Metagenomic Analysis of the Gastrointestinal Microbiota of *Gadus morhua callarias* L. Originating from a Chemical Munition Dump Site

Wojciech Wilczynski 1,2,*, Monika Radlinska 1, Klaus Wysujack 3, Michał Czub 2,4, Tomasz Brzeziński 2, Grzegorz Kowalczyk 2, Jacek Beldowski 4, Pedro Nogueira 3 and Piotr Maszczyk 2

1 Department of Environmental Microbiology and Biotechnology, Institute of Microbiology, Faculty of Biology, University of Warsaw, I. Miecznikowa 1, 02-096 Warsaw, Poland; m.radlinska@biol.uw.edu.pl
2 Department of Hydrobiology, Institute of Functional Biology and Ecology, Faculty of Biology, University of Warsaw, Zwrki i Wigury 101, 02-089 Warsaw, Poland; mczub@iopan.pl (M.C.); t.brzezinski@uw.edu.pl (T.B.); kowalczykg@gmail.com (G.K.); p.maszczyk@uw.edu.pl (P.M.)
3 Thünen Institute of Fisheries Ecology, Herwigstraße 31, 27572 Bremerhaven, Germany; klaus.wysujack@thuenen.de (K.W.); pedro.nogueira@thuenen.de (P.N.)
4 Institute of Oceanology, Polish Academy of Sciences, Powstańców Warszawy 55, 81-712 Sopot, Poland; hyron@iopan.pl
* Correspondence: wk.wilczynski@gmail.com; Tel.: +48-22-55-26518

Abstract: Several hundred thousand tonnes of munitions containing chemical warfare agents (CWAs) are lying on the seafloor worldwide. CWAs have started leaking from corroded munitions, and their presence in the environment and in organisms inhabiting dump sites has been detected. The presence of CWAs in the water negatively affects fish, macrobenthos and free-living bacteria. It can be expected that the presence of CWAs would also affect the gut-associated bacteria in fish, which are vital for their condition. The main aim of this study was to test if the microbiota of cod collected in the Baltic Bornholm Deep (highly polluted with CWAs) is dysregulated. To investigate this, we conducted metagenomic studies based on 16S rRNA gene sequencing. We found that the microbiota of cod inhabiting the dump site was significantly less taxonomically diverse compared to those from a non-polluted reference site. Moreover, taxa associated with fish diseases (e.g., *Vibrionaceae*, *Aeromonadaceae*) were more prevalent, and probiotic taxa (e.g., *Actinobacteriota*, *Rhodobacteraceae*) were less frequent in the guts of individuals from the dump site, than those from the reference site. The differences in vulnerability of various bacterial taxa inhabiting cod gastrointestinal tracts to CWAs were hypothesised to be responsible for the observed microbiota dysregulation.

Keywords: 16S rRNA metagenomics; eastern Baltic cod; Baltic Sea; Bornholm Deep; chemical warfare agents; CWAs; microbiome

1. Introduction

Chemical warfare agents (CWAs) are the toxic components of chemical weapons. They include choking agents designed to impede a victim’s ability to breathe (e.g., phosgene and hydrogen cyanide), vesicant agents designed to inflict chemical burn injuries upon contact with the victim’s skin (e.g., yperite and lewisite) and nerve agents designed to fatally interfere with the victim’s nervous system (e.g., tabun and sarin). During the past century, due to the enormous military potential of CWAs, they were mass produced and often exploited in numerous international conflicts.

The mass disposal of several hundred thousand tons of unwanted, obsolete or captured chemical munitions was costly and problematic. Thus, the most common means of getting rid of unused chemical munitions was dumping them into the seas and the oceans. Sea-dumping operations took place worldwide [1]. Several dumping sites have been documented in European waters. The Baltic Sea, the Skagerrak strait, the Irish Sea and
the Bay of Biscay are the areas with the largest quantities of dumped chemical munitions. In the Skagerrak Strait, at least 170,000 tons of munitions containing CWAs were dumped [2,3], and in the Baltic Sea, at least 50,000 tons were dumped [4,5].

The sea-dumping of chemical munitions continued until the early 1970s. Nowadays, they pose an immense threat to numerous aquatic ecosystems and human well-being [6–8]. Recent studies have reported that most shells and casings collected from the chemical warfare (CW) dump sites are corroded to such an extent that their contents (CWAs) have started to leak into the adjacent water and sediments [7–9] and that their concentrations in the bottom waters will peak in the next decades [6]. Newest research suggests that CWAs are highly toxic to aquatic organisms [10–12] and that their continuous release from the munitions deposited in the bottom waters can negatively influence the benthic biota [13,14].

The CWAs toxicity to demersal fish has long been estimated using mathematical modelling, which screened the risk profiles of various CWAs based on their chemical structure (The Ecological Structure Activity Relationships: ECOSAR) [6]. According to these estimations, the most dangerous CWAs are the organoarsenic CWAs in the bottom waters, up to 4 m from the sediments in the CW dump sites. Although the bioaccumulation potential of CWAs in the tissues of aquatic animals is rather low [15], it has recently been reported that some of the oxidation products of CWAs are bioaccumulated and biotransformed by macrobenthos and fish [14,16,17]. Several CWAs are also known to demonstrate systemic geno- and cytotoxicity [14,18–20]. The latest studies on the ecotoxicity of CWAs have shown the induction of severe DNA damage in the gills of macrobenthos (Blue mussel, Mytilus trossulus), fish from the Mediterranean Sea (Blackbelly rosefish Helicolenus dactylopterus and European conger Conger conger) and fish from the Baltic Sea (European flounder Platichthys flesus, Atlantic herring Clupea harengus and eastern Baltic cod Gadus morhua callarias) [14,16,21].

