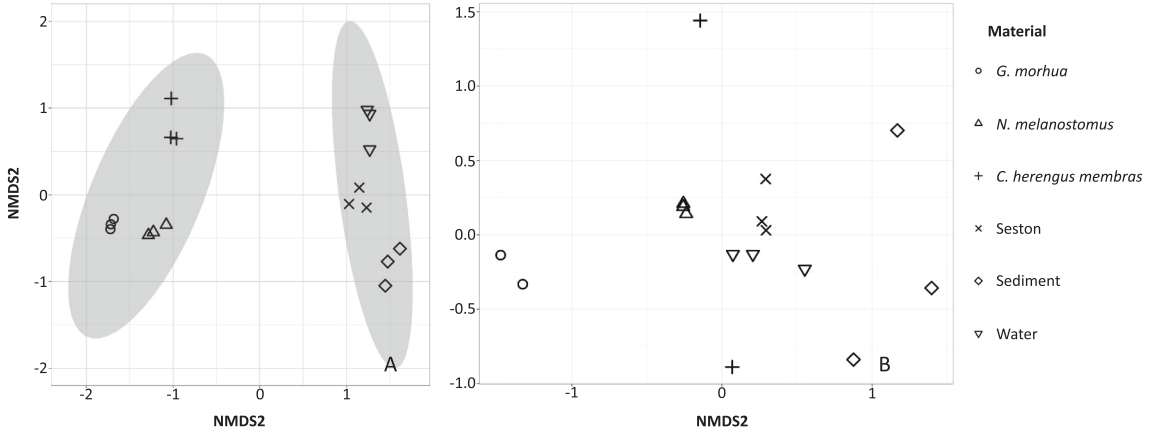

Fig. 6. nMDS plots of the bacterial and *Vibrio* communities in the summer samples collected from Warnemünde in 2015. A: Bacterial communities, stress = 0.05. The two ellipses mark the fish samples and the sediment-seston-water samples. B: *Vibrio* communities, stress = 0.097.