Besides geno- and cytotoxicity, CWAs could exert other negative effects on the aquatic organisms, i.e., the putative effect on the microbiota (microbial consortium) inhabiting the gastrointestinal (GI) tracts of fish. The composition and diversity of GI microbiota play an important role as indicators of both water contamination [22] and the gut health of sampled fish [23]. Balanced GI microbiota plays an important role in the nutrition of fish (i.e., digesting their food and synthesizing vitamins [24,25]), increasing their resistance to pathogens [26] and aiding in the intestinal regeneration by stimulating the proliferation of epithelial cells [27].

The presence of various anthropogenic pollutants in the aquatic environment, such as copper [28], lead [29] and microplastics [30,31], can cause the dysregulation (dysbiosis) of a balanced GI microbiota of fish through modifying its bacterial composition, which could, in turn, impair their performance and lower their fitness by leading to inflammation, chronic illnesses and decreased immunity to secondary infections [32]. It can be expected that the pollution of aquatic environments by CWAs could also lead to dysbiosis in the GI tracts of fish, since it has been shown that the free-living bacteria abundance, biomass and taxonomic diversity is low in CW dump sites as compared to non-contaminated sites, which results from the vulnerability of several bacterial taxa to CWAs [13,18]. The reduced abundance and diversity of free-living bacteria at dump sites are likely to constrain the potential to reinforce the GI microbiota, whereas the uptake of toxics with water and/or food could evoke selective pressure on the bacterial communities in the GI tracts of fish.

Among the fish species that are particularly threatened by CWAs is the eastern Baltic cod (Gadus morhua callarias, Linnaeus, 1758), a keystone species in the Baltic ecosystem and an economically important resource. The major spawning site of the vast majority of the G. morhua callarias population, the Bornholm Deep [33], is also one of the main dump sites of chemical munitions, where 40,000 tons of munitions containing CWAs were deposited [2]. As a demersal species, G. morhua callarias is especially prone to CWAs exposure: individuals of this species live in the vicinity of submerged military objects [8,34]. For this reason, cod from the Bornholm Deep have long been monitored and studied for biomarkers of CWAs.
Toxics 2022, 10, 206

3 of 13

exposure [35,36]. A recent study documented biotransformation and bioaccumulation of the oxidation products of several CWAs by individuals of this species [37,38].

The aim of this study was to analyze the hitherto undescribed GI microbiota of eastern Baltic cod (G. morhua callarias) and to compare it to the GI microbiota of cod collected at a chemical warfare dump site (the Bornholm Deep of the Baltic Sea). We tested two hypotheses: first, that the taxonomic compositions (at the phyla, families and genera levels) of the GI microbiota of cod collected at a CWAs polluted site and at reference site are different and, second, that the taxonomic diversity of the GI microbiota of cod collected at a chemical warfare dump site is altered in comparison to that of cod collected at a reference site.

2. Materials and Methods

2.1. G. morhua callarias Sampling

Specimens of G. morhua callarias were collected on the 18th and 19th of August 2019, using long-distance trawling during ICES monitoring cruise no. 429 of the German Fishery Research Vessel “Walther Herwig III”. The trawlings took place between 8–10 a.m local time (CEST). Two sampling sites were chosen: (1) the designated dumping area in the Bornholm Deep, around 55°18.949′N, 15°34.756′E, where residual cod were previously confirmed to bioaccumulate CWAs-related compounds, which signifies considerable exposure to CWAs contamination [38], and (2) the DAIMON project Bornholm Deep CW dump site reference area, around 55°06.938′N, 18°10.907′E (Figure 1). At each site, cods were sampled from two hauls of 60 min, respectively. Among the available fish, 24 individuals were selected (10 from the reference site and 14 from the CW dump site) based solely on size similarity (28 ± 1 cm in length from the top of the mouth to the tip of the caudal fin). Such a selection decreased the risk of bias associated with size (such as different ages or diets of individuals). Before each haul, the physicochemical parameters (temperature, salinity and dissolved oxygen concentration) of the water column at the sampling stations were measured (Figure 2) using a multiparametric probe (SBE 19plus V2 SeaCAT Profiler CTD).

Figure 1. Confirmed and unconfirmed locations of deep-sea CW dump sites (red) in the Baltic Sea (reprinted with permission from [34]) with indicated sampling stations: the Bornholm Deep CW dump site (yellow circle) and the reference area (blue circle).
2.2. Gastrointestinal Tract and Fecal Matter Extraction

Upon capture and length measurements, individuals of adequate length were stunned by a blow on the head and afterwards put to death by severing the spinal cord and placed on ice. Next, in order to collect the GI tracts, the peritoneal cavity of each fish was aseptically opened using a scalpel, and the intestine was freed from the connective tissue, as well as adjacent internal organs. Afterwards, the esophagus was cut, and the whole GI tract (stomach, pyloric caeca and the intestine) was isolated. In order to extract its contents (fecal matter comprising of digested food and mucus secreted in the intestine), the GI tract was cut lengthwise using a same scalpel. Then, the entire contents were gently transferred to separate sterile “falcon”-type tubes (15 mL), and the samples were homogenized using a hand-held homogenizer. The samples were flash-frozen and kept at −80 °C until further processing. The whole extraction procedure was conducted in sterile conditions, using a new scalpel blade for each individual GI tract.

2.3. DNA Extraction and PCR Amplification

In order to isolate the total DNA from the fecal matter, the FastDNA™ Spin Kit for Feces (MP Biomedicals, Solon, OH, USA) was used, according to the manufacturer’s protocol, with 500 mg (wet mass) of each homogenized fecal matter sample representing individual fish.

The V3–V4 hypervariable region of the 16S rRNA encoding gene was amplified in the samples using universal primers 341f (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and 785r (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGACAGCAG-3′), synthetized by Sigma-Aldrich, Saint Louis, Missouri, MO, USA. The master mixes were prepared using the KAPA HiFi PCR Kit (Roche, Basel, Switzerland), and the PCR was run using the Eppendorf™ Mastercycler nexus X2 (Eppendorf, Westbury, NY, USA). The PCRs were performed in a 25 µL final reaction volume with 26 cycles of 98 °C (20 s), 55 °C (15 s) and 72 °C (15 s). The quality of DNA at each step was checked (including negative controls) by agarose gel electrophoresis and by measuring the double-stranded DNA concentration using the Qubit™ dsDNA HS Assay Kit on the Qubit™ 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the samples containing PCR products were frozen at −20 °C until further analyses. The PCR was run in two repetitions for every individual sample while preparing new DNA template dilutions and new master mixes for each repetition. Before sequencing, two samples containing amplicons representing the microbiota of an individual GI tract (replicates) were pooled.
2.4. Sequencing and Data Processing

The amplicons were sequenced using an MiSeq (Illumina) platform on a single run using the MiSeq Reagent Kit v2 (Illumina, San Diego, CA, USA) and the paired-end method (2 × 300 bp), according to the standard protocols by Genomed (Warsaw, Poland).

Demultiplexing and trimming of Illumina adapter sequences (cutadapt software v3.5 [39]) was performed by the sequencing company (Genomed, Warszawa, Poland). Quality inspection, visualization and assessment of raw fastq files was performed with FastQC [40] and MultiQC [41]. The sequences were processed using the DADA2 plugin within QIIME 2 [42]. Sequences were trimmed at 270 nt, and the first 8 nt were truncated. Amplicon sequence variants (ASVs) and their counts for each sample were acquired. Alpha rarefaction plots confirmed that the number of remaining sequences was sufficient to detect the microbial diversity present. Taxonomies were assigned to the resulting ASVs with the q2-feature-classifier plugin, using the weighted Naive Bayes classifier based on the 16S rRNA silva 138 SILVA SSU gene database at 99% similarity. The Align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin was used to construct a rooted phylogenetic tree using MAFFT. Phylogenetic and non-phylogenetic core diversity metrics, including alpha and beta diversity, were calculated using the Core-diversity-metrics pipeline. Data for this purpose were rarefied to a sampling-depth equal to the lowest frequency among the samples (23,500 reads). Phylloseq [43] and qiime2R [44] were used to plot ordination plots.

The diversity of ASVs between the samples originating from the GI tracts of cod from the reference site and the CW dump site was analyzed. Both α and β diversities, as well as the mean relative abundances of dominant ASVs, were analyzed between the studied variants. To measure α diversity, the Chao1 estimator and Shannon index were calculated. Chao1 is an estimator measuring the total richness, which is particularly useful because of a valid variance, which can be used to calculate associated confidence intervals [45], whereas the Shannon index reflects the species numbers and abundance equality, whereby the greater the species numbers and the evener their abundances, the higher the index value [46]. For the sake of measuring of β diversity, Jaccard, Bray–Curtis, unweighted UniFrac and weighted UniFrac indices were calculated. The Jaccard and unweighted UniFrac indices take into account only the number of observed ASVs, whereas the Bray–Curtis and weighted UniFrac indices consider both the number of observed ASVs and their relative abundance. The UniFrac indices additionally incorporate phylogenetic distances between observed ASVs. Basing on the mean relative abundance of ASVs in the samples, dominant phyla (top five most abundant ASVs in either group), as well as dominant families and genera (top ten most abundant ASVs in either group), were calculated. α diversity indices’ values were statistically compared using the Mann–Whitney U Test (statistical significance threshold was set at \( p \leq 0.05 \)). The bootstrap resampling method (1000 iterations) was used to calculate the confidence intervals for the differences between the mean relative abundances of dominant ASVs (an effect is statistically significant only if the corresponding confidence interval does not include zero). β diversity indices’ values were statistically compared using the permutational analysis of variance (PERMANOVA). The statistical analyses were performed in R Studio [47] and using the QIIME2 bioinformatics platform [42].

3. Results

3.1. Dominant ASVs

Our results have shown that Firmicutes, Fusobacteriota, Proteobacteria, Actinobacteriota and Spirochaetota were the predominant bacteria in the GI tracts of the studied G. morhua callarias (in either group) at the level of the phylum classification. Significant differences in the mean relative abundances between the reference site and the CW dump site were observed for two phyla, Actinobacteriota (whose abundance was decreased) and Spirochaetota (whose abundance was increased), in the gut content of fish from the CW dump site (Figure 3, Table 1).
The predominant bacteria at the family level included *Fusobacteriaceae*, *Mycoplasmataceae*, *Ruminococcaceae*, *Clostridiaceae*, *Acetobacteriaceae*, *Lachnospiraceae*, *Rhodobacteraceae*, *Erysipelotrichaceae*, and *Vibrionaceae*. In the gut content of the fish from the CW dump site, in comparison to the reference site, *Clostridiaceae* and *Rhodobacteraceae* were significantly less abundant, while *Acetobacteriaceae*, *Erysipelotrichaceae*, *Brachyspiraceae* and *Vibrionaceae* were more abundant (Figure 3, Table 1).

The most abundant genera in the GI tracts of the studied *G. morhua callarias* (in either of the two groups) comprised *Cetobacterium*, *Aeromonas*, *Macellibacteroides*, *Sulfitobacter*, *Tyzzerella*, *Escherichia-Shigella*, *Photobacterium*, *Brevinema*, *Aliivibrio* and *Candidatus Bacilloplasma*. Among them, the relative abundances of *Aeromonas*, *Macellibacteroides*, *Brevinema* and *Aliivibrio* were higher, while the relative abundance of *Sulfitobacter* were lower (Figure 3, Table 1), in the gut content of fish from the CW dump site, as compared to the reference site.