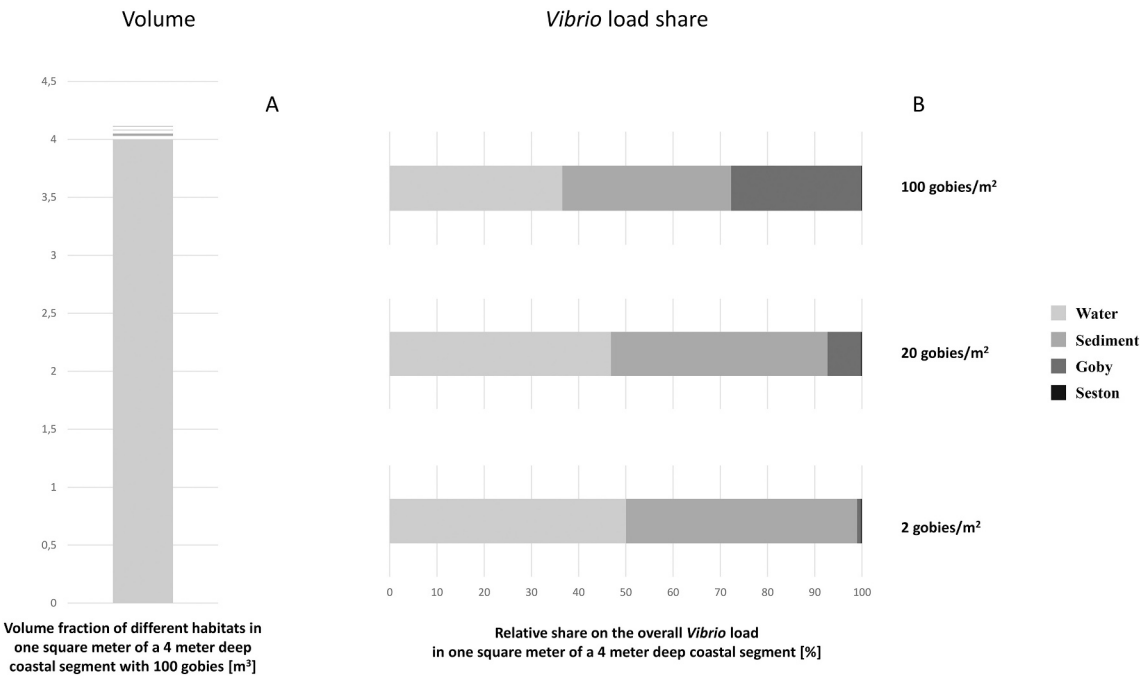

Fig. 7. Theoretical proportion of goby tissue as a *Vibrio* spp. habitat based on samples collected in winter 2015/16 from Warnemünde. The area calculations were set for 1 m^2 and a 4-m depth. A: Volume of water, the first 2 cm of sediment, seston, and goby at a density of 100 gobies per m^2. Other goby densities are not shown, as differences in goby volume is not visible. B: The relative share of each habitat for the overall *Vibrio* spp. cell load, depending on the assumed goby density.

DNA-containing cells will also be detected by PCR (Allmann et al., 1995; Wolffs et al., 2005). Thus, due to the approximately 10 times higher numbers in cod and herring than in the rest of the samples, for these two species the *Vibrio* count was probably overestimated by the ddPCR approach.

Bottom-feeding fish often harbor high *Vibrio* spp. cell numbers (DePaola et al., 1994), such that besides the round goby other bottom-feeding fish species in the Baltic Sea, including the indigenous eelpout *(Zoarces viviparus)*, may also serve as a *Vibrio* reservoir. However, as the invasive round goby may replace native bottom-feeding fish species in coastal areas (Sapota and Skóra, 2005) and reach high coastal abundances (EMI, 2017), a better understanding of its characteristics is essential. The enrichment of *V. vulnificus* in bottom-feeding fish in the study of DePaola et al. (1994) was explained by the consumption of mollusks and crustaceans whose tissues or surfaces were enriched with *Vibrio*. These two food sources represent a major part of the diet of round goby in the Baltic Sea (Skabeikis and Lesutienė, 2015), which could explain the elevated concentrations of *Vibrio* spp. and the detection of *V. vulnificus* in goby tissue in our study. Goby was one of the few sampled materials, and the only fish, in which this potential pathogen, responsible for the vast majority of vibriosis infection at the German Baltic Sea coast, was found. However, none of the strains in our study were of the clinical type, consistent with the observation that the vast majority of *V. vulnificus* strains from the environment lack virulence markers and are considered non-clinical (Han et al., 2009). Nonetheless, environmental strains have been described as still virulent but requiring higher infective doses than clinical strains (DePaola et al., 2003b; Starks et al., 2000; Stelma et al., 1992; Tison and Kelly, 1986)).

Majumdar et al. (2014) investigated the gut, intestines, and gills of several fish species obtained from markets in Bangladesh and showed different *Vibrio* spp. cell abundances in different body parts. Gut and gills were the main *Vibrio* carriers while the concentrations in skin mucus were 10-fold lower. In our study these three compartments also differed in their *Vibrio* concentrations, with gut as the most enriched compartment. In addition, *Vibrio* spp. cell abundances in fish tissue were high both in summer and in winter. In winter, the overall load was smaller but it was still significantly higher than in the surrounding water and sediment. This observation suggests that *Vibrio* overwinters in fish tissue, as also proposed by DePaola et al. (1994) and Givens et al. (2014). Those authors demonstrated that during colder periods *Vibrio* could be detected in fish, but not in sediment or water. The studies were performed in subtropical regions, where water temperatures consistently remain above 10 °C, but our study indicates that a similar scenario is possible in temperate climate zones.