Table 1. Mean relative abundances (± SD) of the dominant bacteria taxa (a) phyla, (b) families and (c) genera (respectively, top 5, 10 and 10 most abundant ASVs in either group) in the gastrointestinal microbiota of *G. morhua callarias* originating from either the reference site (Reference) or the chemical warfare dump site (CW dump site) and the bootstrap confidence intervals for differences in means between the two groups.

| Rank | ASV                                   | Relative Abundance (%) | Difference in Means: Bootstrap Confidence Interval (95%) |
|------|---------------------------------------|------------------------|--------------------------------------------------------|
|      |                                       | Reference  | CW Dump Site                                      |
| Phylum | **Fusobacteriaceae (Fusobacteriota)** | 13.5 ± 11.2 | 27.0 ± 24.4  | −28.603~0.252                                      |
|       | **Firmicutes**                        | 10.7 ± 11.6 | 7.84 ± 9.21  | −5.194~10.839                                      |
|       | **Proteobacteria**                    | 10.6 ± 9.85 | 12.5 ± 9.66  | −9.385~6.089                                       |
| Family | **Ruminococcaceae (Firmicutes)**      | 17.3 ± 23.6 | 0.05 ± 0.15  | 5.360~34.340                                       |
|       | **Clostridiaceae (Firmicutes)**       | 2.56 ± 1.84 | 8.13 ± 8.91  | −10.573~1.269                                      |
|       | **Acetobacteriaceae (Protobacteria)** | 2.26 ± 2.57 | 6.02 ± 8.81  | −9.321~0.277                                       |
|       | **Lachnospiraceae (Firmicutes)**      | 4.41 ± 9.61 | 0.08 ± 0.13  | 0.962~11.383                                       |
|       | **Rhodobacteraceae (Protobacteria)**  | 0.57 ± 0.89 | 6.62 ± 8.69  | −10.837~2.056                                      |
|       | **Erysipelotrichaceae (Firmicutes)**  | 0.04 ± 0.10 | 2.27 ± 4.74  | −4.730~0.080                                       |
|       | **Brachyspiraceae (Spirochaetota)**   | 1.41 ± 3.49 | 7.75 ± 11.1  | −13.013~0.777                                      |
|       | **Vibrionaceae (Protobacteria)**      | 13.5 ± 11.2 | 27.0 ± 24.4  | −28.007~0.472                                      |
| Genus  | **Cetobacterium (Fusobacteriaceae, Fusobacteriota)** | 2.56 ± 1.84 | 8.19 ± 8.90  | −10.296~1.185                                      |
|       | **Aeromonas (Acetobacteriaceae, Proteobacteria)** | 0.04 ± 0.07 | 4.60 ± 15.3  | −13.713~0.100                                      |
|       | **Macellibacteroides (Tannerellaceae, Bacteroidota)** | 3.78 ± 9.75 | 0.06 ± 0.08  | 0.375~10.765                                       |
|       | **Sulfitobacter (Rhodobacteraceae, Proteobacteria)** | 0.00 ± 0.00 | 2.11 ± 7.79  | 0.000~6.762                                        |
|       | **Tyzzerella (Lachnospiraceae, Firmicutes)** | 1.72 ± 5.33 | 1.20 ± 2.72  | −2.238~4.428                                      |
|       | **Escherichia-Shigella (Enterobacteriaceae, Proteobacteria)** | 1.18 ± 3.43 | 3.35 ± 5.74  | −5.842~1.282                                      |
|       | **Photobacterium (Vibrionaceae, Proteobacteria)** | 0.26 ± 0.74 | 2.96 ± 5.34  | −5.582~0.263                                      |
|       | **Brevinema (Brevinemataceae, Spirochaetota)** | 0.21 ± 0.22 | 4.37 ± 7.65  | −8.126~0.650                                      |
|       | **Aliivibrio (Vibrionaceae, Proteobacteria)** | 1.55 ± 1.95 | 0.44 ± 0.98  | −0.034~2.453                                      |
|       | **Candidatus Bacilloplasma (Mycoplasmataceae, Firmicutes)** | 0.10 ± 2.25 | 2.57 ± 6.02  | −9.66~0.000                                      |
Figure 3. Mean relative abundances of the dominant bacteria taxa (a) phyla, (b) families and (c) genera (respectively, top 5, 10 and 10 most abundant ASVs in either group) and other (including unassigned) phyla, families and genera in the gastrointestinal microbiota of G. morhua callarias originating from either the reference site (Ref.) or the chemical warfare dump site (CW dump site). The asterisks (*) indicate statistically significant differences between the two groups.

3.2. α Diversity

The α diversity in the GI microbiota of cod originating from the CW dump site was significantly lower compared to those originating from the reference site, as evidenced by differences in the values of both the Chao 1 estimator (U = 10; Z = 3.48; p = 0.005) and Shannon index (U = 28; Z = 2.43; p = 0.015) (Figure 4).

Figure 4. Boxplots of the (a) Chao 1 estimator and (b) Shannon index calculated on the basis of the number and the relative abundances of ASVs present in the gastrointestinal microbiota of G. morhua callarias from the reference site (blue) and the CW dump site (yellow). The asterisks (*) indicate statistically significant differences between the two groups.

3.3. β Diversity

The ASVs composition in the GI microbiota of G. morhua callarias originating from the CW dump site was notably distinct compared to those from the reference site, as evidenced by dissimilarity indices: Jaccard (F = 0.102; p = 0.001), Bray–Curtis (F = 0.140; p = 0.001),
unweighted UniFrac ($F = 0.144; p = 0.001$) and weighted UniFrac ($F = 0.129; p = 0.002$). The NMDS (Non-metric Multi-dimensional Scaling) plots revealed clear clustering of samples corresponding to the two studied groups (Figure 5).

4. Discussion

The metagenomic analysis, based on 16S rRNA gene sequencing, allowed the indication of the main phyla, families and genera in the GI microbiota of *G. morhua callarias* collected at the reference and the CW dump site (the Bornholm Deep). The dominant taxa observed in our study mostly reflected typical microbiota of marine fish [25,48], with especially close resemblance to the results of metagenomically analyzed microbiota of captive Atlantic cod [22].

We found significant differences between the taxonomic compositions of cod GI microbiota from the CW dump site versus reference site, which allowed us to confirm the first hypothesis of this study (that the taxonomic structure of the GI microbiota of cod collected at the two sites are different). Generally, probiotic bacteria were less abundant, and pathogenic bacteria were more abundant, in the GI tracts of cod from the CW dump site, compared with the reference site. With regard to microorganisms considered to be probiotic, the following groups of bacteria at different taxonomic levels were found to be highly reduced in the GI microbiota of cod from the CW dump site: (i) at the phylum level, *Actinobacteriota*, known for their probiotic mode of action in the guts of animals [49,50]; (ii) at the family level, *Rhodobacteraceae*, which are symbionts known for synthesizing vitamin B12 and their probiotic properties [51], and *Clostridiaceae*, solely associated with plant-based
diets in fish [52–54]; (iii) at the genera level, *Sulfitobacter*, which comprise probiotic bacteria that are capable of inhibiting the growth of bacterial fish pathogens [55]. Simultaneously, the GI microbiota of cod from the CW dump site was characterized by a significant increase in the proportion of harmful bacteria, including the families *Aeromonadaceae*, *Brachyspiraceae* and *Vibrionaceae*, as well as the genera *Aeromonas*, *Brevinema* and *Aliivibrio*, whose various species are opportunistic pathogens associated with fish intestine diseases [56–59].

The second hypothesis of this study (that the taxonomic diversity of the GI microbiota of cod collected at the CW dump site is altered in comparison to those from the reference site) was also confirmed: the values of the $\alpha$ diversity metrics (Chao 1 estimator and Shannon index) were notably lower for the GI microbiota of cod collected at the CW dump site, as compared to cod collected at the reference site, indicating a significant decrease in the microbial diversity in the GI tracts of fish living in the vicinity of sea-dumped chemical munitions. These differences of the microbial diversity together with a significant change in the taxonomic composition of GI microbiota, with probiotic taxa diminishing and pathogenic taxa proliferating, are a symptom of dysbiosis, which is known to have severe consequences for the host. Dysregulation of the taxonomic composition of the GI microbiome in fish impairs their performance, as it is correlated with intestinal inflammation and chronic diseases [28–31].

The values of $\beta$ diversity (the Jaccard index, the Bray–Curtis index, as well as the unweighted UniFrac and weighted UniFrac indices), indicated notable differences in the taxonomic composition (quantitative and qualitative) of the GI microbiota of *G. morhua callarias* originating from the CW dump site, as compared to those originating from the reference site. The similarity of the GI microbiota composition of cod originating from the same site and the dissimilarity of the GI microbiota composition of cod originating from different sites (in biodiversity and functional composition) additionally confirmed that the fish have been living in these sites for a time long enough to develop a site-specific microbiota, originating from adaptation to local habitats (which is the reason why the sampling took place late in the summer, long after the cod spawning season had begun).

Despite the fact that both sites were slightly different in various physico–chemical parameters (such as oxygen concentration, temperature or salinity, Figure 2), one may expect that the presence of CWAs in the dump site was one of the factors shaping the GI microbiota composition of demersal fish. Some of the *G. morhua callarias* collected from the studied dump site were earlier confirmed to come into contact with, as well as bioaccumulate, CWAs-related compounds [38]. The differences in the vulnerability of bacterial taxa to the presence of CWAs and their degradation products either dissolved in the surrounding water or bioaccumulated in the ingested food may be responsible for the observed changes in the taxonomic composition and relative abundances of GI microbiota [13]. An alternative (but not exclusive) explanation may rely on the fact that the immune system of the host also regulates the composition of the GI microbial community [60] and that CWAs and their degradation products are known to affect the immune response and condition of exposed individuals [14,18]. Exposed individuals, in a poor condition, may not be able to maintain homeostasis with their symbionts in the gut or may be more prone to infections, which also would result in changes in the GI microbiota. Although, without further studies, it is impossible to decisively distinguish whether the presence of CWAs or other environmental factors are ultimately responsible for the dysbiosis observed in the GI tracts of cod inhabiting the Bornholm deep, our results suggest that this habitat is suboptimal for adult cod.

Assuming that the results obtained in this study are associated with the presence of CWAs in one site and their absence in the other, one could expect that the CWAs would exhibit a notable selective pressure on the GI microbiota of demersal fish that are exposed to them. This would be in line with the results of previous studies, which have shown that the CWAs shape the free-living bacterial communities in the CW dump sites [13,61]. However, it should be noted that the interpretation of obtained results should be treated with caution, as the results could have been affected by the site-specific differences in the
physico–chemical parameters, other pollutants unrelated to CWAs or slight differences in the diets of the studied cod besides the presence or absence of CWAs.

5. Conclusions

The results of this study were the first to describe the GI microbiota of eastern Baltic cod. The GI microbiota was typical for marine fish and very similar to the GI microbiota of Atlantic cod. However, the taxonomic structure of the GI microbiota of cod collected at the two studied sites was significantly distinct. The two most important differences were (1) cod from the CW dump site had significantly lower abundances of probiotic bacteria, as seen at the phylum (Actinobacteriota), family (Rhodobacteraceae) and genera (Sulfito bacter) levels, and (2) cod from the CW dump site had significantly higher abundances of pathogenic bacteria, as seen at the phylum (potentially, Spirochaetota), family (Aeromonadaceae, Brachyspiraceae and Vibrionaceae) and genus (Aeromonas, Brevinema and Aliivibrio) levels. Moreover, the GI microbiota of cods collected at the CW dump site expressed significantly reduced taxonomic diversity, as well as an overall distinct taxonomic composition (based on the bacterial number, abundance and phylogeny) when compared to those collected at the reference site.

The obtained results could become an important starting point for the future studies on the evanescing populations of this species in the Baltic Sea.

Author Contributions: Conceptualization, W.W., M.R., M.C., T.B., J.B. and P.M.; methodology, W.W., M.R., K.W., G.K. and P.N.; validation, W.W., M.R., K.W., M.C., T.B., G.K., J.B., P.N. and P.M.; formal analysis, W.W., T.B., M.C., J.B. and G.K.; investigation, W.W., M.R. and K.W.; resources, W.W., M.R., K.W., P.N. and P.M.; data curation, W.W. and G.K.; writing—original draft preparation, W.W.; writing—review and editing, W.W., M.R., K.W., M.C., T.B., G.K., J.B., P.N. and P.M; visualization, W.W., M.C., G.K. and P.M.; supervision, W.W., M.R., K.W., P.N. and P.M.; project administration, W.W.; funding acquisition, W.W., P.M. and M.C. All authors have read and agreed to the published version of the manuscript.