4.3. Seston and sediment as potential winter reservoirs and the impact of temperature on Vibrio abundances

Seston may also serve as a retreat for *Vibrio* during cold water periods, as its ability to enrich bacterial populations is well established (Caron et al., 1982) and *Vibrio* species, including *V. cholerae*, are able to form biofilms on chitin particles (Kirschner et al., 2011; Matz et al., 2005). *Vibrio* assemblages on seston have been detected in diverse habitats (Eiler and Bertilsson, 2006; Eiler et al., 2006). In our study, summertime *Vibrio* concentrations on seston were similar to those associated with cod and herring. The cell abundances determined in our study, 10^4–10^5 cells (g dry weight)$^{-1}$, were similar to those reported by Kaneko and Colwell (1973). In their study, cultivation-dependent *Vibrio* spp. cell abundances reached ~10^5 cells g^{-1} at temperatures of ~18 °C and levels of ~10^9 cells g^{-1} were possible at temperatures of about 30 °C. While in our summer samples the concentration of *Vibrio* spp. cells on particles reached levels comparable to those associated with fish, during winter times the concentration dropped by about two magnitudes, such that concentrations were similar to those in the sediment. The strong influence of temperature on seston in the Chesapeake Bay, USA, was also noted by (Kaneko and Colwell, 1973). The sharp drop in *Vibrio* cell numbers on seston at low temperatures indicated that, while seston is a relevant habitat at warmer water temperatures, as a winter habitat it is less viable. In sediment, by contrast, the influence of temperature was less pronounced and *Vibrio* spp. cell numbers were similar in summer and winter, indicating that sediment offers a relatively stable environment for *Vibrio*. A lower temperature sensitivity of *Vibrio* in sediment was observed in other studies as well, as demonstrated by the isolation of *Vibrio* spp. from sediment samples obtained

during winter, when this was no longer possible in the corresponding water samples (Böer et al., 2013). These observations led to the hypothesis that *Vibrio* overwinters in sediment. In our study, *Vibrio* was also detected at a nearly constant level in the sediment, while in water in half of the samples *Vibrio* cell numbers were close to 0 in winter. However, when the water temperature exceeded 20 °C *Vibrio* spp. counts in sediment and water increased by one and two orders of magnitude, respectively. For *Vibrio* spp., a temperature of 20 °C is considered a threshold above which these bacteria begin to proliferate and eventually reach high cell numbers (Böer et al., 2012; Böer et al., 2013; Kaspar and Tamplin, 1993). This behavior is well described and has also been observed in temperate regions. Indeed, the *Vibrio* spp. cell numbers detected in our study were comparable to those reported in other studies in which samples were obtained from similar environmental conditions (Baker-Austin et al., 2013; Hauk and Duty, 2015). At temperatures below 20 °C, *Vibrio* abundances in fish followed a similar pattern as in sediment, water, and seston, but did not increase when the threshold of 20 °C was exceeded. This can be explained by a slower temperature adjustment of the fish body or avoidance by the fish of unfavorable temperature conditions, by moving into other thermal layers (Claireaux et al., 1995). With the limited time points of our study, this behavior could not be excluded but remains to be demonstrated.

4.4. Salinity as a driving factor of the Vibrio community

While temperature is the main driving factor influencing *Vibrio* spp. abundances in temperate regions, salinity is a key parameter affecting *Vibrio* species composition (Takemura et al., 2014). This was the case in our study, in which a salinity-dependent species composition was observed: *V. parahaemolyticus,* with its preference for a salinity of 17–20‰ (Cook et al., 2002; DePaola et al., 2003a; Rivera et al., 1989), was found more frequently at the western stations, characterized by elevated salinities, than at stations in the east. By contrast, both *V. cholerae*, and *V. mimicus*, which prefer salinities ranging from <5‰ to freshwater conditions (Chowdhury et al., 1989; Singleton et al., 1982; Vital et al., 2007), were found in the east. Consistent with our findings, a salinity-driven separation of *Vibrio* species in the North Sea and Baltic Sea was previously described (Böer et al., 2012).

5. Conclusion

Our study showed that fish has the potential to serve as a winter reservoir for *Vibrio* spp. in the southern Baltic Sea. Namely the round goby was one of the few sampling materials in which the potential human pathogenic *V. vulnificus* was detected. As the invasive species round goby can reach high abundances, especially close to the coastal shore, it might serve as a new winter reservoir for distinct coastal *Vibrio* communities and potentially pathogenic species. Therefore, areas of high round goby densities may give rise to pathogenic *Vibrio* blooms in summer underlining the importance of invasive fish species not only a threat to native fauna but also potentially harmful to humans.

Deposit of sequence data

Sequence data are deposed at the European Nucleotide Archive, ENA, under the accession number: PRJEB38826.

Declaration of Competing Interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmarsys.2021.103574.

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