Funding: The research described here was supported by grants no. 2020/37/N/NZ8/04099, 2016/23/D/NZ8/03532, 2021/40/C/NZ8/00125 and 2019/35/B/NZ8/04523 from the National Science Centre, Poland.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We are grateful to the Walther Herwig III’s crew and the technicians of the Thünen Institute for their help with cod sampling.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript or in the decision to publish the results.

References

1. Greenberg, M.I.; Sexton, K.J.; Vearrier, D. Sea-dumped chemical weapons: Environmental risk, occupational hazard. Clin. Toxicol. 2016, 54, 79–91. [CrossRef] [PubMed]
2. HELCOM. Chemical Munitions Dumped in the Baltic Sea. Report of the ad hoc Expert Group to Update and Review the Existing Information on Dumped Chemical Munitions in the Baltic Sea (HELCOM MUNI). In Proceedings of the 2013 HELCOM Ministerial Meeting, Copenhagen, Denmark, 3 October 2013; p. 142.
3. Laurin, F. The Baltic and North Sea dumping of chemical weapons: Still a threat. Chall. Old Chem. Munitions Toxic Armament Wastes 1997, 16, 263–278.
4. Glasby, G.P. Disposal of chemical weapons in the Baltic Sea. Sci. Total Environ. 1997, 206, 267–273. [CrossRef]
5. Missiaen, T.; Henriet, J.P. Chemical Munion Dump Sites in Coastal Environments; Federal Office for Scientific and Cultural Affairs (OSTC): Brussels, Belgium, 2002.
6. Sanderson, H.; Fauser, P.; Thomsen, M.; Sørensen, P.B. Screening level fish community risk assessment of chemical warfare agents in the Baltic Sea. J. Hazards Mater. 2008, 154, 846–857. [CrossRef]
7. Beldowski, J.; Klusek, Z.; Szubsk, M.; Turja, R.; Bulczak, A.; Rak, D.; Brenner, M.; Lang, T.; Kotwicki, L.; Grzelak, K.; et al. Chemical munitions search & assessment—An evaluation of the dumped munitions problem in the Baltic Sea. Deep. Sea Res. Part II Top. Stud. Oceanogr. 2016, 128, 85–95. [CrossRef]

8. Czub, M.; Kotwicki, L.; Lang, T.; Sanderson, H.; Klusek, Z.; Grabowski, M.; Szubsk, M.; Jakacki, J.; Andrzejewski, J.; Rak, D.; et al. Deep sea habitats in the chemical warfare dumping areas of the Baltic Sea. Sci. Total Environ. 2018, 616, 1485–1497. [CrossRef]

9. Vanninen, P.; Östin, A.; Beldowski, J.; Pedersen, E.A.; Söderström, M.; Szubsk, M.; Grabowski, M.; Siedlewicz, G.; Czub, M.; Popiel, S.; et al. Exposure status of sea-dumped chemical warfare agents in the Baltic Sea. Mar. Environ. Res. 2020, 161, 105112. [CrossRef]

10. Brzeziński, T.; Czub, M.; Nawala, J.; Gordon, D.; Dziedzic, D.; Dawidziuk, B.; Popiel, S.; Maszczyn, P. The effects of chemical warfare agent Clark I on the life histories and stable isotopes composition of Daphnia magna. Environ. Pollut. 2020, 266, 115412. [CrossRef]

11. Czub, M.; Nawala, J.; Popiel, S.; Dziedzic, D.; Brzeziński, T.; Maszczyn, P.; Sanderson, H.; Fabisiak, J.; Beldowski, J.; Kotwicki, L. Acute aquatic toxicity of sulfur mustard and its degradation products to Daphnia magna. Mar. Environ. Res. 2020, 161, 105077. [CrossRef]

12. Czub, M.; Nawala, J.; Popiel, S.; Brzeziński, T.; Maszczyn, P.; Sanderson, H.; Maszer, E.; Gordon, D.; Dziedzic, D.; Dawidziuk, B.; et al. Acute aquatic toxicity of arsenic-based chemical warfare agents to Daphnia magna. Aquat. Toxicol. 2021, 230, 105693. [CrossRef]

13. Medvedeva, N.; Polyak, Y.; Kankaanpää, H.; Zaytseva, T. Microbial responses to mustard gas dumped in the Baltic Sea. Mar. Environ. Res. 2009, 68, 71–81. [CrossRef] [PubMed]

14. Höher, N.; Turja, R.; Brenner, M.; Nyholm, J.R.; Östin, A.; Leffler, P.; Butrimavičienė, L.; Baršienė, J.; Halme, M.; Karjalainen, M.; et al. Toxic effects of chemical warfare agent mixtures on the mussel Mytilus trossulus in the Baltic Sea: A laboratory exposure study. Mar. Environ. Res. 2019, 145, 112–122. [CrossRef] [PubMed]

15. Amato, E.; Alcaro, L.; Corsi, I.; Dela Torre, C.; Farchi, C.; Focardi, S.; Marino, G.; Tursi, A. An integrated ecotoxicological approach to assess the effects of pollutants released by unexploded chemical ordnance dumped in the southern Adriatic (Mediterranean Sea). Mar. Biol. 2006, 149, 17–23. [CrossRef]

16. Dela Torre, C.; Petochi, T.; Corsi, I.; Dinaro, M.M.; Baroni, D.; Alcaro, L.; Focardi, S.; Tursi, A.; Marino, G.; Frigeri, A.; et al. DNA damage, severe organ lesions and high muscle levels of As and Hg in two benthic fish species from a chemical warfare agent dumping site in the Mediterranean Sea. Sci. Total Environ. 2010, 408, 2136–2145. [CrossRef]

17. Niemikoski, H.; Söderström, M.; Vanninen, P. Detection of chemical warfare-agent-related phenylarsenic compounds in marine biota samples by LC-HESI/MS/MS. Anal. Chem. 2018, 90, 11129–11134. [CrossRef] [PubMed]

18. Munro, N.B.; Talmage, S.S.; Griffin, G.D.; Waters, L.C.; Watson, A.P.; King, J.F.; Hauschild, V. The sources, fate, and toxicity of chemical warfare agent mixtures in rainbow trout liver cell line RTL-W1. Aquat. Toxicol. 2021, 241, 106075. [CrossRef] [PubMed]

19. Amata, R.; Beblo, D.A.; Rosemond, Z.A. Toxological Profile for Sulfur Mustard; US Department of Health and Human Services Public Health Service Agency for Toxic Substances and Disease Registry: Atlanta, GA, USA, 2003; Volume 80.

20. Niemikoski, H.; Lehtonen, K.K.; Ahvo, A.; Heiskanen, I.; Vanninen, P. Metabolism and cytotoxicity of diphenylarsinic acid, a degradation product of sea-dumped chemical warfare agents, in a rainbow trout liver cell line RTL-W1. Aquat. Toxicol. 2019, 216, 105993. [CrossRef]

21. Baršienė, J.; Butrimavičienė, L.; Grygriel, W.; Lang, T.; Michailovas, A.; Žukauskas, T. Environmental genotoxicity and cytotoxicity in Deep sea habitats in the chemical warfare dumping areas of the Baltic Sea. Sci. Total Environ. 2014, 495, 56–67. [CrossRef]

22. Bagi, A.; Riser, E.S.; Molland, H.S.; Star, B.; Havercamp, T.H.; Sydnes, M.O.; Papamnin, D.M. Gastrointestinal microbial community changes in Atlantic cod (Gadus morhua) exposed to crude oil. BMC Microbiol. 2018, 18, 25. [CrossRef]

23. Egerton, S.; Culloty, S.; Whooley, J.; Stanton, C.; Ross, R.P. The gut microbiota of marine fish. Front. Microbiol. 2018, 9, 873. [CrossRef]

24. Nayak, S.K. Probiotics and immunity: A fish perspective. Fish Shellfish Immunol. 2010, 29, 2–14. [CrossRef]

25. Sullam, K.E.; Essinger, S.D.; Lozupone, C.A.; O’Connor, M.P.; Rosen, G.L.; Knight, R.; Kilham, S.S.; Russell, J.A. Environmental and ecological factors that shape the gut bacterial communities of fish: A meta-analysis. Mol. Ecol. 2012, 21, 3363–3378. [CrossRef] [PubMed]

26. Gómez, G.D.; Balcázar, J.L. A review on the interactions between gut microbiota and innate immunity of fish. FEMS Immunol. Med. Microbiol. 2008, 52, 145–154. [CrossRef] [PubMed]

27. Rawls, J.F.; Samuel, B.S.; Gordon, J.I. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc. Natl. Acad. Sci. USA 2004, 101, 4596–4601. [CrossRef] [PubMed]

28. Meng, X.-L.; Li, S.; Qin, C.-B.; Zhu, Z.-X.; Hu, W.-P.; Yang, L.-P.; Lu, R.-H.; Li, W.-J.; Nie, G.-X. Intestinal microbiota and lipid metabolism responses in the common carp (Cyprinus carpio L.) following copper exposure. Ecotoxicol. Environ. Saf. 2018, 160, 257–264. [CrossRef] [PubMed]

29. Giri, S.S.; Park, S.C. Therapeutic effect of intestinal autochthonous Lactobacillus reuteri P16 against waterborne lead toxicity in Cyprinus carpio. Front. Immunol. 2018, 9, 1824. [CrossRef] [PubMed]

30. Jin, Y.; Xia, J.; Pan, Z.; Yang, J.; Wang, W.; Fu, Z. Polystyrene microplastics induce microbiota dysbiosis and inflammation in the gut of adult zebrafish. Environ. Pollut. 2018, 235, 322–329. [CrossRef]
31. Qiao, R.; Deng, Y.; Zhang, S.; Wolosker, M.B.; Zhu, Q.; Ren, H.; Zhang, Y. Accumulation of different injury and gut microbiota dysbiosis in the gut of zebrafish. *Chemosphere* **2019**, *236*, 124334. [CrossRef]
32. Egan, S.; Gardiner, M. Microbial dysbiosis: Rethinking disease in marine ecosystems. *Front. Microbiol.* **2016**, *7*, 991. [CrossRef]
33. Tomkiewicz, J.; Lehmann, K.M.; John MA, S. Oceanographic influences on the distribution of Baltic cod, *Gadus morhua*, during spawning in the Bornholm Basin of the Baltic Sea. *Fish. Oceanogr.* **1998**, *7*, 48–62. [CrossRef]
34. Beldowski, J.; Fabisiak, J.; Popiel, S.; Ostín, A.; Olsson, U.; Vanninen, P.; Lastumaki, A.; Lang, T.; Fricke, N.; Brenner, M.; et al. CHEMSEA Findings; Institute of Oceanology Polish Academy of Sciences: Sopot, Poland, 2014.
35. Beldowski, J.; Jakacki, J.; Grabowski, M.; Lang, T.; Weber, K.; Kotwicki, L.; Paka, V.; Rak, D.; Golenko, M.; Czub, M.; et al. Best practices in monitoring. In *Towards the Monitoring of Dumped Munitions Threat (MODUM)*. NATO Science for Peace and Security Series C: Environmental Security; Beldowski, J., Been, R., Turmus, E., Eds.; Springer: Dordrecht, The Netherlands, 2018. [CrossRef]
36. Lang, T.; Kotwicki, L.; Czub, M.; Grzelak, K.; Weirup, L.; Straumer, K. The health status of fish and benthos communities in chemical munitions dumpsites in the Baltic Sea. In *Towards the Monitoring of Dumped Munitions Threat (MODUM)*; Springer: Dordrecht, The Netherlands, 2018; pp. 129–152. [CrossRef]
37. Niemikoski, H.; Koske, D.; Kammann, U.; Lang, T.; Vanninen, P. Studying the metabolism of toxic chemical warfare agent-related phenylarsenic chemicals in vitro in cod liver. *J. Hazards Mater.* **2020**, *391*, 122221. [CrossRef] [PubMed]
38. Niemikoski, H.; Strauman, K.; Ahvo, A.; Turja, R.; Brenner, M.; Rautanen, T.; Lang, T.; Lehtonen, K.K.; Vanninen, P. Detection of chemical warfare agent related phenylarsenic compounds and multimarker responses in cod (*Gadus morhua*) from munition dumpsites. *Mar. Environ. Res.* **2016**, *202*, 105160. [CrossRef] [PubMed]
39. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **2011**, *17*, 10–12. [CrossRef]
40. Andrews, S. FastQC: A Quality Control Tool for High Throughput Sequence Data. 2010. Available online: [https://www.bioinformatics.babraham.ac.uk/projects/fastqc/](https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (accessed on 3 April 2022).
41. Ewels, P.; Magnusson, M.; Lundin, S.; Käller, M. MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* **2016**, *32*, 3047–3048. [CrossRef] [PubMed]
42. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef]
43. Bisanz, J.E. Qiime2R: Importing QIIME2 Artifacts and Associated Data into R Sessions. 2018. Available online: [https://github.com/bisanz/qiime2R](https://github.com/bisanz/qiime2R) (accessed on 15 November 2021).
44. McMurdie, P.J.; Holmes, S. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef]
45. Chao, A. Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* **1987**, *43*, 783–791. [CrossRef]
46. Guinane, C.M.; Tadrous, A.; Fouhy, F.; Ryan, C.A.; Dempsey, E.M.; Murphy, B.; Andrews, E.; Cotter, P.D.; Stanton, C.; Ross, R. Microbial composition of human appendices from patients following appendectomy. *MBio* **2013**, *4*, e00366–12. [CrossRef]
47. RStudio Team. RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA. 2022. Available online: [http://www.rstudio.com/](http://www.rstudio.com/) (accessed on 10 March 2022).
48. Tarnecki, A.M.; Burgos, F.A.; Ray, C.L.; Arias, C.R. Fish intestinal microbiome: Diversity and symbiosis unravelled by metagenomics. *J. Appl. Microbiol.* **2017**, *123*, 2–17. [CrossRef]
49. Das, S.; Ward, L.R.; Burke, C. Prospects of using marine actinobacteria as probiotics in aquaculture. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 419–429. [CrossRef]
50. Carter, G.R.; Cole, J.R., Jr. (Eds.) *Diagnostic Procedure in Veterinary Bacteriology and Mycology*; Academic Press: Cambridge, MA, USA, 2012.
51. Zhang, Y.; Wen, B.; David, M.A.; Gao, J.Z.; Chen, Z.Z. Comparative analysis of intestinal microbiota of discus fish (*Symphysodon harelly*) with different growth rates. *Aquaculture* **2021**, *540*, 736740. [CrossRef]
52. Hao, Y.T.; Wu, S.G.; Jakovlici, I.; Zou, H.; Li, W.X.; Wang, G.T. Impacts of diet on hindgut microbiota and short-chain fatty acids in grass carp (*Ctenopharyngodon idellus*). *Aquac. Res.* **2017**, *48*, 5595–5605. [CrossRef]
53. Huang, Q.; Sham, R.C.; Deng, Y.; Mao, Y.; Wang, C.; Zhang, T.; Leung, K.M. Diversity of gut microbiomes in marine fishes is shaped by host-related factors. *Mol. Ecol.* **2020**, *29*, 5019–5034. [CrossRef] [PubMed]
54. Serra, C.R.; Oliva-Teles, A.; Enes, P.; Tavares, F. Gut microbiota dynamics in carnivorous European seabass (*Dicentrarchus labrax*) fed plant-based diets. *Sci. Rep.* **2021**, *11*, 447. [CrossRef] [PubMed]
55. Sharifah, E.N.; Eguchi, M. Benefits of live phytoplankton, *Chlorella vulgaris*, as a biocontrol agent against fish pathogen *Vibrio anguillarum*. *Fish. Sci.* **2012**, *78*, 367–373. [CrossRef]
56. Godoy, F.A.; Miranda, C.D.; Wittwer, G.D.; Aranda, C.P.; Calderon, R. High variability of levels of *Alivibrio* and lactic acid bacteria in the intestinal microbiota of farmed Atlantic salmon *Salmo salar* L. *Ann. Microbiol.* **2015**, *65*, 2343–2353. [CrossRef]
57. He, X.; Chaganti, S.R.; Heath, D.D. Population-specific responses to interspecific competition in the gut microbiota of two Atlantic salmon (*Salmo salar*) populations. *Microb. Ecol.* **2018**, *75*, 140–151. [CrossRef]
58. Wang, C.; Sun, G.; Li, S.; Li, X.; Liu, Y. Intestinal microbiota of healthy and unhealthy Atlantic salmon *Salmo salar* L. in a recirculating aquaculture system. *J. Oceanol. Limnol.* **2018**, *36*, 414–426. [CrossRef]
59. Brown, R.M.; Wiens, G.D.; Salinas, I. Analysis of the gut and gill microbiome of resistant and susceptible lines of rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* **2019**, *86*, 497–506. [CrossRef]

60. Hooper, L.V.; Littman, D.R.; Macpherson, A.J. Interactions between the microbiota and the immune system. *Science* **2012**, *336*, 1268–1273. [CrossRef]

61. Sanderson, H.; Fauser, P.; Thomsen, M.; Vanninen, P.; Soderstrom, M.; Savin, Y.; Khalikov, I.; Hirvonen, A.; Niiranen, S.; Missiaen, T.; et al. Environmental hazards of sea-dumped chemical weapons. *Environ. Sci. Technol.* **2010**, *44*, 4389–4394. [CrossRef] [PubMed]